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Translation of *trpG* in *Bacillus subtilis* Is Regulated by the *trp* RNA-Binding Attenuation Protein (TRAP)

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***trpG* of *Bacillus subtilis* encodes a glutamine amidotransferase subunit that is involved in the synthesis of both folic acid and L-tryptophan. Expression of *trpG* is negatively regulated by tryptophan even though this gene is located within a folic acid biosynthetic operon. Examination of both transcriptional and translational gene fusions to *lacZ* involving *trpG* and direct measurements of *trpG* mRNA levels and TrpG polypeptide accumulation demonstrated that translation of *trpG* is regulated by tryptophan whereas transcription is not. These studies also show that this regulation is mediated by the *trp* RNA-binding attenuation protein. Deletion and point mutations indicated that regulation is dependent on a series of G/UAG trinucleotide repeats surrounding the putative ribosome-binding site for *trpG*. Our results are consistent with a model in which the tryptophan-activated *trp* RNA-binding attenuation protein and ribosomes compete for binding to *trpG* mRNA.**

In *Bacillus subtilis*, there are seven genes involved in tryptophan biosynthesis. Six of these are clustered in the *trpEDCFBA* operon (9), while the other, *trpG*, is located within a folate operon containing *pab*, *trpG*, *pabC*, and *sul* (26). *trpG* encodes a glutamine amidotransferase subunit which is involved in the biosynthesis of both folic acid and tryptophan (14). The *p*-aminobenzoate synthase enzyme in the folic acid synthesis pathway is composed of subunits of TrpG and Pab, whereas anthranilate synthase, which catalyzes the first step in tryptophan biosynthesis, is a complex of TrpG and TrpE polypeptides.

Expression of the *trp* operon is negatively regulated in response to tryptophan by a transcription attenuation mechanism involving the *trp* RNA-binding attenuation protein (TRAP) (4, 6, 15, 24), which is the product of the *mtrB* gene (7). Expression of *trpG* is also negatively regulated by tryptophan (13); however, it does not appear that the other genes in the folate operon are controlled by tryptophan. Slock et al. (26) proposed that translation of *trpG* is regulated in response to tryptophan. They suggested that this regulation is mediated by tryptophan-activated TRAP binding to a site in the *trpG* mRNA that overlaps the *trpG* ribosome-binding site, thus preventing ribosomes from initiating translation. In this report, we demonstrate that while TrpG polypeptide levels vary significantly in *B. subtilis* in response to the presence of TRAP, *trpG* mRNA levels vary only slightly. Furthermore, we used transcriptional and translational gene fusions between *trpG* and *lacZ* to demonstrate that tryptophan-activated TRAP does regulate translation of *trpG*.

MATERIALS AND METHODS

Bacterial strains and transformations. The bacterial strains used in this study are described in Table 1. *Escherichia coli* JM107 was used as the host for plasmid constructions, and TG1 was used for site-directed mutagenesis. *B. subtilis* BG2087 (*argC4*) and BG4233 (*argC4* Δ*mtrB*) were used as hosts for transformation and integration of gene fusions. BG4233 was created by Dennis Henner (Genentech) and contains a deletion of *mtrB*, which we have determined extends from codons 7 to 62. Measurements of *trpG* mRNA and TrpG polypeptide levels were done with *B. subtilis* 1012 (*leuA8 metB5* r_M^- m_M^-) and IBC72 (*leuA8 metB5* r_M^- m_M^- *mtrB*). The *mtrB* gene was disrupted in strain IBC72 by homologous integration of a chloramphenicol resistance marker (Fig. 1). DNA fragments containing the 5′ or 3′ end of *mtrB* were generated by PCR with oligonucleotide primers AB1 to AB4 (Table 2). The PCR product of primers AB1 and AB2 introduces a TGA stop codon after the first 19 amino acids of *mtrB* and creates an *Eco*RI site. The 3′ *mtrB* fragment was created by PCR with primers AB3 and AB4 (Table 2), which also introduced an *Eco*RI restriction site at the 5′ end of the fragment. The two PCR fragments were cloned into pBluescript SK+ (Stratagene), and then a chloramphenicol resistance cassette from pBEST401 (12) was inserted into the *Eco*RI site between the 5′ and 3′ *mtrB* fragments to create pFP2A (Fig. 1). This plasmid was linearized with *Sca*I and transformed into wild-type *B. subtilis* 1012, and chloramphenicol-resistant transformants were selected. Transformants in which the *mtrB* gene was disrupted by homologous recombination were identified by Southern blotting. All such mutants were found to result from double-crossover events and did not contain any vector sequences. The resulting strain was named IBC72.

E. coli was transformed by the calcium heat shock procedure (5), and transformants were selected on Luria-Bertani plates containing either 100 μg of ampicillin per ml or 12.5 μg of chloramphenicol per ml. *B. subtilis* was transformed by using natural competence (1) modified as described in reference 15. Transformants were selected on plates containing 0.2% acid-hydrolyzed casein, 0.2% glucose, 10 μg of L-arginine per ml, 1× minimal salts (28), 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml, and 5 μg of chloramphenicol per ml.

Plasmids and gene fusions. The plasmids used in this study are listed in Table 1. Figure 2 outlines the strategies used to create transcriptional or translational fusions between *trpG* and *lacZ* driven by either the native folate promoter or the heterologous *spac* promoter (30).

