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cis-Acting Sites in the Transcript of the *Bacillus subtilis trp* Operon Regulate Expression of the Operon

MITZI I. KURODA,[1] DENNIS HENNER,[2] AND CHARLES YANOFSKY[1]*

Department of Biological Sciences, Stanford University, Stanford, California 94305-5020,[1] and Genentech Corp., South San Francisco, California 94080[2]

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Transcription of the *trp* operon of *Bacillus subtilis* is regulated by attenuation. A *trpE'-'lacZ* gene fusion preceded by the wild-type *trp* promoter-leader region was used to analyze regulation. Overproduction of the *trp* leader transcript in *trans* from a multicopy plasmid caused constitutive expression of the chromosomal *trpE'-'lacZ* fusion, presumably by titrating a negative regulatory factor encoded by the *mtr* locus. Subsegments of the *trp* leader region cloned onto the multicopy plasmid were examined for their abilities to elevate β-galactosidase activity. An RNA segment spanning the portion of the leader transcript that forms the promoter-proximal strand of the proposed antiterminator structure was most active in this *trans* test. The data suggest that the *mtr* gene product, when activated by tryptophan, binds to this RNA segment and prevents formation of the antiterminator. In this manner, the *trans*-acting factor promotes formation of the RNA structure that causes transcription termination. Secondary-structure predictions for the leader segment of the *trp* operon transcript suggest that if the *mtr* factor bound this RNA segment in a nonterminated transcript, the ribosome-binding site for the first structural gene, *trpE*, could be sequestered in a stable RNA structure. We tested this possibility by comparing transcriptional and translational fusions containing the initial segments of the *trp* operon. Our findings suggest that the *mtr* product causes both transcription attenuation and inhibition of translation of *trpE* mRNA. Inhibition of translation initiation would reduce ribosome density on *trpE* mRNA, perhaps making it more labile. Consistent with this interpretation, the addition of tryptophan to *mtr*$^+$ cultures increased the rate of *trpE'-'lacZ* mRNA decay.

In *Bacillus subtilis*, transcription of the *trp* operon is regulated by transcription attenuation in response to the availability of tryptophan. However, unlike the attenuation mechanism used to control amino acid biosynthetic operons in enterobacteria, in which synthesis of a leader peptide regulates transcription termination in the leader region (16), in *B. subtilis* a tryptophan-activated *trans*-acting regulatory factor appears to bind to the leader transcript and cause transcription termination (25). The *trans*-acting factor is presumed to be the product of the *mtr* (methyltryptophan resistance) locus, since mutations in this gene result in constitutive expression of the operon (13).

Overproduction of the *trp* leader transcript in *trans* also results in a constitutive phenotype (24). The *trp* promoter is dispensable if replaced by another promoter (24). Presumably the *mtr* product is titrated out by multiple copies of its target site, i.e., a segment of the leader transcript (or the transcription elongation complex containing this RNA segment). To locate the target site of the *mtr* product more precisely, we constructed multicopy plasmids carrying subsegments of the *trp* leader region and used these plasmids to identify the RNA segments that elevate expression of the chromosomal *trpE'-'lacZ* gene fusion.

Predictions of RNA secondary structures that could form in the leader segment of the *trp* operon transcript suggested that the ribosome-binding site for the first polypeptide specified by the operon could be blocked when the *mtr* product attached to its leader transcript-binding site. To test this hypothesis, we compared the regulation of a transcriptional *trp* operon *lacZ* fusion with the regulation of the *trpE'-'lacZ* translational fusion. We also examined *lacZ* mRNA turnover in *mtr*$^+$ and *mtr* strains.

MATERIALS AND METHODS

Bacterial strains and transformations. *B. subtilis* CYBS12 {*argC4 amyE*::[*trpp*-(*trpE'-'lacZ*) Em^r]} was constructed by integration of a segment of ptrpBG3 into the *amyE* locus of strain BG2087 (28). ptrpBG3 is a derivative of ptrpBG1 (26) that carries an erythromycin resistance gene rather than a chloramphenicol acetyl transferase gene (D. Henner, unpublished observations). CYBS12 was transformed by a published procedure (1), except that cells were diluted twofold instead of 10-fold into stage II medium. Transformants carrying pMK104 and its derivatives were selected on tryptose blood agar base plates (Difco Laboratories) containing chloramphenicol (5 μg/ml). Resistance to the tryptophan analog 5-fluorotryptophan was examined by replica plating colonies onto plates containing 0.2% glucose, 0.2% acid-hydrolyzed casein, 1× minimal salts (26), and 200 μg of DL-5-fluorotryptophan per ml. Two derivatives of CYBS12, designated CYBS12A (*trpA mtr*$^+$) and CYBS12B (*trpA mtr-264*), were used in mRNA measurement experiments. *Escherichia coli* CY15073 (*supE supF hsdR hsdM*$^+$ *met lacY*) was used for plasmid constructions. *E. coli* transformations were performed by the calcium shock procedure (5). Transformants were selected on LB plates containing chloramphenicol (12.5 μg/ml).

Plasmid constructions. pMK104 was constructed from pBS42 (2) by cleavage at the unique *Xba*I site, treatment with Klenow fragment, and addition of *Hin*dIII linkers. The resulting plasmid was cut with *Eco*RI and *Hin*dIII, and the ca. 600-base-pair (bp) *Eco*RI-*Hin*dIII fragment was replaced with the polylinker from pUC18 (20).

* Corresponding author.

Segments of the *B. subtilis trp* operon promoter and transcribed leader region were cloned into the polylinker of pMK104. *Eco*RI-*Hin*dIII fragments were isolated from several previously described deletion plasmids. pMK105 contains the 730-bp *Eco*RI-*Hin*dIII fragment from pUCtrpE2 (25). pMK106 contains the 730-bp *Eco*RI-*Hin*dIII fragment from pUCKpnI (25). pMKdH4 contains the 131-bp *Eco*RI-*Hin*dIII fragment from ptrpdH4 (24). pMKdR4 contains the promoterless 320-bp *Eco*RI-*Hin*dIII fragment from ptrpdR4 (24). pMK107 was constructed by linearizing pMK106 with *Kpn*I restriction endonuclease, by digesting the plasmid, and by filling in the single-stranded ends with T4 DNA polymerase and deoxynucleoside triphosphates, followed by religation and transformation into *E. coli*.

pMK66R contains the 477-bp *Eco*RI-*Kpn*I fragment from pUCKpnI (25). *Eco*RI-*Kpn*I deletion fragments were generated from pUCKpnI by cleavage at the unique *Kpn*I site created at position +65 and BAL 31 exonuclease digestion, followed by *Kpn*I linker addition (5'-GGGTACCC-3'). Two of the resulting *Eco*RI-*Kpn*I fragments that extend from the *Eco*RI site at position −412 to position +14 or +29, respectively, were cloned into pMK104 and named pMK14R and pMK29R.

