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Novel Form of Transcription Attenuation Regulates Expression of the *Bacillus subtilis* Tryptophan Operon

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Transcription of the *trp* operon of *Bacillus subtilis* is regulated in response to the availability of tryptophan. The first structural gene of the operon is preceded by a 204-base-pair transcribed leader region that contains a segment with the features of a procaryotic termination site. Transcription of the leader region was analyzed in vivo and in vitro to determine whether this putative termination site was used to regulate operon expression. When RNA was isolated from wild-type cells grown in the presence of excess tryptophan, transcripts of the operon ended at the putative termination site. In contrast, RNA isolated from cells grown in the absence of tryptophan or from a mutant strain which is constitutive for *trp* operon expression contained *trp* transcripts that extended beyond the termination site into the structural genes. To assess termination quantitatively in vivo, a *trpE-lacZ* fusion was constructed in which the *trp* promoter and leader region controls hybrid β-galactosidase formation. The effects on hybrid β-galactosidase levels of point mutations and deletions introduced into this leader region were determined. The results obtained establish that transcription of the *trp* operon structural genes is regulated in the leader region. This regulation appears to be mediated by the formation of alternative secondary structures of the leader transcript. In vitro transcription studies with wild-type and mutant templates provided additional evidence that the identified alternative RNA secondary structures regulate transcription termination. We hypothesize that binding of a tryptophan-activated regulatory protein to a specific segment of the nascent leader transcript prevents formation of one of the alternative secondary structures, thereby directing RNA polymerase to terminate transcription.

Bacillus subtilis is a gram-positive bacterium that has been studied extensively as an example of an organism with a simple developmental pathway, i.e., sporulation (18, 26). Perhaps because of this emphasis, little has been learned about regulation of vegetative genes in this organism. Genetic and biochemical studies with *B. subtilis* have established that a single operon encodes polypeptides that catalyze the terminal sequence of reactions in tryptophan biosynthesis (7, 37). We felt that the *trp* operon in *B. subtilis* would be an ideal model system with which to study gene regulation in this gram-positive organism. Accordingly, we isolated overlapping clones of the *trp* operon of *B. subtilis* and determined the nucleotide sequence of the entire operon (4, 12). The *trp* promoter was localized by deletion analysis and S1-mapping experiments; the start of transcription was 204 base pairs before *trpE*, the first structural gene (32, this study). The leader region of the operon contains a region of dyad symmetry, followed by a T-rich segment. These features resemble those of ρ-independent transcription termination sites of *E. coli* and are reminiscent of the termination site involved in transcription attenuation in the *E. coli trp* operon (16, 17). However, there is no homology between the *trp* leader region of *B. subtilis* and that of *E. coli* nor is there a coding region for a Trp-rich leader peptide that could modulate termination translationally, as occurs in *E. coli* (25, 40, 46).

The introduction into *B. subtilis* of a high-copy plasmid containing the *B. subtilis trp* promoter and leader region results in derepression of the chromosomal *trp* operon, presumably by sequestering a regulatory protein present in limiting amounts (32). Analysis of promoter and leader region deletions and point mutations led to the hypothesis that the presumed regulatory protein binds to the nascent transcript and that this binding results in transcription termination (32). According to this hypothesis, the leader region of the *B. subtilis trp* operon should contain a transcription termination site which is functional in the presence of tryptophan but not in its absence. Short terminated transcripts should be produced when expression of the *trp* operon is turned off by the presence of excess tryptophan. In this paper we demonstrate the existence of the expected terminated transcripts. We also provide in vivo and in vitro evidence for the existence of alternative secondary structures in the nascent leader transcript: a termination structure and an antitermination structure. We have analyzed the functions of these presumed structures in regulating transcription termination both in vitro with purified RNA polymerase and in vivo with a gene fusion to the *E. coli lacZ* gene.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* MM294 (F⁻ *supE44 endA1 thi-1 hsdR4*) was used as a host for plasmid constructions (3). The *B. subtilis* strains used in this study are listed in Table 1. Plasmids ptrpIFId1 and its deletion derivatives ptrpdH1, ptrpdH2, ptrpdH3, and ptrpdR4 (32), pJH101-TrpE2 (4), pUC18 (24), and pSP64 (21) were used in construction of the derivative plasmids described here.

In vitro transcription with SP6 RNA polymerase. *Eco*RI-*Hin*dIII fragments containing various regions of the *trp* leader region were derived from plasmids ptrpIFId1, ptrpdH1, ptrpdH2, ptrpdH3, and ptrpdR4. These fragments were cloned into the *Eco*RI and *Hin*dIII sites of plasmid pSP64. SP6 RNA polymerase (6) was obtained from Promega Biotec. pSP64-derived plasmids containing the *trp* leader region were cleaved with *Eco*RI. The linear DNA