The folate operon promoter has been mapped to approximately 60 bp upstream of the translational start of *pab* with the main transcription initiation site at 33 nucleotides upstream of the ATG codon (5a). We used PCR to amplify two 1.8-kbp *Bam*HI-*Bgl*II fragments containing the promoter, *pab*, and the first 24 codons of *trpG* (Fig. 2). The product from oligonucleotides A and G was ligated into *Bam*HI-digested pDH32 (21) to create a transcriptional fusion with *lacZ*. In this fusion, a UAA stop codon was created after codon 24 of *trpG*, which is followed by 61 untranslated nucleotides and then the start codon of *lacZ*. A translational *trpG′-′lacZ* fusion was constructed with the PCR product of oligonucleotides A and F (Fig. 2). This fragment was ligated into *Bam*HI-cut pPDG120, which is similar to pDH32 but contains the polycloning site of pRS552 (25) prior to *lacZ*.

The *spac* promoter is a modified version of the *E. coli lac* promoter that functions in *B. subtilis* (30). We cloned several PCR fragments containing *trpG′* under control of this heterologous promoter in pDH88 (8). The *trpG′* PCR fragments were the products of oligonucleotides C and F (translation fusion) or C and G (transcriptional fusion) (Fig. 2). After digestion with *Hin*dIII-*Bgl*II, these fragments contain the first 25 codons of *trpG* preceded by 71 nucleotides to a naturally occurring *Hin*dIII site at position 1668 in *pab* (26). *Eco*RI-*Bgl*II fragments containing the *trpG′* fragments following the *spac* promoter were excised from pDH88, and *lacZ* fusions were constructed as described above for fusions under control of the native folate promoter.

We also constructed a translational *trpG′-′lacZ* fusion to test the effect of

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TABLE 1. Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Description or genotype	Reference or source
E. coli strains		
JM107	*supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ(*lac-proAB*)	29
TG-1	*supE hsd*Δ*5 thi*(*lac-proAB*), F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	
B. subtilis strains		
1012	*leuA8 metB5* r_M^- m_M^-	22
IBC72	*leuA8 metB5* r_M^- m_M^- *mtrB* Cm^r	This study
BG2087	*argC4*	D. Henner
BG4233	*argC4* Δ*mtrB* (deletion from positions 1001 to 1176)[a]	D. Henner
PGBS11	*argC4 amyE*::[P_{pab}-(*pab-trpG′-′lacZ*)] Cm^r translational fusion	This study
PGBS12	*argC4 amyE*::[P_{spac}-(*′pab-trpG′-′lacZ*)] Cm^r "coupled" translational fusion	This study
PGBS13	*argC4 amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r translational fusion	This study
PGBS14	*argC4 amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r (*trpG′* −36 deletion)	This study
PGBS15	*argC4 amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r (*trpG′* −20 deletion)	This study
PGBS16	*argC4 amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r (*trpG′* A-1720 → G)	This study
PGBS17	*argC4 amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r (*trpG′* G-1721 → T)	This study
PGBS18	*argC4 amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r (*trpG′* A-1722 → G)	This study
PGBS19	*argC4 amyE*::[P_{pab}-(*pab-trpG′-′lacZ*)] Cm^r transcriptional fusion	This study
PGBS20	*argC4 amyE*::[P_{spac}-*lacZ*] Cm^r	This study
PGBS21	*argC4 amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r transcriptional fusion	This study
PGBS31	*argC4* Δ*mtrB amyE*::[P_{pab}-(*pab-trpG′-′lacZ*)] Cm^r translational fusion	This study
PGBS32	*argC4* Δ*mtrB amyE*::[P_{spac}-(*′pab-trpG′-′lacZ*)] Cm^r "coupled" translational fusion	This study
PBGS33	*argC4* Δ*mtrB amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r translational fusion	This study
PGBS34	*argC4* Δ*mtrB amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r (*trpG′* −36 deletion)	This study
PBGS35	*argC4* Δ*mtrB amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r (*trpG′* −20 deletion)	This study
PBGS36	*argC4* Δ*mtrB amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r (*trpG′* A-1720 → G)	This study
PGBS37	*argC4* Δ*mtrB amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r (*trpG′* G-1721 → T)	This study
PGBS38	*argC4* Δ*mtrB amyE*::[P_{spac}-(*trpG′-′lacZ*)] Cm^r (*trpG′* A-1722 → G)	This study
PGBS39	*argC4* Δ*mtrB amyE*::[P_{pab}-(*pab-trpG′-lacZ*)] Cm^r transcriptional fusion	This study
PGBS40	*argC4* Δ*mtrB amyE*::[P_{spac}-*lacZ*] Cm^r	This study
PGBS41	*argC4* Δ*mtrB amyE*::[P_{spac}-(*trpG′-lacZ*)] Cm^r transcriptional fusion	This study
Plasmids		
pBluescript SK+	Amp^r cloning vector	
pFP2	Amp^r contains 5′ and 3′ fragments of *mtrB* cloned in pBluescript SK+	This study
pBEST401	Cm^r cassette plasmid	12
pFP2A	Amp^r Cm^r; contains Cm^r cassette from pBEST401 cloned into *Eco*RI site between 5′ and 3′ fragments of *mtrB* in pFP2	This study
pDH32	Cm^r *lacZ* transcriptional fusion vector	21
pDH88	Cm^r P_{spac} vector	8
pDG120	Cm^r *lacZ* translational fusion vector	This study
pMY11	Cm^r *pab-trpG′-′lacZ* translational fusion	This study
pMY12	Cm^r *′pab-trpG′-′lacZ* translational fusion	This study
pMY13	Cm^r *trpG′-′lacZ* translational fusion	This study
pMY14	Cm^r *trpG′-′lacZ* translational fusion (*trpG′* −36 deletion)	This study
pMY15	Cm^r *trpG′-′lacZ* translational fusion (*trpG′* −20 deletion)	This study
pMY16	Cm^r *trpG′-′lacZ* translational fusion (*trpG′* A-1720 → G)[b]	This study
pMY17	Cm^r *trpG′-′lacZ* translational fusion (*trpG′* G-1721 → T)[b]	This study
pMY18	Cm^r *trpG′-′lacZ* translational fusion (*trpG′* G-1721 → T)[b]	This study
pMY19	Cm^r *pab-trpG′-lacZ* transcriptional fusion	This study
pMY20	Cm^r P_{spac}-*lacZ* fusion	This study
pMY21	Cm^r *trpG′-lacZ* transcriptional fusion	This study