pMKdH4 contained the *trp* leader segment from position −50 to position +81. During construction of the original deletion plasmid, ptrpdH4 (24), an *Eco*RI linker was ligated adjacent to one end of the fragment at position −50, and the other end of the fragment (position +81) was ligated into the *Hin*cII site of the pUC18 polylinker. The resulting *trp* leader segment is flanked by an *Eco*RI site at position −50 and a *Hin*dIII site 15 bp 3′ of position +81, at the end of the polylinker. *Kpn*I-*Hin*dIII fragments were generated from pMKdH4 after cleavage at the unique *Eco*RI site, BAL 31 exonuclease digestion, and *Kpn*I linker addition. The resulting *Kpn*I-*Hin*dIII fragments start at various positions within the transcribed leader region and extend to the *Hin*dIII site 15 bp past position +81. Deletion fragments were cloned into M13mp10 or M13mp11 (18) and sequenced by the dideoxynucleotide chain termination method of Sanger et al. (22).

Plasmid ptrpBG1, containing a *trpE'-'lacZ* fusion in a single-copy integration vector, has been described previously (23). A transcriptional fusion was constructed from ptrpBG1 as follows. ptrpBG1 was digested with *Hin*dIII, repaired to flush ends with the Klenow fragment of DNA polymerase I and deoxynucleoside triphosphates, and then digested with *Cla*I, removing the front portion of the *lacZ* gene at its fusion point to the *trpE* gene. This fragment of ptrpBG1 was ligated to an approximately 900-bp *Sma*I-*Cla*I fragment of pJM783 (9) that encoded a *spoVG'-'lacZ* translational fusion with its own ribosome-binding site. The resulting plasmid, pDH37, contains the *trp* promoter and leader region and the first 40 codons of the *trpE* gene, followed by 8 codons derived from the linker, a stop codon, and 33 untranslated nucleotides; the *spoVG'-'lacZ* fusion follows. The exact sequence of the junction is as follows:

trpE' (37 amino acids) Glu Lys Leu Gly Ile Pro Ser Leu Leu Ile His STOP

-GAG AAG CTG GGG ATC CCC AGC TTG TTG ATA CAC TAA

TGCTTTTATATA GGGAAAAGGT GGTGAACTAC T GTG GAA-

Met Glu *spoVG'-'lacZ*

Plasmid pDH37 was linearized with *Pst*I and transformed into strain W168 by selection for chloramphenicol resistance. The resultant strain, BG4228, has a single copy of the transcriptional fusion integrated in the *amyE* gene (28). Strain BG2199 is an isogenic stain carrying the translation fusion of ptrpBG1 (23).

β-Galactosidase assays. CYBS12 isolates containing pMK104 and its derivatives were grown in 0.2% glucose, 0.2% acid-hydrolyzed casein, 1× minimal salts (26), and 5 μg of chloramphenicol per ml in the presence or absence of 50 μg of tryptophan per ml. Each culture (1 ml) was harvested by centrifugation at an optical density of 0.4 to 0.6 at 600 nm and suspended in 1 ml of 10 mM Tris hydrochloride (pH 7.5) on ice. Toluene (10 μl) was added, and cells were vortexed vigorously. Following incubation on ice for 20 min, 0.1 ml of cells was added to 0.9 ml of Z buffer (19) and assayed for β-galactosidase activity (19). Activity is presented in Miller units and represents the average of two or more independent experiments. In the experiments described in Table 3, β-galactosidase was assayed as described by Ferrari et al. (8) on cultures grown as indicated in Table 3.

mRNA measurements. Cultures used for mRNA measurements were grown at 37°C in minimal medium containing 0.05% acid-hydrolyzed casein, 20 μg of L-tryptophan per ml, and 0.3% glucose. At a turbidity of 80 (Klett colorimeter, 660 filter), cells were harvested by filtration and washed on the filter with warm medium lacking tryptophan. Cells were then suspended in the same warm medium lacking tryptophan and shaken in a water bath at 37°C. After 6 min, a solution of 7-aza-DL-tryptophan was added to a final concentration of 50 μg/ml and shaking was continued for 6 min. Aliquots (20 ml) were then removed to separate flasks shaking at 37°C and were treated as follows. To one aliquot, 0.4 ml of [^{3}H]uridine (28 mCi/mmol) was added at time 0. At 15 s, 60 μl of a rifampin solution (50 mg/ml in methanol) was added, and at 2 min, the culture was poured into frozen killing mix (27). An identically treated aliquot was harvested at 4.25 min. To a third aliquot, tryptophan (to 20 μg/ml) was added at 0.75 min, and the cells were killed at 4.25 min. In experiments using CYBS12B, an aliquot with tryptophan added at 0.75 min was harvested at 2 min. RNA was extracted essentially as described elsewhere (27), treated with RNase-free DNase, phenol extracted, alcohol precipitated, and dissolved in 0.01 M Tris (pH 7.3) containing 0.5 M KCl. Samples (0.4 ml) were placed in vials containing 13-mm BA85 nitrocellulose filters, onto which 2 μg of denatured pACYC177 DNA or placZ1 DNA (pACYC177 containing *lacZ*) had been deposited by filtration. Before denaturation, the DNAs had been linearized by treatment with *Bam*HI, phenol extracted, and alcohol precipitated. The filters were baked in a vacuum oven at 80°C for 2 h immediately before use. Sealed vials containing one filter of each type and the RNA solution were incubated for 18 h at 66°C. The filters were then washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), incubated at room temperature in

2× SSC containing 20 μg of RNase per ml for 1 h with gentle shaking, washed, resuspended in 2× SSC, and incubated at 66°C for 30 min. The filters were washed and dried, and the radioactivity was counted. Hybridizations were performed in duplicate.

Computer analyses. RNA secondary structures were predicted by using the program of Zuker and Stiegler (29) through the BIONET computer resource (Intelligenetics, Inc.). Prediction of the theoretically most stable RNA structures is based on published values of stacking and destabilizing energies as compiled by Salser (21).