TABLE 1. *B. subtilis* strains

Strain	Genotype	Source
W168	Prototroph	J. A. Hoch
I168	*trpC2*	J. A. Hoch
1A72	*mtr-264*	BGSC[a]
BG2143	Δ(*trpEp-trpE'*)*1*[b]	D. J. Henner
BG2199	*amyE*::[*trpEp* Φ(*trpE'-lacZ'*) Cm^r]	This study
BG2200	*amyE*::[*trpEp* (Kpn)[c] Φ(*trpE'-lacZ'*) Cm^r]	This study
BG2201	*amyE*::[*trpEp* (Kpn-C)[d] Φ(*trpE'-lacZ'*) Cm^r]	This study
BG2202	*amyE*::[*trpEp* Δ(nucleotide 65–147) Φ(*trpE'-lacZ'*) Cm^r]	This study
BG2203	*amyE*::[*trpEp* Δ(nucleotide 29–95) Φ(*trpE'-lacZ'*) Cm^r]	This study

[a] *Bacillus* Genetic Stock Center, Ohio State University, Columbus, Ohio.
[b] This strain has a chromosomal deletion of the *trp* promoter and a part of the *trpE* gene region (*Hae*III-*Hin*dIII region in Fig. 1) (D. Henner, unpublished data).
[c] The Kpn derivative of *trpEp* changes nucleotide 65 from G to C.
[d] The Kpn-C derivative of *trpEp* changes nucleotide 65 from G to C and nucleotide 106 from C to G.

templates were transcribed in the presence of [α-^{32}P]CTP (Amersham Corp.) and SP6 RNA polymerase, as described previously (21). After RNA synthesis, the DNA template was digested with DNase I (final concentration, 40 U/ml; Cooper Biomedical, Inc.) in the presence of vanadyl ribonucleoside complex (final concentration, 2 mM, Bethesda Research Laboratories, Inc.). After a 30-min incubation at 37°C, the reaction was extracted with phenol and chloroform and precipitated by the addition of 2.5 volumes of ethanol. The RNA probes were stored in 70% ethanol and precipitated immediately before use.

Preparation of RNA. *B. subtilis* W168 or 1A72 cells were grown in 5 ml of L broth at 37°C. At an optical density at 600 nm of approximately 1.0, the culture was poured into a hot phenol mixture containing 5 ml of phenol and 2 ml of 1 M NaCl–0.25 M Tris hydrochloride (pH 7.5)–20 mM EDTA–0.4% sodium dodecyl sulfate. The mixture was incubated at 90°C for 10 min with shaking. After centrifugation, the aqueous phase was collected and RNA was precipitated by adding 3 volumes of ethanol. Ethanol precipitation was repeated, and the precipitate was washed twice with 70% ethanol.

RNA from *B. subtilis* cells grown in minimal-CH medium (30) was prepared as follows. *B. subtilis* W168 or I168 cells were grown in 100 ml of minimal-CH medium supplemented with 50 μg of Trp per ml. At an optical density at 600 nm of approximately 0.5, cells were harvested and washed with minimal-CH medium. The cells were suspended in 50 ml of minimal-CH medium in the presence or absence of 50 μg of Trp per ml and grown at 37°C for 7 min for W168 and 15 min for I168 and harvested by centrifugation (3 min with an SS34 rotor, Sorvall RC-5B). The cells were suspended in 5 ml of cold TE buffer (10 mM Tris hydrochloride [pH 7.5]–0.1 mM EDTA) and poured into a hot phenol mixture containing 5 ml of RNA extraction buffer (0.5 M NaCl, 10 mM EDTA, 125 mM Tris hydrochloride [pH 7.5], 0.2% sodium dodecyl sulfate), 5 ml of chloroform, and 5 ml of phenol. The mixture was incubated at 75°C for 10 min with shaking. After centrifugation, RNA was precipitated as described above.

Solution hybridization and RNA mapping. A schematic representation of the RNA mapping experiment is shown in Fig. 2. An [α-^{32}P]CTP-labeled RNA probe and 100 μg of cellular RNA were dissolved in 40 μl of hybridization buffer {80% formamide, 20 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid] [pH 6.4], 400 mM NaCl, 1 mM EDTA}. The mixture was heated at 90°C for 10 min and incubated at 40°C for 3 h. After addition of 200 μl of H_2O and 200 μl of 2× S1 buffer (60 mM sodium acetate [pH 4.8], 100 mM NaCl, 2 mM $ZnCl_2$, 10% glycerol), the reaction mixture was treated with 5,000 U of S1 nuclease (Bethesda Research Laboratories) for 1 h at 37°C. The reaction was terminated by phenol extraction. The reaction products were precipitated with ethanol. The precipitate was dissolved in 20 μl of the loading dye mix (80% formamide, 1 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol), heated at 90°C for 2 min, and electrophoresed on a 5% polyacrylamide–8 M urea gel.

S1 mapping experiment. The restriction fragment containing the *trp* promoter and leader region was obtained from plasmid ptrpIFId1 (32). After digesting ptrpIFId1 with *Xmn*I, the 5' end was labeled with polynucleotide kinase in the presence of [γ-^{32}P]ATP (19) and cleaved with *Eco*RI. The *trp* promoter and leader fragment (the region from −48 to +120) which had been labeled with ^{32}P at the 5' end of the *Xmn*I site was isolated by polyacrylamide gel electrophoresis and used as a hybridization probe. This fragment and 20 μg of the bacterial RNA were hybridized and digested with S1 nuclease by the method of Aiba et al. (1) and electrophoresed on a 7% polyacrylamide–8 M urea gel. Size standards were created with G- and C-specific cleavage reactions for the same labeled fragment (20).