[a] Numbers refer to nucleotides as designated in reference 7.
[b] Numbers refer to nucleotides as designated in reference 26.

translating the distal portion of the preceding *pab* gene on regulation of *trpG*. The *′pab-trpG′* fragment contains an artificial ribosome-binding site created in oligonucleotide B (Fig. 2A), followed by the last 41 codons of the *pab* gene beginning at Met-508 and then the first 25 codons of *trpG*. This *Xba*I-*Bgl*II fragment was cloned into similarly digested pDH88 (8), and then a translational fusion with *lacZ* was created in pPDG120 as described above.

Ligation mixtures containing the fusion plasmids described above were cut with *Sca*I or *Pst*I and transformed directly into *B. subtilis* BG2087 (*argC4*) or BG4233 (*argC4* Δ*mtrB*). (Transforming any of the gene fusion plasmids into *E. coli* resulted in unwanted rearrangements). The gene fusions were integrated into the *amyE* locus by homologous recombination (23). Transformants were selected as chloramphenicol-resistant blue colonies on X-Gal plates and were further characterized as lacking amylase activity (23). Southern blotting with an *Nru*II-*Eco*RV fragment of *amyE* probe (23) confirmed that all of the strains carried single-copy integrations.

Cells were grown in morpholinepropanesulfonic acid (MOPS) minimal medium containing 40 mM MOPS (pH 7.0), 50 mM KCl, 0.5 mM $MgSO_4$, 4 mM Tricine, 10 mM NH_4Cl, 10 mM glucose, 1.32 mM K_2HPO_4, 10 μM $FeCl_3$, 5 μg of L-arginine per ml, and 5 μg of chloramphenicol per ml at 37°C until the optical density at 600 nm reached 0.4 to 0.6. β-Galactosidase activity was then assayed as described previously (15).

Deletions and site-directed mutagenesis. The −36 and −20 deletions were

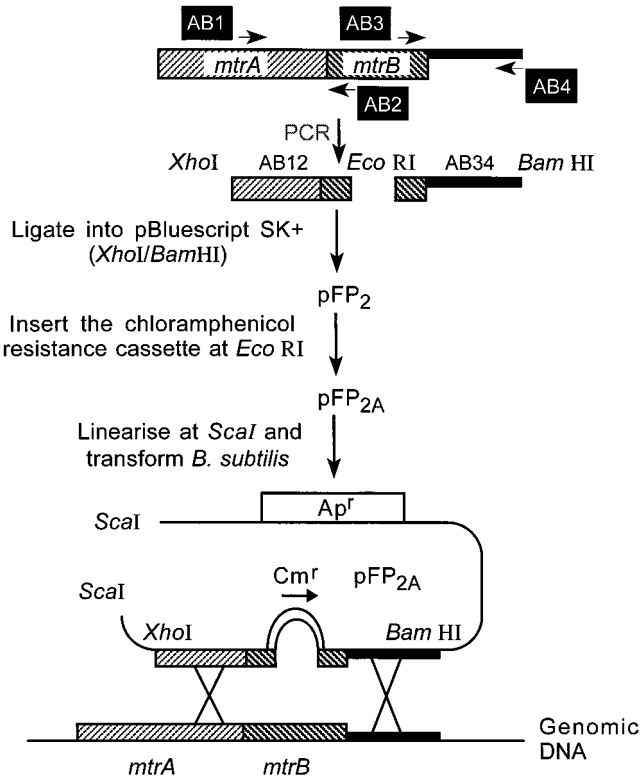

FIG. 1. *mtrB* gene disruption. Construction of plasmid pFP_{2A}. The PCR fragment generated by primers AB1 and AB2 (see Table 2) contains *Xho*I and *Eco*RI sites on the 5′ and 3′ ends, respectively, and the product of primers AB3 and AB4 contains *Eco*RI and *Bam*HI sites on its 5′ and 3′ ends, respectively. These two fragments were ligated into *Xho*I-*Bam*HI-cut pBlueScript SK+ to create pFP_2. The chloramphenicol resistance cassette from pBESTA401 (12) was then inserted into the *Eco*RI site of pFP_2 to create pFP_{2A}, which was linearized with *Sca*I and used to transform *B. subtilis*, resulting in disruption of the *mtrB* gene.

constructed by PCR with primers that bind 36 (oligonucleotide D) or 20 (oligonucleotide E) bp upstream of the ATG codon of *trpG* (see Fig. 5) together with oligonucleotide F. Point mutations at A-1720, G-1721, and A-1722 were created by site-directed mutagenesis (Amersham Sculptor kit) by using a mutagenic oligonucleotide containing random nucleotides at these three positions. Translational *trpG′-′lacZ* fusions containing these deletions and point mutations were prepared as described above.

mRNA measurements. *B. subtilis* total RNA was prepared by the phenol-grinding method of Maes and Messens (17) with the following modifications. *B. subtilis* cells were pelleted and frozen in liquid nitrogen for 5 min prior to starting the procedure, and an additional DNase treatment, followed by phenol extraction and ethanol precipitation, was included at the end of the preparation. The resulting RNA was dissolved in H_2O and stored at −70°C. We obtained approximately 1 mg of total RNA from 50 ml of culture grown to an optical density at 600 nm of 0.8.