RESULTS

Transcription of the *trp* operon leader region in *trans* causes constitutive expression of a chromosomal *trpE'-'lacZ* fusion. Transcription of the *trp* leader region of *B. subtilis* or *Bacillus pumilus* from a multicopy plasmid confers 5-fluorotryptophan resistance to analog-sensitive *B. subtilis* cells (15, 23). Anthranilate synthase activity is elevated in resistant cells, indicating that analog resistance is due to increased expression of the chromosomal *trp* operon (15). We assayed β-galactosidase expression from a single-copy *trpE'-'lacZ* gene fusion to verify that expression of genes controlled by the *trp* regulatory region is elevated when strains carry multiple copies of the plasmid-borne *trp* leader region (Table 1). The components of our assay system are diagrammed in Fig. 1. A single-copy *trpE'-'lacZ* translational fusion was used to assay expression of the chromosomal *trp* operon. ptrpBG3, a derivative of ptrpBG1 (23) that carries an erythromycin resistance gene rather than a chloramphenicol resistance gene (D. Henner, unpublished observations), was integrated in single-copy form into the *amyE* locus of strain BG2087 (23). In this construct, expression of β-galactosidase is controlled by the *trp* promoter, leader, and *trpE* translational signals. The resulting strain, CYBS12, has an intact *trp* operon at its normal chromosomal location and a *trpE'-'lacZ* gene fusion controlled by a second copy of the *trp* regulatory region at the *amyE* locus. When CYBS12 is grown in the presence of 50 μg of L-tryptophan per ml, β-galactosidase activity is not detectable above background (less than 0.5 Miller units). CYBS12 grown in the absence of exogenous tryptophan produces approximately 132 Miller units of β-galactosidase (Table 1).

The second component diagrammed in Fig. 1 is pMK105, a plasmid that carries the *trp* promoter and a segment of the *trp* leader region. The vector used, pMK104, is an *E. coli-B. subtilis* shuttle vector derived from pBS42 (2). pMK104 contains the origins of replication from pBR322 (4) and pUB110 (11), the polylinker segment from pUC18 (20), and the chloramphenicol acetyltransferase gene from pC194 (7).

TABLE 1. Titration of the *mtr* product by overproduced *trp* leader transcripts

Strain	Integrated plasmid	Multicopy plasmid	β-Galactosidase activity[a]	
			+Trp	−Trp
BG2087			<0.5	<0.5
CYBS12	ptrpBG3		<0.5	132
CYBS12	ptrpBG3	pMK104	<0.5	160
CYBS12	ptrpBG3	pMK105	904	1,123

[a] Expressed in Miller units (see Materials and Methods). Activity was measured for cells grown in medium containing 0.2% glucose, 0.2% acid-hydrolyzed casein, and 1× minimal salts, with 50 μg of tryptophan per ml (+Trp) or without tryptophan (−Trp).

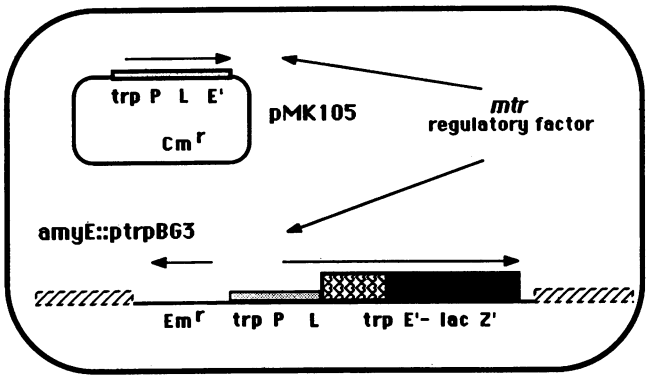


FIG. 1. Diagram of an in vivo assay for the *trp* regulatory factor target site. Components are described in Results. P, Promoter; L, leader; E', first 40 codons of *trpE*; lacZ', *lacZ* gene lacking the first 7 codons; Cm^r, chloramphenicol acetyltransferase gene from pC194 (7); Em^r, erythromycin resistance determinant from pE194 (14); amyE, *B. subtilis* amylase gene (28). One copy of ptrpBG3 is inserted in the chromosome; plasmid pMK105 is present in approximately 50 copies per cell.

The pUB110 origin dictates a copy number of about 50 DNA molecules per cell when present in *B. subtilis* (11). pMK105 contains the *trp* promoter-leader segment and a portion of *trpE*. The insert extends from the *Eco*RI site at position −412 to the *Hin*dIII site at position +318 relative to the *trp* operon transcription start site (25). When CYBS12 is transformed with pMK104, β-galactosidase activity from cells grown in the presence or absence of exogenous tryptophan is the same as in strains lacking any plasmid (Table 1). However, when CYBS12 cells contain pMK105, β-galactosidase activity is elevated independently of the presence of tryptophan in the medium. In the absence of exogenous tryptophan, β-galactosidase levels are approximately seven times higher in cells with pMK105 than in cells containing pMK104. This high level of expression is maintained when cells carrying pMK105 are grown in the presence of 50 μg of tryptophan per ml, conditions under which β-galactosidase synthesis by the control strain is undetectable. The level of β-galactosidase activity observed in cells with pMK105 may reflect *trp* operon expression approaching its maximum synthetic capacity. β-Galactosidase expression in cells with pMK105 can be compared with expression in an *mtr* mutant lacking pMK105. In the *mtr* mutant, β-galactosidase activity was intermediate (600 to 700 Miller units) (23), consistent with the conclusion that the *mtr* allele used probably is not fully constitutive (13).

Localization of the *trp* leader segment(s) required for elevation of chromosomal *trpE'-'lacZ* expression. Overproduction of the *trp* leader transcript in *trans* could cause elevated expression of the *trp* operon by sequestering a negative regulatory factor present in limiting amounts (24). We wished to define more precisely the *trp* leader segment(s) required for the observed regulatory effect. We therefore tested a number of plasmids carrying subsegments of the *trp* leader region for their abilities to elevate *trp* operon expression.

Two deletion series of the *trp* leader region were prepared and tested. Plasmids listed in Fig. 2 were mutant or deletion derivatives of pMK105. One of these plasmids, pMKdH4, was retreated with BAL 31 exonuclease to generate a second set of deletions (Fig. 3). These plasmids contained pMKdH4 subsegments adjacent to an *Eco*RI-*Kpn*I fragment (positions −412 to +29) carrying the *trp* promoter.