β-Galactosidase assays. *B. subtilis* cells containing the *trpE-lacZ* fusion were grown in minimal-CH medium (30) containing 0.05% Casamino Acids (Difco Laboratories), 0.5% glucose, and minimal salts (34) in the presence or absence of 50 μg of Trp per ml and supplemented with 5 μg of chloramphenicol per ml. Cells were harvested at an optical density at 600 nm of 0.5 by centrifugation, frozen, and stored at −20°C (44). The cells were thawed and assayed for the specific activity of β-galactosidase by the method of Miller (22).

In vitro transcription with *B. subtilis* RNA polymerase. *Eco*RI-*Hin*dIII restriction fragments containing the *trp* promoter and leader region of *B. subtilis* were used as templates. The restriction fragments were derived from pJH101-TrpE2 (4) or the *trp* leader deletion derivatives of pUCKpnI described in this paper. [α-^{32}P]GTP was purchased from Amersham Corp. *B. subtilis* vegetative type (σ43) RNA polymerase was the kind gift of J. Helmann and M. Chamberlin. Reactions were carried out for 30 min at 30°C in 120 mM KCl–4 mM $MgCl_2$–40 mM Tris hydrochloride (pH 8.0)–10 mM β-mercaptoethanol–4 mM spermidine–2.5% glycerol–2.7 mM ATP–1.1 mM GTP–6 μCi of [α-^{32}P]GTP–1.4 mM UTP–0.7 mM CTP–10 to 20 nM restriction fragment–0.64 μg of RNA polymerase holoenzyme. The final volume of each reaction mixture was 10 μl. Reactions were stopped by addition of an equal volume of 0.025% xylene cyanol–0.025% bromphenol blue–0.1% sodium dodecyl sulfate–14 M urea. Each reaction mixture was electrophoresed on a 6% polyacrylamide–7 M urea gel.

DNA manipulations. Plasmids were isolated from *E. coli* by the cleared lysate procedure and purified on Bio-Gel A50 columns (10), or small-scale preparations of plasmids were isolated as described by Birnboim and Doly (5). DNA sequencing was conducted by the dideoxy chain termination method of Sanger et al. (31). Certain DNA regions on the plasmids were sequenced by the supercoil sequencing method (9). The *Eco*RI-*Hin*dIII fragment from pJH101-TrpE2 was cloned into the *Eco*RI and *Hin*dIII sites of pUC18 (24) vector, and this plasmid was designated pUCtrpE2.

Oligonucleotide-directed mutagenesis was performed by the published procedures (43) to create a *Kpn*I site in the pUCtrpE2 plasmid. Oligonucleotides (*Kpn*I primer, 5'-CATTCTCTGGTACCCTATT-3', and *Kpn*I-C primer, 5'-CTTCTTTTGGGTACAATAAACATAATGTC-3') were provided by the Genentech organic synthesis group. BAL 31 exonuclease (Bethesda Research Laboratories) deletions were constructed as described previously (16). DNA blot hybridization analysis was performed by the method of Southern (33).

Bacterial transformations. *E. coli* transformations were performed by the calcium shock procedure (11). Transformants were selected on L-broth plates supplemented with ampicillin at 50 μg/ml or chloramphenicol at 12.5 μg/ml. *B. subtilis* was transformed by a published procedure (2), except that the cells were diluted 2-fold in the stage II medium instead of 10-fold. Transformants were selected on tryptose blood agar base (Difco Laboratories) plates supplemented with chloramphenicol at 5 μg/ml.

RESULTS

In vivo RNA mapping. The nucleotide sequence of the *B. subtilis* promoter region is shown in Fig. 1A. The promoter was defined by BAL 31 deletion analysis and analysis of mutations in the promoter region (32). The transcription start point for this promoter was defined by S1 analysis with a DNA fragment labeled at the *Xmn*I site at +120 (Fig. 1B). Between the transcription start site and the *trpE* structural gene, there is a 204-base-pair leader region that contains a segment of dyad symmetry, followed by a T-rich segment (shown as arrows C and D in Fig. 1A). Transcripts of this region could form the type of secondary structure that is typical of procaryotic termination structures (29).

In vivo RNA mapping experiments were performed to determine whether this structure functions as a termination site. A schematic representation of these experiments is shown in Fig. 2. Two antisense ^{32}P-labeled RNA probes were used. The first, designated probe a, extends from −48 to +323. The second, probe b, extends from −48 to +182. When RNA from *B. subtilis* W168 was used in the RNA mapping experiment, two primary RNA species were observed of approximately 130 nucleotides and 180 nucleotides in length (Fig. 3, lanes 1 and 2). These bands were observed with both probes. When the RNA was derived from *B. subtilis* 1A72, a methyltryptophan-resistant (*mtr*) strain which constitutively synthesizes the enzymes of the *trp* operon (14, 15), three major RNA species were detected with probe a, approximately 320, 180, and 130 nucleotides in length (Fig. 3, lane 3). When probe b was used, only the 180- and 130-nucleotide species were detected (Fig. 3, lane 4).