RNase A/T1 protection was done as described by Melton et al. (18). Fifteen or 30 μg of total RNA was mixed with 75,000 cpm of a gel-purified antisense RNA probe and dried in a Speed-Vac (Savant). The dried pellets were dissolved in 20 μl of hybridization solution [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.7), 80% formamide, 0.4 M NaCl, 1 mM EDTA), heated to 90°C for 5 min, and then incubated at 45°C overnight. A 200-μl volume of an RNase A/T1 mixture (10 mM Tris-Cl [pH 7.5], 5 mM EDTA, 0.3 M NaCl, 80 μg of RNase A [Boehringer] per ml, 50 U of RNase T1 [Boehringer] per ml) was added, and the mixture was incubated at 26°C for 40 min. One microliter of 20% sodium dodecyl sulfate (SDS) and 3 μl of proteinase K (10 mg/ml) were added, and the tubes were incubated for 15 min at 37°C. A 20-μg sample of glycogen was added as a carrier, and the reaction was precipitated with ethanol. The pellet was then dissolved in formamide RNA loading dye and run on a 6% sequencing gel. The gel was fixed, dried, and exposed with a Molecular Dynamics Phosphorimager. ^{32}P-end-labelled *Hin*fI-digested ϕX174 DNA was used as size markers. The probe used was obtained by in vitro transcription of *Hin*dIII-cut pPT1 with incorporation of [α-^{32}P]UTP. Plasmid pPT1 was created by cloning a PCR fragment containing the 3′ end of *pab* (*Hin*dIII at position 1668 [26]) and the 5′ portion of *trpG*, to nucleotide 1941 (followed by a *Sac*I restriction site), into pBluescript SK^-.

Antibody preparation. TrpG and Pab fusion proteins were constructed and expressed in *E. coli* M15 by using the pDSHis6 system (27) and purified under denaturing conditions on an Ni-NTA column (Qiagen). The eluates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and the recombinant proteins were subsequently electroeluted from the gel. Purified proteins showed single bands on SDS-PAGE and were used to raise polyclonal antibodies in mice. Crude antisera were used at a dilution of 1/20,000. Western blots (immunoblots) were developed with the ECL system of Amersham.

RESULTS

Transcription of *trpG*. To examine the mechanism of regulation of *trpG*, we compared control of transcription and translation of this gene. We first examined whether transcription of *trpG* is regulated by either tryptophan or TRAP. Expression of a transcriptional fusion of the folate promoter to *lacZ* was not affected by tryptophan in either the wild type or an *mtrB* mutant strain (Table 3), indicating that the folate promoter is not regulated by tryptophan.

Expression of the transcriptional fusion was, however, approximately twofold higher in BG4233 (Δ*mtrB*) than in BG2087 ($mtrB^+$) irrespective of tryptophan (Table 3). Moreover, an RNase protection assay, using an antisense probe covering the region between the 3′ end of *pab* and the 5′ portion of *trpG*, showed that the intracellular level of *pab-trpG* mRNA is approximately 2.7-fold higher in *mtrB* mutant strain

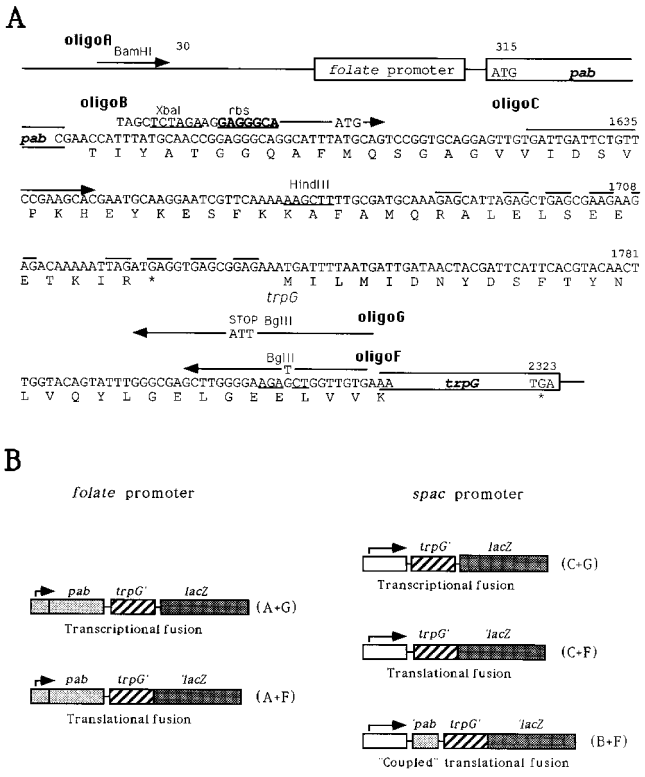

FIG. 2. (A) Regulatory regions of *trpG* used in this study. The nucleotide sequence of the 3′ end of *pab*, the intergenic region, and the 5′ portion of *trpG* is shown. The numbering conforms to that in reference 26. Oligonucleotides (oligos) used to create PCR-generated fragments for gene fusions are shown as arrows. Nucleotides altered to create restriction sites or regulatory features are indicated. Short overlines indicate the trinucleotide repeats involved in TRAP recognition. rbs, ribosome-binding site. (B) Schematic diagram of the *trpG′-lacZ* gene fusions under control of the folate and *spac* promoters. The pair of oligonucleotides (shown above) used to create the *trpG′* fragment by PCR is in parentheses to the right of each construct. Promoters are indicated by arrows.