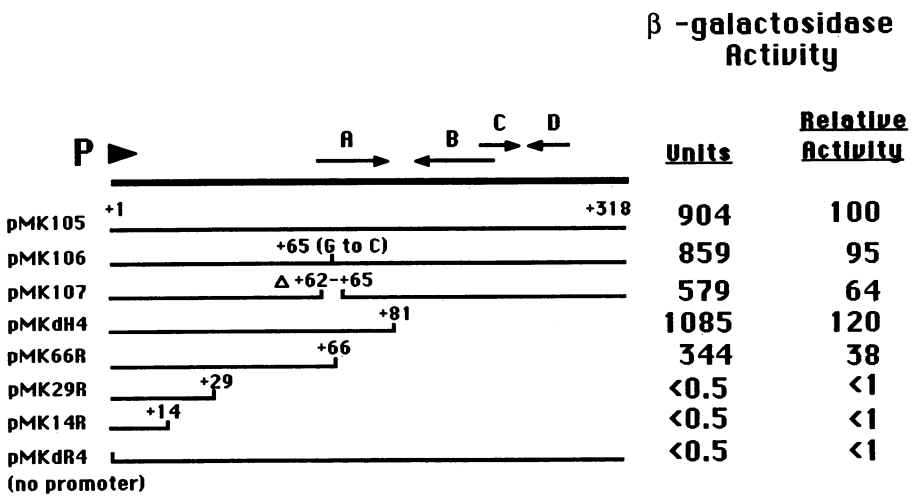


FIG. 2. Diagram of *trp* leader deletion plasmids and resultant β-galactosidase activities from CYBS12 isolates containing each plasmid, grown in media containing 50 μg of tryptophan per ml. The top line represents the *trp* leader region. Arrows represent inverted repeats that may participate in transcript secondary structures (25) (see Fig. 5). A and B, Complementary segments that may form an antiterminator; C and D, complementary segments that form a transcription terminator. Bars below represent sequences that are present in each plasmid. Units are Miller units (19). Relative activity was calculated by arbitrarily setting pMK105 activity to 100. P, Promoter.

Figure 2 lists results of β-galactosidase assays on CYBS12 strains carrying *trp* leader deletion plasmids. Cells containing these plasmids were grown in minimal medium supplemented with 0.2% acid-hydrolyzed casein and 50 μg of tryptophan per ml (see Materials and Methods). These strains were also grown in the absence of exogenous tryptophan to ensure that the *trpE'-'lacZ* gene fusion was present and functional (data not shown). We compared *trp* leader deletion plasmids with pMK105 for their abilities to elevate chromosomal *trpE'-'lacZ* expression (Fig. 2). We found that several constructs were inactive, confirming the results of Shimotsu and Henner (24) that promoter activity was required for *trp* leader segments to affect chromosomal *trp* operon expression in *trans*. The inactive plasmids were pMKdR4 (positions −2 to +318), which lacks a promoter, and pMK14R (positions −412 to +14) and pMK29R (positions −412 to +29), which contain the promoter but only a short 5′ segment of the transcribed region. The active constructs all contained the *trp* promoter and a portion of the transcribed leader region. They were (i) pMK105, containing the full *trp* leader segment; (ii) pMK106, which is identical to pMK106 except for a G→C change at position +65 that creates a *Kpn*I site (25); (iii) pMK107, which was derived from pMK106 by a 4-bp deletion of the *Kpn*I site (positions +62 to +65); (iv) pMKdH4, which contains a *trp* leader insert from positions −50 to +81; and (v) pMK66R, which contains an insert from positions −412 to +66.

Since pMKdH4 appeared to contain all of the sequences required for activation of the *trpE'-'lacZ* fusion in *trans*, we examined deletions of the pMKdH4 construct (positions −50 to +81) to localize further the subsegment that was responsible for elevation of *trpE'-'lacZ* gene expression. This second set of subsegments tested is indicated in Fig. 3. Each *Kpn*I-*Hin*dIII fragment was cloned into pMK29R so that the insert was transcribed as an RNA sequence fused to the first 29 nucleotides from the 5′ portion of the transcript. Plasmids were named according to the nucleotides deleted from the pMKdH4 transcript; e.g., pdH4Δ30-71 has residues 30 to 71 deleted. The segment from residues +72 to +81 had no detectable titration activity, but segments from residues +64 to +81, +52 to +81, and +48 to +81 all elevated chromosomal *trpE'-'lacZ* gene expression. Finally, pdH4Δ30-35, the plasmid with the largest insert (positions +36 to +81), was approximately three times as active as the plasmids with smaller inserts. This plasmid is essentially a reconstruction of pMKdH4 with the substitution of a *Kpn*I linker for the sequences from positions +30 to +35.

Sequences at the 5′ end of deletion transcripts affect their activities. We used two different *Eco*RI-*Kpn*I fragments containing the *trp* promoter in constructs designed to test titration activity of *trp* leader subsegments (Fig. 4). Each fragment extended from the *Eco*RI site at position −412 past the transcription start site (position +1). A *Kpn*I linker was ligated adjacent to bp +14 in plasmid pMK14R and to bp +29 in plasmid pMK29R. A series of *trp* leader subsegments was inserted into the *Kpn*I and *Hin*dIII sites of the polylinker such that transcription from the *trp* promoter extended through the insert to be tested, as described above. Our results are summarized in Table 2. We found that *trp* leader segments were much less active when cloned adjacent to position +14 than when joined to position +29. Neither promoter fragment alone exhibited any activity in *trans* (Fig. 2).

Comparison of transcriptional and translational fusions. All previous studies of regulation of the *trp* operon were performed by using ptrpBG1, a translational *trpE'-'lacZ* fusion (23). To determine whether there was a translational component to overall regulation of *trp* operon expression, we prepared a transcriptional fusion (see Materials and Methods) and compared it with the *trpE'-'lacZ* translational fusion. Isogenic strains carrying the translational (BG4288) and transcriptional (BG2199; see Materials and Methods) fusions were grown in the presence and absence of tryptophan, and β-galactosidase activity was determined (Table 3). There was a 14-fold difference in the induction ratios (without tryptophan/with tryptophan) for the two strains. This finding suggests that excess tryptophan causes both transcription termination in the leader region and inhibition of translation of the *trpE* coding region.