To ensure that these bands were specific transcripts from the *trp* promoter region, a similar experiment was performed with RNA from strain BG2143 (Table 1). This strain has a chromosomal deletion of the entire *Hae*III-*Hin*dIII fragment used to make the probe. With this RNA, no bands were detected on the gel (data not shown). The 320-nucleotide transcript detected with probe a is the expected size for a transcript extending from the *trp* promoter to the *Hin*dIII site at +323 (Fig. 3, lane 3). As expected, this transcript is truncated to 180 nucleotides when probe b is used (Fig. 3, lane 4). The 180- and 130-nucleotide transcripts are potential candidates for terminated transcripts.

Fine-structure mapping of the *trp* transcripts. To determine more precisely the origin of the 180- and 130-nucleotide transcripts detected in the above experiments, RNA probes complementary to different segments of the leader region were used. RNA probes which are complementary to the regions −48 to +323 (probe a), −48 to +182 (probe b), −48 to +144 (probe c), −48 to +112 (probe d), and −2 to +323 (probe e) were prepared with the SP6 transcription system (Fig. 4). Each probe was hybridized to total cellular RNA from strain W168 grown in L broth, and the RNA-RNA complexes were treated with S1 nuclease. When RNA probes a, b, and c were used for hybridization, both the 180- and 130-nucleotide transcripts were observed (Fig. 4, lanes a, b, and c). These results indicate that neither the 180- nor the 130-nucleotide transcript extends past position +144. When RNA probe d was used, both the 180- and the 130-nucleotide transcripts were truncated to approximately the same extent (Fig. 4, lane d). This finding suggests that both the 180- and the 130-nucleotide transcripts terminate at the same position, near the end of the termination structure. When RNA probe e was used, the 180-nucleotide transcript disappeared; it presumably comigrated with the 130-nucleotide transcript. These results indicate that the 130-nucleotide transcript must originate at the previously characterized initiation site and terminate with the termination structure, while the 180-nucleotide transcript must initiate at an upstream promoter and terminate with the same termination structure (Fig. 4).

Transcripts detected during tryptophan limitation. The previous results indicate that in wild-type cells grown in L broth, transcription initiates from the previously defined *trp* promoter and from an unidentified promoter upstream. Both of these transcripts terminate near the end of the termination structure in the *trp* leader region. In 1A72, a strain that synthesizes the *trp* biosynthetic enzymes constitutively, some of the transcripts extend past the terminator into the *trp* structural genes (Fig. 3, lane 3). Similar RNA mapping experiments were performed with RNA extracted from strains grown under conditions of tryptophan limitation. When the RNA was extracted from either W168 or I168 (*trpC2*) grown in minimal-CH medium plus tryptophan, the 130- and 180-nucleotide transcripts were detected (Fig. 5, lanes 2 and 4). When the RNA was extracted from W168 grown in the absence of tryptophan or from I168 starved for tryptophan, the readthrough transcript of 320 nucleotides was also present (Fig. 5, lanes 1 and 3). An additional band of 370 nucleotides is also apparent in lane 3. This is the expected size for a readthrough transcript from the upstream promoter which produces the 180-nucleotide terminated transcript. It is difficult to be certain of the amount of the 370-nucleotide transcript, as this is the size of the probe and any undigested probe will also appear to detect a 370-nucleotide transcript.

These results establish that transcription occurs from the *trp* promoter in L broth or minimal-CH media in the presence or absence of tryptophan; i.e., it is a constitutive promoter. However, only during conditions of tryptophan limitation or in a constitutive strain do these transcripts extend into the *trp* structural genes (320-nucleotide transcript) (Fig. 3, lane 3 and Fig. 5, lanes 1 and 3).

Construction of a *trpE-lacZ* fusion. To analyze the mechanism of this regulated termination event, we constructed a *trpE-lacZ* fusion in which the *trp* promoter and leader region directs hybrid β-galactosidase formation. This fusion contains the *trp* promoter and leader region and the first 40 codons of *trpE* fused in the correct reading frame to codon 8 of *lacZ* (8). Since in previous studies it had been shown that the *trp* promoter was not regulated properly when placed on a high-copy plasmid (32), we integrated this *trpE-lacZ* fusion into the chromosome as a single copy. Thus, only two copies

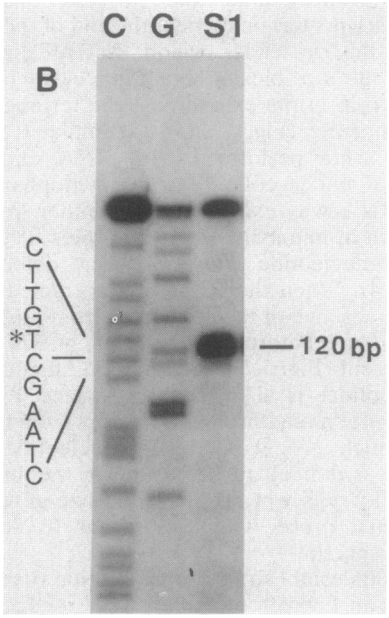

FIG. 1. DNA sequence of the *trp* leader region. (A) The DNA sequence is numbered relative to the mRNA start site. The sequences constituting the −10 and −35 region of the *trp* promoter are underlined. The termination structure (C-D) and the antitermination structure (A-B) discussed in the text are indicated by arrows. (B) For determination of the transcription start site by S1 analysis, the [^{32}P]DNA probe labeled at its 5′ *Xmn*I end was hybridized to total cellular RNAs and treated with S1 nuclease. The G- or C-specific degradation reactions (20) with the same DNA fragment provides size markers. The sequence (nontranscribed strand) surrounding the transcription initiation site is also shown. The A or G residues located at positions +1 and +2 were identified as the transcription start sites.