TABLE 2. Oligonucleotides used for amplification

Oligonucleotide designation	Sequence[a]	Location and purpose[b]
A	5′-TCTCGGAtcCATCTCATC-3′	260 bases upstream of *pab* start site (position 5) to create *pab-trpG′* fragment
B	5′-tagctctagaaGGAGGGCAGGCATTT**ATG**CAG-3′	138 bases upstream of *pab* stop codon (position 1585) to create "coupled" *′pab-trpG′* fragment
C	5′-GATTGATTCTGTTCCGAAGCACG-3′	100 bases upstream of *pab* stop codon (position 1623) to create *trpG′* fragment
D	5′-GAGtctAGAAGAGACAAAAA-3′	41 bases upstream of *trpG* start codon (position 1698) to create *trpG′* −36 deletion fragment
E	5′-AAATcTAGATGAGGTGAGCG-3′	24 bases upstream of *trpG* start codon (position 1715) to create *trpG′* −20 deletion fragment
F	5′-CACAACCAGaTCTTCCCCAAGC-3′	80 bases downstream of *trpG* start codon (position 1822) to create 3′ end of *trpG′* for translational fusions
G	5′-CGATAAAGaTCT**a**CCCCAAGCTCGCC-3′	73 bases downstream of *trpG* start codon (position 1815) to create 3′ end of *trpG′* for transcriptional fusions
AB1	5′-gtcctcgAGTATTCTCCGGCTTGAATGAAG-3′	445 bases upstream of *mtrA* stop codon (position 521) to create fragment AB12
AB2	5′-gtcgaattctcaCACTCCGTCCTCAACGGC-3′	54 bases downstream of *mtrB* start codon (position 1046) to create fragment AB12
AB3	5′-caggaATTCAAACCGCGTACGGAGAAATG-3′	37 bases upstream of *mtrB* stop codon (position 1176) to create fragment AB34
AB4	5′-ctcggatCCGCTGAAATAATCGCCCGCGAG-3′	465 bases downstream of *mtrB* stop codon (position 1681) to create fragment AB34

[a] Underlined bases designate restriction sites, lowercase letters represent mismatches, and boldface bases in oligonucleotides B and G indicate the locations of the artificial *′pab* start codon and the artificial *trpG′* stop codon, respectively.
[b] The position numbers refer to nucleotides as designated in references 26 (oligonucleotides A to G) and 7 (oligonucleotides AB1 to 4).

IBC72 than in wild-type strain 1012 when the bacteria are grown in rich medium (Fig. 3, compare lanes 1 and 2 and lanes 3 and 4). These results correlate well and indicate a slight increase in transcription of *trpG* in the absence of functional TRAP. There is, however, no tryptophan-specific control of *trpG* transcription.

Translational control of *trpG*. In contrast to the results seen with the transcriptional fusion, expression of the translational *trpG′-′lacZ* fusion was regulated by tryptophan, but only in an *mtrB*$^+$ strain. β-Galactosidase expression from the *trpG′-′lacZ* fusion in BG2087 (*mtrB*$^+$) was sevenfold lower in the presence of tryptophan (8 U) than in cells grown in the absence of tryptophan (56 U) (Table 3). Expression of this fusion was not affected by tryptophan in Δ*mtrB* strain BG4233 (Table 3). These results suggest that translation of *trpG* is regulated by tryptophan and that this regulation depends on TRAP.

The level of expression from the *trpG′-′lacZ* fusion in BG4233 (Δ*mtrB*), in either the absence or the presence of tryptophan (≈250 U), is approximately fivefold higher than that seen in wild-type strain BG2087 grown in the absence of added tryptophan (56 U). This difference presumably reflects the intracellular levels of tryptophan present in BG2087, which is *trp*$^+$, when these cells are grown in the absence of exogenous tryptophan. Comparing the values seen in the absence of any regulation in BG4233 (Δ*mtrB*; 250 U) with that obtained in the wild-type strain in the presence of tryptophan (8 U) indicates that tryptophan-activated TRAP regulates translation of *trpG* approximately 35-fold, although half of this regulation may reflect changes in transcription, as shown above.

We also examined the steady-state TrpG and Pab polypeptide levels directly by using polyclonal antibodies and Western blotting. We compared the levels of TrpG and Pab present in wild-type *B. subtilis* 1012 and *mtrB* mutant *B. subtilis* IBC72 grown in rich medium. TrpG polypeptide (21.7 kDa) was detectable only in *mtrB* mutant strain IBC72 and was not seen in wild-type strain 1012 grown under these conditions (Fig. 4B). In contrast, disruption of *mtrB* did not affect the translation of *pab*; similar amounts of the Pab polypeptide were observed for both strains (Fig. 4A). These striking results show that translation of *trpG* is regulated by TRAP, whereas that of the preceding *pab* gene is not.

***cis*-acting regulatory sequences.** To further characterize the *cis*-acting sequences involved in translational control of *trpG*, we created *trpG′-′lacZ* gene fusions under control of the heterologous *spac* promoter (Fig. 2) (30). Since our *B. subtilis*

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

Promoter and type of fusion	L-Tryptophan[a]	β-Galactosidase activity (U)[b]	
		BG4233 (Δ*mtrB*)	BG2087 (*mtrB*$^+$)
Folate			
Transcriptional	−	66 ± 9	26 ± 5
	+	56 ± 1	26 ± 4
Translational	−	277 ± 19	56 ± 2
	+	250 ± 19	8 ± 3
spac			
Transcriptional	−	143 ± 18	133 ± 7
	+	132 ± 6	124 ± 15
Translational	−	371 ± 18	176 ± 18
	+	362 ± 13	63 ± 6
Translational ("coupled")	−	355 ± 23	166 ± 15
	+	361 ± 20	54 ± 4

[a] With (+) or without (−) 50 μg of L-tryptophan per ml as a growth supplement.
[b] Each value is the average (± the standard deviation) of two different experiments, each done in triplicate.