Effect of *trpE* mRNA translation inhibition on mRNA stability. The findings described in the previous section suggest that in *mtr*$^+$ cultures growing in the presence of tryptophan, translation initiation at the *trpE* ribosome-binding site may

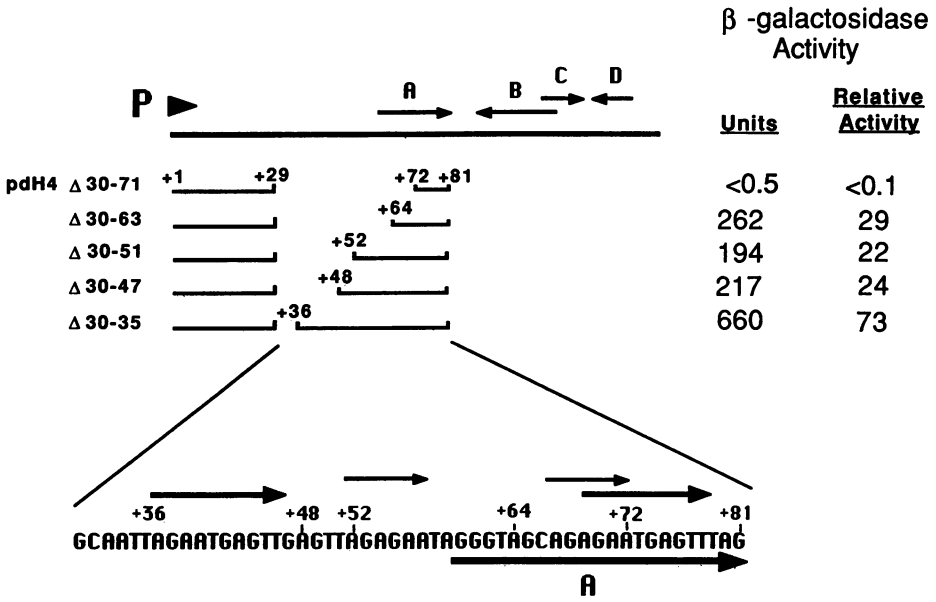


FIG. 3. Diagram of *trp* leader deletion plasmids and resultant β-galactosidase activities from CYBS12 isolates containing each plasmid, grown in media containing 50 μg of tryptophan per ml. The top line represents the *trp* leader region as described in the legend to Fig. 2. Bars below represent the *trp* leader transcript sequences present in each plasmid. Endpoints of each fragment are numbered relative to their positions in the wild-type *trp* leader transcript. A *Kpn*I linker is present between position +29 and the left end of each adjoining *trp* leader segment. The DNA sequence from positions +30 to +81 is displayed below the diagram of the set of deletions. Arrows above the DNA sequence indicate pairs of direct repeats. The large arrow below the sequence indicates the first segment of the potential antiterminator structure (25) (see Fig. 5). β-Galactosidase activity was calculated in Miller units (19). Relative activity was calculated by arbitrarily setting pMK105 activity to 100. P, Promoter.

be inhibited. In this section, we ask whether the addition of tryptophan to an *mtr*$^+$ culture causes an increased rate of *trp* mRNA decay. To measure mRNA decay conveniently, we used the *trpE'-'lacZ* translational fusion and measured *lacZ* mRNA by quantitative filter hybridization. To perform these experiments, it was desirable to devise a procedure that would prevent transcription termination in the *trp* leader region. In *mtr*$^+$ strains growing in the presence of tryptophan, most transcription of the leader region terminates at the attenuator. The strategy that we used was as follows. We used a *trpA* auxotroph that could be starved of tryptophan, thereby preventing transcription termination in the leader region. However, starved tryptophan auxotrophs barely incorporate [^{3}H]uridine into RNA. To restore labeled uridine incorporation into mRNA, we added the tryptophan analog 7-azatryptophan. This analog is incorporated into protein by *B. subtilis*, although poorly, but it does not replace tryptophan as an activator of transcription termination at the attenuator (data not shown). However, it does restore protein synthesis sufficiently well to allow incorporation of [^{3}H]uridine into RNA, thereby permitting labeling of newly synthesized RNA. The design of the experiment was to label *trpE'-'lacZ* mRNA in cells in the presence of 7-azatryptophan and then add rifampin to block initiation of transcription. Subsequently, when all transcribing polymerase molecules had moved beyond the leader region, tryptophan was either added or not added, and the decay of labeled *lacZ* mRNA was measured. As a control, an isogenic *mtr trpA* double mutant was prepared and labeled by using the same protocol. This protocol and the results of these experiments are summarized in Table 4. In a preliminary experiment, we

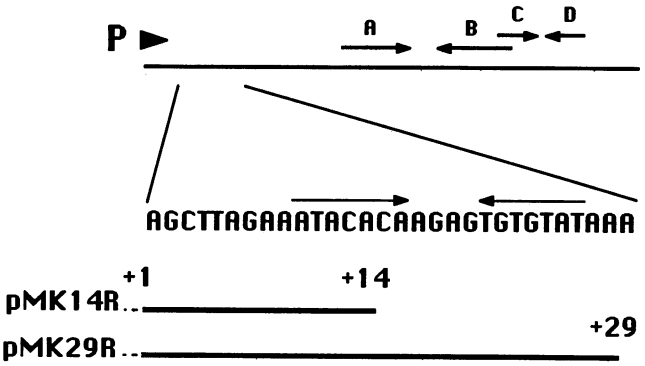


FIG. 4. DNA sequence of the *trp* leader region transcribed from pMK14R and pMK29R. The top line represents the *trp* leader region as described in the legend to Fig. 2. Arrows above the sequence indicate inverted repeats. Bars represent transcribed sequences that are present in pMK14R and pMK29R. When pMK14R or pMK29R contains a *Kpn*I-*Hin*dIII insert, transcription presumably initiates at position +1 and continues past position +14 or +29, respectively, through the *Kpn*I linker sequence into the insert to be tested. P, Promoter.

TABLE 2. Effect of 5' sequences on titration activity

Insert (residues)	β-galactosidase activity[a]	
	pMK14R	pMK29R
72–81	<0.5	<0.5
64–81	<0.5	262
52–81	9	194
48–81	93	217
36–81	199	660

[a] Expressed in Miller units (see Materials and Methods).

TABLE 3. β-Galactosidase activity of transcriptional and translational fusions

Strain	Fusion	Trp[a]	β-Galactosidase activity (ratio)[b]	
			Expt 1	Expt 2
BG2199	Translation	+	0.32 ± 0.02	0.42 ± 0.03
		−	282 ± 11 (882)	248 ± 2 (590)
BG4288	Transcriptional	+	3.0 ± 0.3	3.7 ± 0.2
		−	260 ± 11 (67)	264 ± 3 (71)

[a] With (+) or without (−) tryptophan (Trp) as a growth supplement.
[b] Cultures were grown in minimal medium supplemented with 0.05% Casamino Acids (Difco) and 50 μg of tryptophan per ml and were harvested at an optical density between 0.5 and 0.6 at 550 nm. β-Galactosidase activity was determined as described by Ferrari et al. (8). Each value is the average of duplicate or triplicate determinations. Induction ratios were determined by dividing the β-galactosidase specific activity determined for cultures grown in the absence of tryptophan by that determined for cultures grown in the presence of tryptophan. See Materials and Methods for the structures of the two types of fusions.

determined that in the absence of tryptophan there was 50% degradation of labeled *lacZ* mRNA 4.25 min after the addition of label. The addition of tryptophan to the *mtr*$^+$ strain caused a marked increase in *lacZ* mRNA decay, from 50% survival to 12% survival at the time of sampling. By contrast, the addition of tryptophan to an *mtr* mutant had no noticeable effect on *lacZ* mRNA degradation. In other experiments we showed that under the conditions we used, rifampin inhibition of transcription initiation was complete; i.e., labeled uridine added 2 min after rifampin did not label *lacZ* mRNA. We conclude from these experiments that the addition of tryptophan to *mtr*$^+$ cultures increases the rate of *trpE'-'lacZ* mRNA decay.