of the *trp* promoter region were present, one in the *trp* operon and the other preceding the *trpE-lacZ* fusion. Use of the vector to integrate the *trpE-lacZ* fusion will be described elsewhere (H. Shimotsu and D. J. Henner, Gene, in press); briefly it entails introducing the *trpE-lacZ* fusion along with a chloramphenicol resistance determinant into the middle of the previously cloned *B. subtilis* amylase gene (39). Transformation with a linearized plasmid results in the integration of a single copy of the chloramphenicol resistance determinant and the *trpE-lacZ* fusion. All analyzed transformants were examined by DNA blot hybridization to ensure that a single copy of the *trpE-lacZ* fusion was inserted into the chromosome (data not shown). A strain (BG2199) carrying the *trpE-lacZ* fusion was grown in minimal-CH medium in the presence or absence of exogenous tryptophan, and the levels of β-galactosidase were measured. In the minimal-CH medium lacking tryptophan, there were 90 U of β-galactosidase activity, while in the presence of tryptophan, there was less than 1 U. These analyses establish that the β-galactosidase levels vary in response to the presence of tryptophan in the culture medium.

Site-directed mutagenesis and deletion of a potential antitermination structure. Studies of the *E. coli trp* operon leader region have shown that formation of alternative RNA secondary structures directs transcribing RNA polymerase either to terminate transcription in the leader region or to

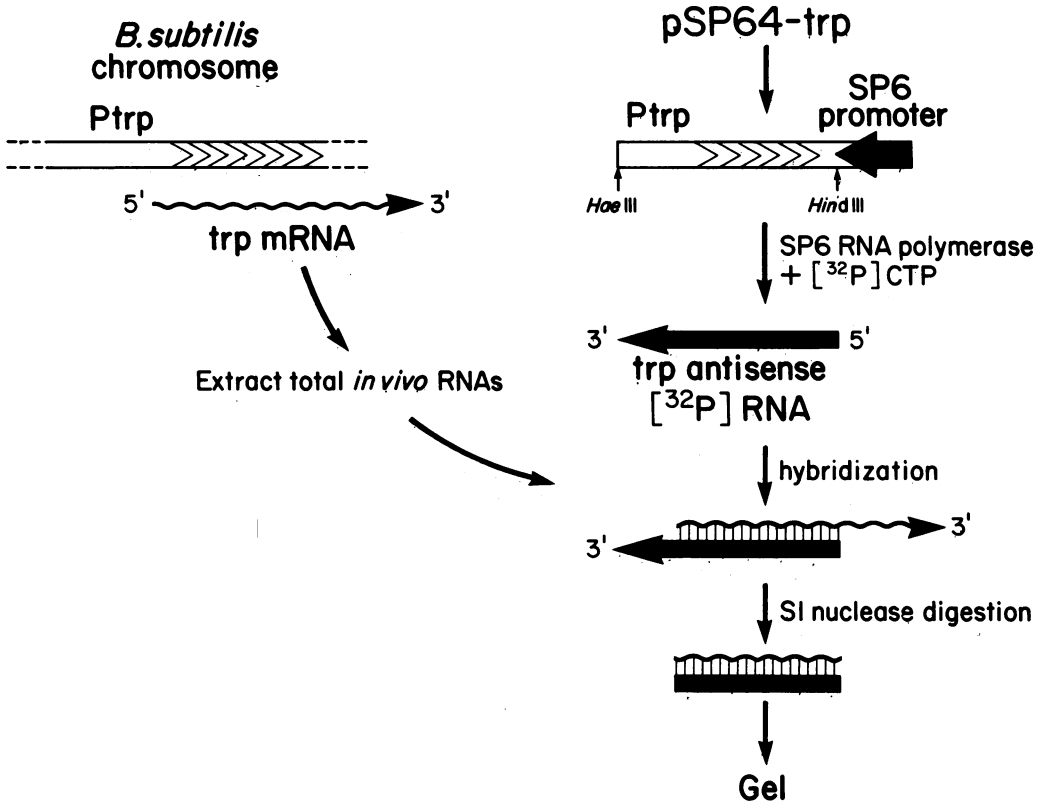

FIG. 2. Schematic representation of the RNA mapping experiment. Experimental procedures are detailed in Materials and Methods. Ptrp, trp promoter (*trpEp*).

read through into the structural genes (25, 40). In the transcript of the *B. subtilis trp* leader region, there also are presumptive alternative secondary structures (Fig. 6). Segments A and B could base pair to form one stable structure, while segments C and D could form an alternative secondary structure (Fig. 6). However, since segments B and C have a four-base overlap, prior formation of structure A-B could transiently prevent formation of structure C-D. Since structure C-D has been shown to function as a terminator, structure A-B could act as an antiterminator.