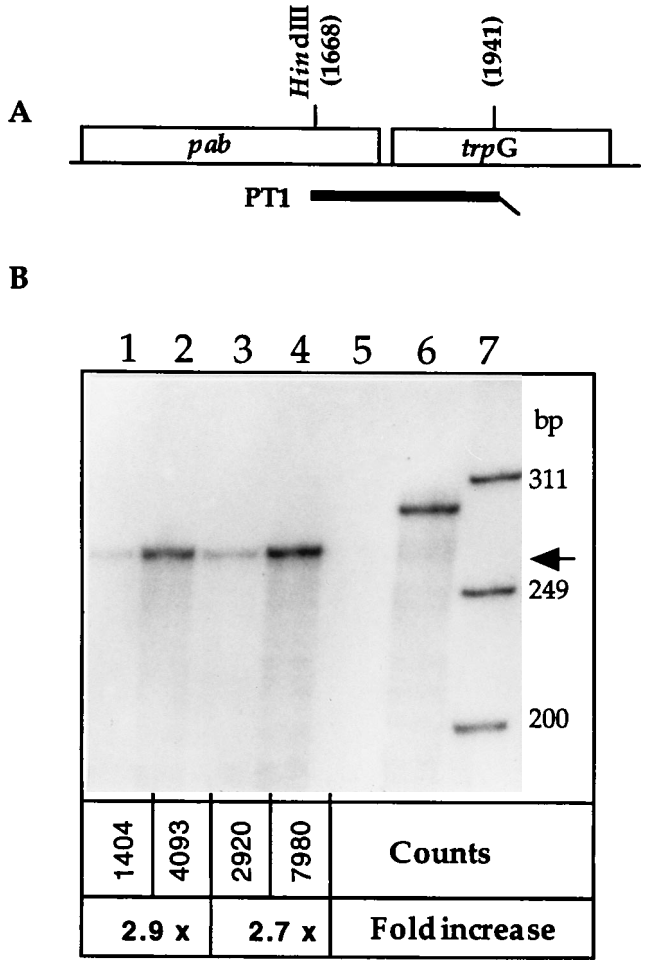

FIG. 3. RNase mapping of *pab-trpG* mRNA. (A) Partial restriction map of the folate operon. The black bar (PT1) indicates the antisense RNA probe used. The line extending the probe represents the pBluescript sequence transcribed from pPT1, which is not homologous with the *trpG* gene. PT1 is 298 nucleotides long, while the sequence homologous to the folate operon is 280 nucleotides long. (B) Phosphorimager scan from the RNase mapping experiment. Lane 7 contained the ^{32}P-labelled DNA marker. The antisense probe annealed in the presence of 20 μg of yeast tRNA and treated with RNase A/T1 mix (lane 5, showing no protected fragment) and the antisense probe alone (lane 6) are also shown. The arrow indicates the position of the transcript obtained when the PT1 probe was annealed with 15 or 30 μg of total RNA (lanes 1 and 2 and 3 and 4, respectively). RNA was prepared from wild-type strain *B. subtilis* 1012 (lanes 1 and 3) and from *mtrB* mutant strain IBC72 (lanes 2 and 4). Quantification of the signals was performed with a Phosphorimager.

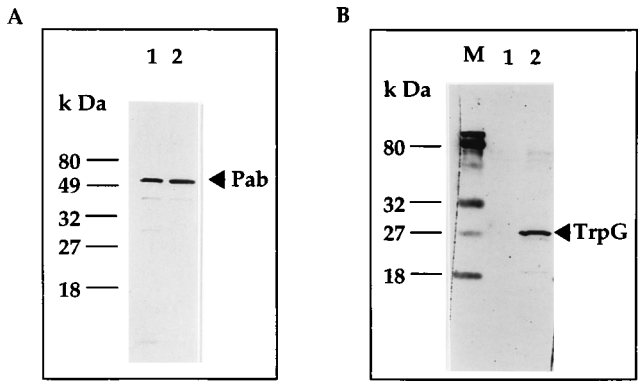

FIG. 4. Comparison of expression of *pab* and *trpG* in 1012 and IBC72. Protein extracts were separated by SDS-PAGE (16% acrylamide) and transferred to a nitrocellulose membrane, and proteins were visualized by using antisera against Pab (A) and TrpG (B). Each lane contained 20 μg of total protein. Lanes: 1, *B. subtilis* 1012; 2, *B. subtilis* IBC72. Sizes of molecular weight markers (in lane M) are indicated to the left of each panel.

strains do not carry the *lac* repressor, transcription from this promoter is not regulated by lactose or isopropyl-β-D-thiogalactopyranoside (IPTG). As expected, a transcriptional *lacZ* fusion containing the first 24 codons of *trpG* driven by the *spac* promoter was not regulated by either tryptophan or TRAP (Table 3). However, the translational *trpG'-'lacZ* fusion driven by the *spac* promoter was regulated by tryptophan in the presence of wild-type TRAP (Table 3). In this case, expression of the *trpG'-'lacZ* fusion was threefold lower in the presence of tryptophan (63 U) than in its absence (176 U). This amount of regulation by tryptophan was somewhat less than the sevenfold seen under control of the folate promoter described above. We believe that TRAP may be limiting when the translational fusion is expressed from the *spac* promoter because of higher levels of *trpG'-'lacZ* mRNA from this promoter (Table 3, compare β-galactosidase activities of the transcriptional fusions in BG2087 under control of the folate promoter [26 U] with that under control of the *spac* promoter [124 U]).