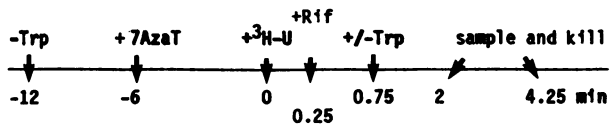
DISCUSSION

The *trp* operon of *B. subtilis* is regulated by transcription attenuation in response to changes in the intracellular level of tryptophan (25). Tryptophan presumably activates the product of the *mtr* locus, and this factor interacts either with a specific segment of the leader transcript or perhaps with the transcription complex containing that portion of the transcript. Such an interaction is believed to influence formation of the secondary structures in the nascent *trp* operon transcript that are the key participants in the attenuation decision.

TABLE 4. Effect of tryptophan on *trpE'-'lacZ* mRNA turnover in *mtr*$^+$ and *mtr* strains[a]

Strain[b]	Relevant markers	Tryptophan supplement	% of labeled RNA that is *lacZ* mRNA (% decay) at:	
			2 min	4.25 min
CYBS12A	*mtr*$^+$ *trpA*	−	0.65	0.32 (51)
		+	ND[c]	0.079 (88)
CYBS12B	*mtr trpA*	−	0.54	0.24 (56)
		+	0.51	0.20 (61)

[a] Protocol:



[b] Both strains contained the same *trpE'-'lacZ* translational fusion.
[c] Not determined. The 2-min culture without tryptophan was the reference culture.

Characterization of the regulatory target site(s) in the leader transcript. Our results confirm previous observations (15, 23) that high-level production of the *trp* leader transcript in *trans* causes appreciable deregulation of the chromosomal copy of the *trp* operon. We sought to define the segment of the leader transcript that interacts with the *mtr* gene product. Toward this end, we tested a number of plasmids carrying subsegments of the *trp* leader region for their abilities to elevate *trp* operon expression in *trans*. For example, a plasmid (pdH4Δ30-63) containing a segment from positions +64 to +81 elevated chromosomal *trpE'-'lacZ* expression about one-third as well as the plasmid containing the entire *trp* leader region, pMK105 (Fig. 2). Longer segments from positions +52 to +81 (pdH4Δ30-51) and from positions +48 to +81 (pdH4Δ30-47) were not more active than pdH4Δ30-63 (Fig. 3). Therefore, it appears that an element contained in the segment from positions +64 to +81 has significant regulatory-factor titration activity. In addition, pdH4Δ30-35 was more active than pdH4Δ30-63, pdH4Δ30-51, or pdH4Δ30-47 (Fig. 3). This finding suggests that a second site for regulatory-factor binding is present between positions +36 and +48. However, our assay is not strictly quantitative, since we cannot distinguish between improvement in regulatory-factor binding and enhancement of deletion transcript stability (see below). Different plasmid copy numbers between the various constructs is another potential complication, although we find this unlikely because all the inserts are very closely related and derived from the same parent vector. Furthermore, there was no indication of plasmid instability.

The *trp* leader transcript contains several repeated sequences within the segment from positions +36 to +81 (24). Two sets of direct repeats are indicated by arrows over the DNA sequence displayed in Fig. 3. There are five repeats of the sequence T/A GAG (and two more between positions +81 and +91). The results (Fig. 3) suggest that the repeated sequence AGAATGAGTT at positions +37 and +69 in *B. subtilis* may be most important in regulatory-factor interaction with the leader transcript. Plasmids with either copy of the repeat, such as pMK66R, pdH4Δ30-63, pdH4Δ30-51, and pdH4Δ30-47, had partial activity in our titration assay. Plasmids with both copies, such as pMKdH4 and pdH4Δ30-35, were more active.

We have compared the *B. subtilis trp* leader sequence with that of *B. pumilus* (15) because *B. pumilus* leader RNA was partially active in our titration assay (ca. 20% of pMK105; values not shown). The region from positions +36 to +81 is highly conserved (15). This includes a perfect match to the *B. subtilis* sequence TAGGGTAG at positions +58 to +65. However, we do not believe this sequence to be important because pMK106, pMK107, and pdH4Δ30-63 all contain alterations of this sequence, yet they retain titration activity. In *B. pumilus*, there are two degenerate copies of the large direct repeats of AGAATGAGTT that appear important for regulatory factor interaction in *B. subtilis*. They are AGAT GAGAA at position +47 and AGATGAGTA at position +73 of the *B. pumilus* transcript (15). The *B. pumilus* leader region also contains eight copies of the T/A GAG repeat.

The RNA segments from positions +36 to +81 in both *B. pumilus* and *B. subtilis* cannot form stable internal base-paired secondary structures. Since deletion transcripts lacking the downstream complementary segments increase expression of the chromosomal *trpE'-'lacZ* gene fusion, we believe that the postulated negative regulatory factor is interacting with single-stranded RNA rather than recognizing one of the specific RNA secondary structures that are involved in attenuation (25).

Significance of a potential RNA hairpin at the 5′ end of the leader transcript. In addition to the demonstrated activity in *trans* attributable to the *trp* leader segment from positions +36 to +81, we also found that the 5′ end of the leader transcript contributed to the elevation of chromosomal *trpE′-′lacZ* expression. When we used a *trp* promoter insert that contained only 14 bp past the transcription start site, deletion constructs were much less active than when the promoter fragment contained 29 bp past the transcription start site (Table 2). Neither of these promoter fragments alone had any activity in *trans*. The sequence from positions +10 to +27 contains a perfect inverted repeat (Fig. 4). It has been demonstrated that potential hairpin structures at the 5′ ends of some transcripts, such as *E. coli ompA* mRNA (10), contribute to stability. It is likely that the differences in activity between the two 5′ end cassettes that we used reflect such a difference in mRNA stability. However, although the region from positions +1 to +29 has no titration activity when tested alone, we have not ruled out the possibility that the activity of this sequence is dependent on the presence of additional regulatory sites within the segment from positions +36 to +81.