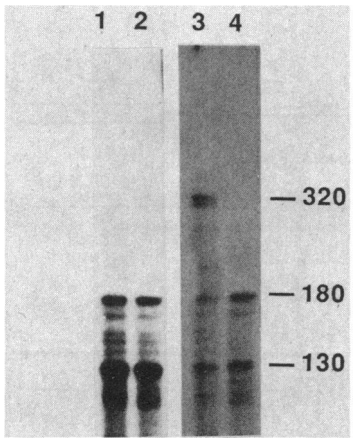

FIG. 3. In vivo RNA mapping. Probe a extends from −48 to +323; probe b extends from −48 to +182. RNAs generated by the SP6 system were used as size markers; these ranged from 170 to 370 nucleotides (not shown). Lanes: 1, RNA from W168 hybridized to the RNA probe a; 2, RNA from W168 hybridized to the RNA probe b; 3, RNA from 1A72 (*mtr*) hybridized to the RNA probe a; 4, RNA from 1A72 (*mtr*) hybridized to the RNA probe b.

To test whether these structures play a role in regulation of the *trp* operon, we produced a series of deletions that removed key segments of the leader region. To provide a convenient site from which to create these deletions, a unique *Kpn*I site was introduced into segment A by site-directed mutagenesis (G → C at position 65 in Fig. 6B); this plasmid was designated pUCKpnI. Deletions were then generated by cleavage with *Kpn*I, followed by digestion with BAL 31 exonuclease. After the BAL 31 digestion, the *Kpn*I site was restored by ligation of a *Kpn*I linker (sequence 5′ GGGTACCC 3′) to the deletion endpoints. Restriction analysis was used to select plasmids with deletions of the desired lengths, and the leader regions were sequenced to determine the exact deletion endpoints. Both unidirectional and bidirectional deletions were generated from the *Kpn*I site by ligating altered restriction fragments into the appropriately digested parental plasmid pUCKpnI. Two deletions were chosen for introduction into the *trpE-lacZ* fusion. Deletion Δ(nucleotide 65–147) removed the entire termination structure, while deletion Δ(nucleotide 29–95) removed segments A and B of the antitermination structure. The endpoints of these deletions are indicated in Fig. 6A. Each deletion derivative was used to replace the wild-type *trp* promoter and leader fragment of the *trpE-lacZ* fusion plasmid and was transformed into *B. subtilis*.

The strain containing the Δ(nucleotide 65–147) derivative, which lacked the termination structure, showed constitutive production of β-galactosidase. Growth in minimal-CH medium lacking tryptophan gave only a slight increase in the

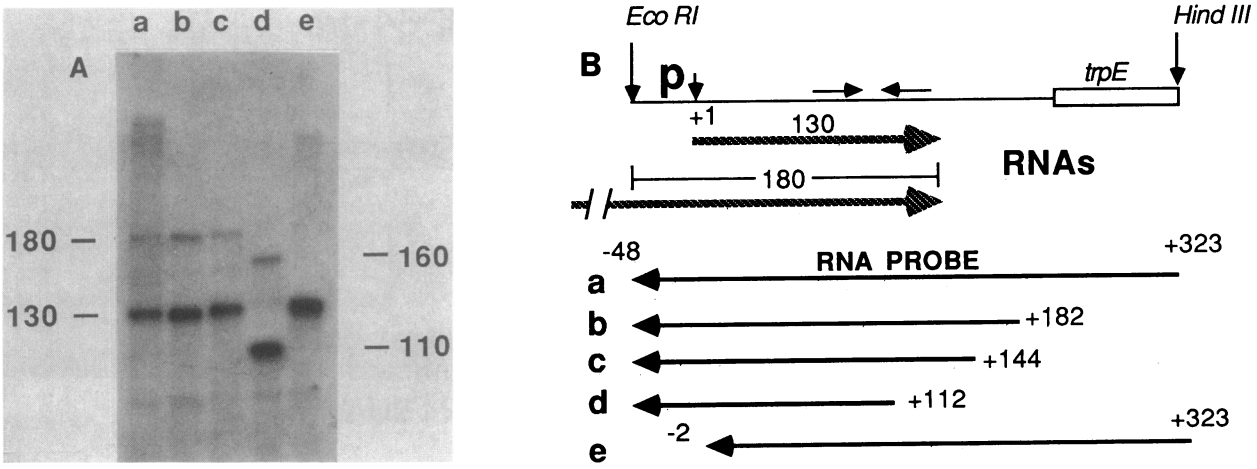

FIG. 4. Fine-structure RNA mapping. (A) In vivo RNA from strain W168 grown in L broth was used for this experiment. Lanes a to e correspond to probes a to e. Estimated sizes are given in nucleotides at the left and the right. (B) The five RNA probes used for the experiments shown in lanes a to e are shown in the lower portion of the figure. An interpretation of the origin of the transcripts detected is shown by the rightward heavy checkered arrows. P, *trp* promoter; thin arrow, termination structure; open box, *trpE* coding region.

β-galactosidase level (Table 2). The strain containing the Δ(nucleotide 29–95) derivative (Fig. 6A), which lacks the antitermination structure, showed no detectable β-galactosidase activity above the background level obtained with a strain that does not carry the β-galactosidase gene (Table 2).