Slock et al. (26) first proposed that translational control of *trpG* is mediated by TRAP because of the presence of a nine-base sequence (AGAUGAGGU) that overlaps the putative ribosome-binding site of *trpG* (Fig. 5). This sequence is very similar to a previously proposed TRAP-binding site present twice in the leader region of the *trp* operons from both *B. subtilis* (15) and *B. pumilus* (16). Recent studies, however, suggest that TRAP recognizes a series of 11 repeated G/UAG elements in the *trp* leader RNA (2, 3). Babitzke et al. (3) pointed out eight or nine similar elements surrounding the start of *trpG* (Fig. 5) and showed that TRAP binds to a segment of the *trpG* transcript that includes the ribosome-binding site.

The previously mentioned *trpG'-'lacZ* fusions begin 72 residues upstream of the *trpG* AUG start codon and contain all of these potential regulatory elements (Fig. 2). To test which sequences are required for translational control of *trpG*, we made several deletions and site-directed mutations and examined their effects on regulation of translational *trpG'-'lacZ* fusions driven by the *spac* promoter. Deleting to 36 bp prior to the *trpG* start codon removes three GAG repeats but does not alter the region surrounding the ribosome-binding site (Fig. 5). Tryptophan regulation of this fusion was only 50% (1.5-fold) of that seen with the full-length construct (Fig. 5). A deletion that maintains the previously proposed nine-base element but leaves only four G/UAGs eliminated tryptophan regulation entirely (Fig. 5). Moreover, several point mutations that alter the regulatory region proposed by Slock et al. (26) but do not affect the proposed ribosome-binding site have little or no effect on tryptophan regulation (Fig. 5, A-1720 to G, G-1721 to T, and A-1722 to G). Although the A-1722-to-G change lowered expression of the fusion approximately 40%, the regulation by tryptophan was still about threefold.

The UGA stop codon of *pab* overlaps the putative ribosome-binding site for *trpG* (Fig. 2), suggesting that translational coupling could be involved in regulation of *trpG*. However, we found nearly identical expression and regulation of *trpG'-'lacZ* translational fusions whether or not the final 41 amino acids of *pab* were translated (Table 3), indicating that translational coupling does not play a role in regulating *trpG*.

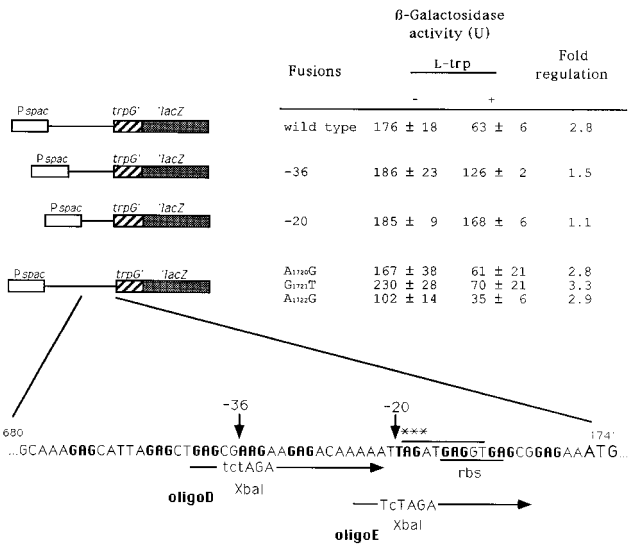

FIG. 5. Examination of *cis*-acting sites involved in translational regulation of *trpG*. The deletion and point mutants examined are schematically represented. The nucleotide sequence of the regulatory region is shown. The trinucleotide (G/UAG) repeats are in boldface. The previously proposed nine-base TRAP-binding site (26) is identified by a line above the sequence, and the putative ribosome-binding site (rbs) is underlined. Oligonucleotides D and E, used to generate deletions by PCR, are indicated, as are the nucleotide changes used to create *Xba*I restriction sites. The residues altered by point mutations are indicated by asterisks. β-Galactosidase activities of translation fusions with *lacZ* grown in the absence or presence of added tryptophan are shown. Fold regulation is defined as the value obtained without tryptophan divided by that obtained in the presence of 50 μg of L-tryptophan (L-trp) per ml.

DISCUSSION

Kane first showed in 1977 (13) that expression of *trpG* (then designated *trpX*) is regulated by tryptophan. Later, Slock et al. (26) determined that *trpG* is located within an operon involved in folic acid biosynthesis and pointed out that it did not seem logical for tryptophan to control transcription of this entire operon. They proposed a possible explanation for this apparent paradox by suggesting that translation of *trpG* could be controlled by tryptophan. This hypothesis was supported by the presence of a sequence (AGAUGAGGU) with homology to a previously proposed TRAP-binding site in the *trp* leader transcript (15) that overlaps the proposed *trpG* ribosome-binding site. Tryptophan-activated TRAP binds to the *trp* leader transcript (20) to cause transcription attenuation (4); therefore, Slock et al. (26) proposed that tryptophan-activated TRAP regulates translation of *trpG* by binding to its mRNA and preventing ribosomes from initiating translation. This mechanism is similar to that by which RegA regulates translation of several genes in bacteriophage T4 (19).

Our results demonstrate that translation of *trpG* is regulated by tryptophan and that this regulation is mediated by TRAP. In cells grown in rich medium, steady-state levels of *trpG* mRNA are only slightly affected by the presence of TRAP whereas accumulation of TrpG polypeptide is strongly inhibited by TRAP. Furthermore, translational gene fusions between *trpG* and *lacZ* under control of either the native folate promoter or the heterologous *spac* promoter are regulated by tryptophan in wild-type strains but not in *mtrB* mutant strains. In contrast, transcription from either promoter was not affected by tryptophan.