The *trp* leader segment defined by *trans* activity is normally a *cis*-acting regulatory site essential for transcription through the *trp* operon attenuator. Previous deletion analyses suggested that the transcript segment from positions +60 to +111 was required to allow transcription to proceed through a strong transcription terminator at positions +108 to +133 (25). This sequence could form an alternative RNA secondary structure, the antiterminator, that would compete with formation of the terminator. The two competing structures are displayed in Fig. 5. Our results suggest that the *mtr* gene product interacts with segment A of the antiterminator. We believe that interaction with the segment including residues +60 to +81 would be effectively equivalent to deleting it; i.e., the antiterminator could not form and therefore would no longer be available to compete with terminator formation. In vitro transcription experiments with purified *B. subtilis* RNA polymerase indicate that when the antiterminator is present, very little termination occurs at position +140. However, if a segment of the antiterminator is deleted, the segment containing residues +108 to +133 is an extremely efficient terminator (25). Since the *mtr* gene product was absent from these reactions, our results demonstrate that it is not essential for transcription termination. We believe that the primary role of the *mtr* gene product is to allow the formation of the RNA secondary structure that functions as a transcription terminator by blocking formation of the alternative, competing structure, the antiterminator.

Binding to the leader RNA segment from positions +36 to +81 could regulate both transcription termination in the *trp* leader region and subsequent translation of any *trpE* mRNA that is produced. Wild-type *B. subtilis* cells that contain a single-copy *trpE′-′lacZ* gene fusion or a single copy of the *trp* operon produce very low levels of β-galactosidase or anthranilate synthase activity when grown in the presence of exogenous tryptophan (Table 1) (15). There is considerable evidence that transcription attenuation is an important component of this negative regulation by tryptophan (25). However, it would be surprising if the 1,000-fold regulation observed for this operon (Table 1) were solely due to transcription termination control; regulation of transcription termination in other examples of attenuation is never more than 100-fold (16). Previous regulatory studies with the *B. subtilis trp* operon did not examine the possibility that excess tryptophan causes some additional form of regulation. Since these earlier studies used a gene fusion, the measured β-galactosidase activities could reflect translational as well as transcriptional inhibition resulting from the presence of tryptophan.

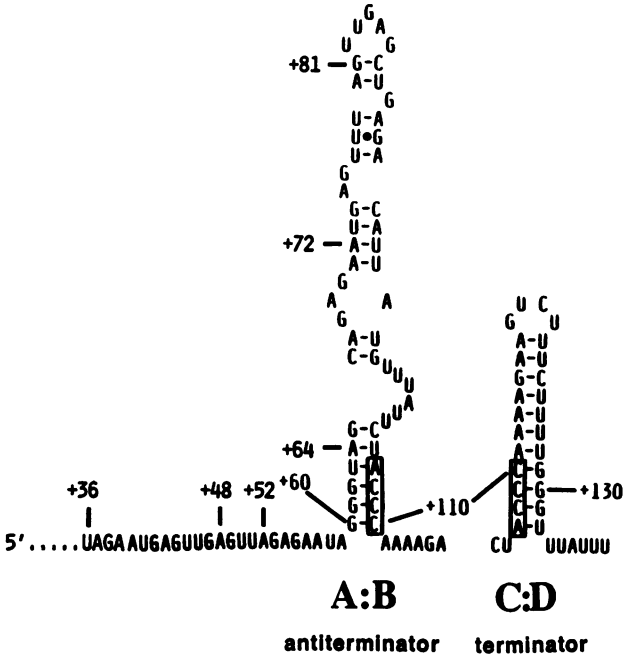


FIG. 5. Predicted transcript secondary structures involved in transcription attenuation in the *B. subtilis trp* operon (25). Numbers indicate the nucleotide positions relative to the transcription start site (position +1). A:B, Antiterminator structure formed by complementary segments A and B (see Fig. 2); C:D, terminator structure formed by complementary segments C and D. The boxed sequence 5′-ACCC-3′ at position +107 indicates the sequence overlap between the two potential structures. The structures have the following calculated free energies of formation (29): A:B, ΔG = −14.3 kcal/mol; C:D, ΔG = −15.3 kcal/mol.

Coding regions from gram-positive bacteria such as *Staphylococcus aureus* and *B. subtilis* are efficiently translated only when the translation start codon is preceded by a highly conserved ribosome-binding site (2, 17). Inducible antibiotic resistance genes, such as those responsible for erythromycin resistance (3, 12, 14) and chloramphenicol resistance (6), are regulated in these organisms by modulation of the ability of ribosomes to initiate translation. In both cases, it appears that the ribosome-binding site can be sequestered in a secondary structure.

Figure 6 shows the two most stable predicted secondary structure conformations of a mature *trp* leader transcript that has escaped premature termination at the attenuator and is allowed to refold to form an equilibrium structure. In these diagrams, we have labeled four RNA segments, A, B, C, and D (Fig. 5), previously demonstrated to form structures critical for transcription attenuation control (25). In addition, two RNA segments that are distal to the attenuator are labeled E and F. Segment F contains the ribosome-binding site for *trpE*. Segment E is complementary to both segment A (Fig. 6A, inset) and segment F (Fig. 6B, inset). The overall conformations in Fig. 6A and B are identical, except that in Fig. 6A, part of segment A (from positions +69 to +76) is base paired to segment E (from positions +174 to +182) and segment F is free, whereas in Fig. 6B, segment A is free and segment E (positions +173 to +185) is base paired to