Introduction of the *Kpn*I site into the leader region lowers the predicted stability of the presumed antitermination structure from −14.3 kcal (−59.8 kJ)/mol to −12.4 kcal (−51.9 kJ)/mol (36, 45). This reduction in stability could facilitate formation of the termination structure. This "*Kpn*" fragment was introduced into the *trpE-lacZ* fusion plasmid and integrated into the *B. subtilis* chromosome. When this strain (BG2200) was grown in minimal-CH medium lacking tryptophan, β-galactosidase activity decreased threefold (Table 2). To restore the stability of the antitermination structure, an additional base change was introduced in segment B (C → G at positions 106, Fig. 6B); this mutation was designatd "Kpn-C." When it was introduced into the *trpE-lacZ* fusion plasmid and the fusion was integrated into the chromosome and examined, the mutation restored wild-type levels of β-galactosidase activity in minimal-CH medium (Table 2). Unexpectedly, this Kpn-C mutant was partially constitutive for β-galactosidase production, even in the presence of exogenous tryptophan (Table 2).

In vitro transcription of wild-type and mutant *trp* leader DNA templates. To examine the roles of the putative antitermination and termination structures more directly, we compared the transcripts produced in vitro when restriction fragments containing the wild-type or deletion derivatives were used as templates. When *Eco*RI-*Hin*dIII restriction fragments from the wild type were transcribed with *B. subtilis* σ43 RNA polymerase, two RNA bands were observed (Fig. 7A, lane 1). A prominent RNA species (approximately 320 nucleotides long) appeared at the position expected for transcription from the *trp* promoter to the end of the fragment. A faint band also appeared, at the position

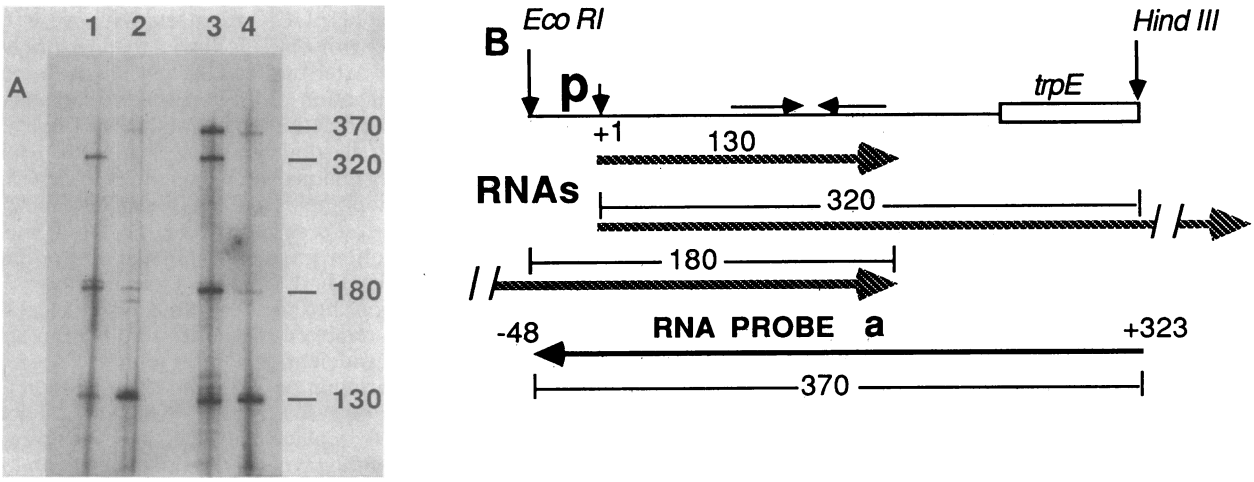

FIG. 5. Detection of transcripts from cultures limited for tryptophan. (A) All RNAs were hybridized to the RNA probe a. Lanes: 1, RNA from strain W168 grown in the absence of tryptophan; 2, RNA from W168 grown in the presence of tryptophan; 3, RNA from strain I168 (*trpC2*) starved for tryptophan; 4, RNA from I168 grown in the presence of tryptophan. Estimated sizes are given in nucleotides at the right. (B) Diagram of the RNA probe (leftward arrow) and the interpretation of the origin of the transcripts detected (heavy checkered rightward arrows). Symbols are as defined in the legend to Fig. 4.

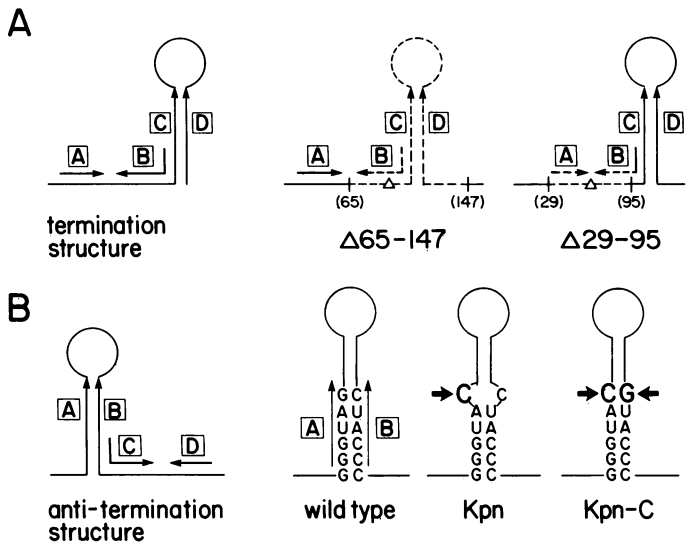

FIG. 6. Schematic representation of deletion and single-base mutants of the *trp* leader. (A) Termination structure along with the structures of deletion mutants Δ(nucleotide 65–147) and Δ(nucleotide 29–95). Deleted regions are indicated by broken lines and triangles. Numbers in parentheses indicate the start and end points of the deletions. (B) Antitermination structure along with the sequence of the wild type, Kpn, and Kpn-C. The single-base changes introduced by oligonucleotide-directed mutagenesis are indicated by arrows. Boxed letters indicate segments.