Both direct mRNA measurements and examination of transcriptional fusions of the folate promoter (including *pab* and *trpG'*) to *lacZ* indicated that transcription of *trpG* is 2- to 2.9-fold higher in the absence of TRAP (Fig. 3 and Table 3). In both folate biosynthesis and tryptophan biosynthesis, chorismate is an intermediate. In the *mtrB* mutant strain, the *trp* operon is constitutively overexpressed (10, 24). We therefore suspect that these results reflect induction of the folate promoter in response to decreased availability of chorismate rather than a direct effect of TRAP.

The TRAP-binding site in the *pab-trpG* mRNA overlaps the last 13 codons of *pab*, and one might suspect that TRAP binding would inhibit the completion of translation of *pab*. However, it does not appear either that the amount of Pab is decreased or that the size of the Pab polypeptide is affected in the wild-type strain compared with that in the *mtrB* mutant (Fig. 4), suggesting that TRAP binding affects translation of *trpG* but not that of *pab*.

Slock et al. (26) proposed that TRAP down-regulates translation of *trpG* on the basis of the presence of an apparent TRAP-binding site overlapping the *trpG* ribosome-binding site. This 9- or 10-base sequence is present twice in the leader regions of the *trp* operon in both *B. subtilis* (15) and *B. pumilus* (16) and was proposed to be important for attenuation control of these operons by TRAP (15). TRAP has been shown to bind specifically to the *trp* leader RNA from either *Bacillus* species (11, 20); however, recent studies (2, 3) have shown that TRAP recognition involves a series of 11 small GAG or UAG repeats separated by two or three residues. Babitzke et al. (3) pointed out the presence of seven GAG repeats, one UAG repeat, and one AAG repeat near the start of translation of *trpG*, several of which overlap the ribosome-binding site. Furthermore, they showed that tryptophan-activated TRAP binds to a segment of the *trpG* transcript that includes both proposed TRAP recognition elements. Our results indicate that regulation of translation of *trpG* depends on the trinucleotide repeats proposed by Babitzke et al. (3) rather than the nine-base element proposed by Slock et al. (26).

There are several unusual features of the proposed TRAP-binding site in *trpG* compared with those in the *trp* leaders of *B. subtilis* and *B. pumilus*. Both the *B. pumilus trp* leader and the *trpG* transcript contain an AAG as part of the binding site. In vivo competition studies have shown that the first position of the trinucleotide repeat is significantly less important for TRAP recognition than the last two positions (2). Therefore, it is not surprising that an AAG can be accommodated as part of the binding site. In both *trp* leader sequences, the G/UAG repeats are always separated by two or three "spacer" residues, whose sequence is not conserved. In *trpG*, six of the spacer regions contain two residues but one set of repeats is separated by eight residues and another set is separated by five (although this set could be reinterpreted to be separated by three on one side and four on the other by using an alternative UAG as the repeat; see Fig. 5). Our studies (2) have shown that the G/UAG repeats must be separated by at least two residues; spacings of no or one residue are not recognized, but we have not investigated the effects of longer spacers on TRAP binding. RNA footprint analysis of a truncated *trp* leader transcript (3) showed that several of the spacer residues were hypersensitive to nuclease or chemical cleavage in the presence of TRAP, suggesting that these regions are looped out in the TRAP-RNA complex. This suggests that larger spacers between repeats could be accommodated by looping out. However, it should be pointed out that in the footprinting experiments these hypersensitive residues were always in spacer elements containing two residues. Residues in spacer elements with three nucleotides were generally not hypersensitive, so there is

no clear relationship between the length of the spacer and its being looped out.

The three-dimensional structure of TRAP has recently been solved (2) and reveals that TRAP consists of 11 identical subunits arranged in a ring or "doughnut-like" structure. The finding that the TRAP-binding site consists of 11 trinucleotide repeats has lead us to propose a model in which each TRAP subunit, or combination of adjacent subunits, interacts with each G/UAG in the RNA. This suggests that in the case of *trpG*, nine TRAP subunits would interact with the repeats present in the mRNA, leaving two free subunits. The unusually long 8-nucleotide spacer region between repeats 5 and 6 in the *trpG* regulatory region may mean that in this case one subunit is "skipped" when TRAP interacts with *trpG* mRNA. Both we (2) and Babitzke et al. (3) have shown that TRAP interacts well with RNAs containing nine properly spaced repeats. The use of 9 rather than 11 repeats, as well as the unusual spacing between repeats, may moderate TRAP binding so as to allow for proper regulation of *trpG* translation by tryptophan but still allow enough TrpG to be produced for *p*-aminobenzoate synthase to produce folic acid.

TRAP has been shown to be quite a versatile gene regulator. It regulates transcription attenuation by binding to a site in the *trp* leader transcript and altering the secondary structure of the leader RNA to produce a transcription terminator (4, 6, 15, 20, 24). TRAP has also been shown to regulate translation of two *trp* genes, apparently by different mechanisms. For *trpE*, the first structural gene of the *trp* operon, TRAP binding to transcripts that have escaped attenuation has been proposed to result in formation of another secondary structure that sequesters the ribosome-binding site (15). We have shown that TRAP also regulates the translation of *trpG*. While we have not demonstrated the mechanism for the regulation of *trpG*, our data are consistent with a model in which tryptophan-activated TRAP and ribosomes compete directly for binding to *trpG* mRNA.

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