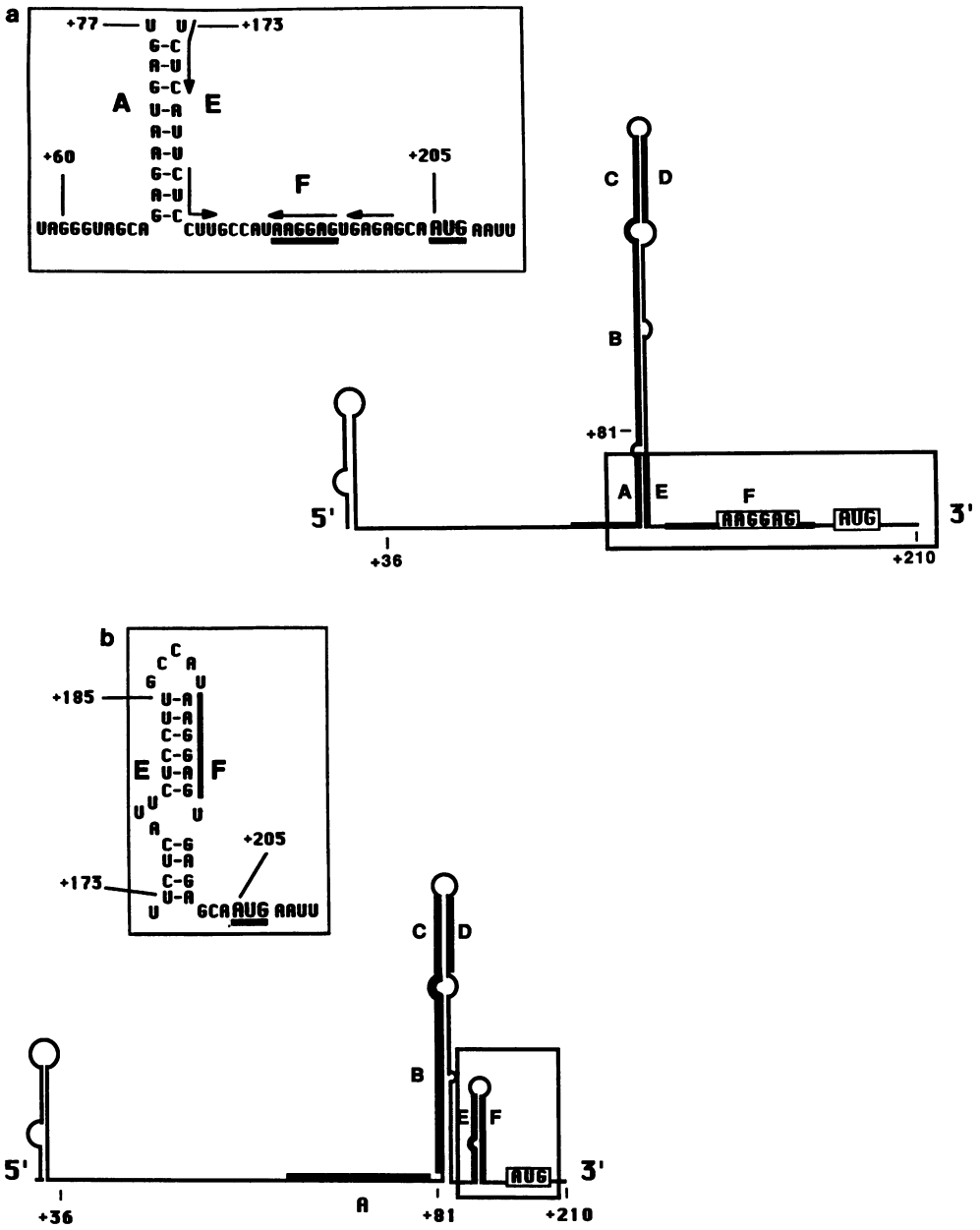


FIG. 6. (a and b) Diagrams of the two most stable equilibrium structures predicted to form in the mature transcript segment upstream of the *trpE* translation start codon. Numbers indicate the nucleotide positions relative to the 5′ end of the transcript. Segment A is the portion of the predicted antiterminator that appears to participate in regulatory-factor binding. A, B, C, and D are RNA Segments critical for attenuation control (see Fig. 5). Segment E is complementary to segment A (panel a) and segment F (panel b). Segment F contains the *trpE* ribosome-binding site. Boxed sections contain the ribosome-binding site and the translation start codon. Magnifications of the boxed regions (insets) show the nucleotide sequence of the transcript and predicted base pairing. Arrows in panel a (inset) indicate inverted repeats that form the secondary structure pictured in the panel b inset. In both insets, the ribosome-binding site 5′-AAGGAG-3′ and the *trpE* start codon are underlined. The structures have the following calculated free energies of formation (29): 5′ hairpin, $\Delta G = -11.3$ kcal/mol; large central hairpin (ABC:DE) (panel a), $\Delta G = -42.3$ kcal/mol; large central hairpin (BC:DE) (panel b), $\Delta G = -30.2$ kcal/mol; structure (E:F) that contains the ribosome-binding site (panel b), $\Delta G = -12.9$ kcal/mol. The summation of free energies of formation for each conformation: $\Delta G = -53.6$ kcal/mol (panel a), $\Delta G = -54.4$ kcal/mol (panel b).

segment F, containing the ribosome-binding site. Thus, as in the antibiotic resistance transcripts mentioned above (6, 12, 14), the *trpE* ribosome-binding site could be sequestered in a theoretically stable RNA secondary structure by base pairing to segment E. Interestingly, we have provided evidencesuggesting that the tryptophan-activated *mtr* gene product binds to a segment of the leader transcript containing a repeated sequence, 5′-AGAAUGAGUU-3′, from positions +69 to +78. Binding of the *mtr* gene product to this segment of the mature mRNA would block the conformation shown in Fig. 6A and favor the conformation shown in Fig. 6B, e.g., the conformation that blocks the *trpE* ribosome-binding site.

Our model is based on the assumption that at least some of the readthrough transcripts, which initially formed an

antiterminator, refold to equilibrium structures with the lowest free energy of formation (depicted in Fig. 6). The most stable predicted conformation for a transcript in which the antiterminator (segment A paired to segment B) persisted would sequester the *trpE* ribosome-binding site, as in Fig. 6B, and have a predicted free energy of formation of $\Delta G = -38.5$ kcal/mol (data not shown). The free energies of formation for the conformations predicted to form at equilibrium are $\Delta G = -53.6$ kcal/mol (Fig. 6A) and -54.4 kcal/mol (Fig. 6B).

We compared regulation of a transcriptional *trp-lacZ* fusion to regulation of a translational *trpE'-'lacZ* fusion as an initial test of translational control of *trpE* expression. We found that there was a greater than 10-fold difference in induction ratios with the two fusions (Table 3). This result is consistent with a model in which binding of the tryptophan activated *mtr* gene product inhibits translation initiation at the *trpE* ribosome-binding site, as predicted from the hypothetical RNA secondary structures and the relative position of the *mtr*-binding site.

Inhibition of translation initiation at the *trpE* ribosome-binding site would reduce ribosome density on *trpE* mRNA, perhaps making this segment of mRNA more labile. To examine this possibility, we compared the turnover of the *lacZ* portion of *trpE'-'lacZ* mRNA in *mtr*$^+$ and *mtr* cultures. We showed that the presence of tryptophan significantly enhanced decay of *trpE'-'lacZ* mRNA in *mtr*$^+$ strains. This finding is consistent with the assumption that ribosome density on the fusion RNA is reduced because of secondary structure blockage of the *trpE* ribosome-binding site. It is also conceivable that the enhanced mRNA decay observed in these studies is propagated along the intact *trp* mRNA so that segments distal to *trpE* mRNA also are degraded more rapidly. If this occurred, then the yield of all the polypeptides encoded in *trp* mRNA would be reduced as a consequence of tryptophan-mediated inhibition of *trpE* mRNA translation. This possibility has not been tested.

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