corresponding to a transcript approximately 140 nucleotides long. Analysis of a complete RNase T_1 digest of these transcripts was consistent with an interpretation that both transcripts originated at the determined transcription start site; the 320-nucleotide transcript was a runoff transcript to the end of the template, while the 140-nucleotide transcript ended near the 3′ end of the termination structure. The apparent 10-nucleotide difference between the in vivo and in vitro terminated transcripts is probably not significant. No size standards were used in common in the two experiments.

We compared the transcripts produced in vitro from Kpn and seven deletion templates (Fig. 7A). Endpoints of deletions are shown in Fig. 7B. Note that the sizes of terminated and readthrough transcripts varied in accordance with the extent of the deletion in each template. The relative molar ratio of terminated transcript to readthrough transcript was determined for each template and is expressed as percent termination in Fig. 7B. Small deletions that removed sequences 5′ or 3′ of RNA segment A increased termination at the hairpin structure [templates Δ(nucleotide 65–95), and Δ(nucleotide 29–65); Fig. 7, lanes 3 and 6), while larger rightward deletions Δ(nucleotide 65–124) and Δ(nucleotide 65–147) abolished termination (Fig. 7, lanes 4 and 5). The bidirectional deletion Δ(nucleotide 29–95) resulted in complete termination (Fig. 7, lane 7). Transcripts from deletion templates Δ(nucleotide 29–124) and Δ(nucleotide 29–147) extended from the promoter to the end of the restriction fragment (Fig. 7, lanes 8 and 9). These data show that the leader region contains sequences that can either promote or prevent transcription termination in vitro at position 140. The segments responsible for these events have been localized to those corresponding to RNA secondary structures A-B and C-D.

TABLE 2. β-Galactosidase levels in strains with leader deletions and base changes

Strain	Promoter derivatives	β-Galactosidase level (Miller units) for growth medium[a]	
		min-CH	min-CH + Trp
BG2199	Wild type	90	<1
BG2202	Δ(nucleotide 65–147)	121	96
BG2203	Δ(nucleotide 29–95)	<1	<1
BG2200	Kpn	34	<1
BG2201	Kpn-C	115	17
W168	No integrant	<1	<1

[a] Miller units are from reference 22. Data shown are the average of three independent assays. Cultures were grown in minimal CH medium (min-CH) or the same medium supplemented with 50 μg of Trp per ml (min-CH + Trp) and assayed as described in Materials and Methods. The activity assayed is that of the product of a *trpE-lacZ* fusion.

DISCUSSION

Regulated termination model. Our current model of regulation of transcription of the *B. subtilis trp* operon is presented schematically in Fig. 8. We propose that transcription of the *trp* operon is regulated by formation of alternative RNA secondary structures in the leader transcript, a termination structure, C-D, and an antitermination structure, A-B. In the presence of tryptophan, the terminator forms and causes transcription termination, while in the absence of tryptophan, the antitermination structure forms, allowing transcriptional readthrough beyond the termination site. We postulate that a *trp* regulatory protein, activated by tryptophan facilitates formation of the terminator by binding to segment A of leader mRNA, an RNA segment that is part of the antiterminator. When cells are starved for tryptophan, the inactive *trp* regulatory protein cannot bind to segment A, and therefore antitermination structure A-B forms. This allows RNA polymerase to continue transcription into the structural genes of the operon. In the following sections we will discuss findings which support this model.

In vivo RNA analyses. An RNA probe complementary to *trp* leader mRNA (antisense RNA) was used to identify in vivo transcripts initiated at the *trp* promoter. Two short transcripts were detected in total cellular RNA of wild-type *B. subtilis* cells grown in the presence of excess tryptophan. The origin of these transcripts was demonstrated with RNA probes complementary to different segments of the leader region. The 130-nucleotide transcript originated at the previously defined *trp* promoter, while the 180-nucleotide transcript originated at an undefined upstream promoter. Both transcripts terminated at approximately +130 with the RNA termination structure C-D. An alternative explanation for the existence of these short transcripts is that they are stable degradation products of rapidly degraded larger transcripts. However, our in vitro evidence that RNA segments C and D form a very effective terminator [in the absence of the antitermination structure, i.e., the Δ(nucleotide 29–95) template] makes this interpretation unlikely. The existence of these short transcripts shows unequivocally that the *trp* promoter is an active site of transcription initiation in the presence or absence of tryptophan. When tryptophan is present, the wild-type strain does not show transcription past the termination structure. In the absence of tryptophan or in a constitutive strain, transcripts are detected which extend past the termination structure into the structural genes. These findings indicate that expression of the *B. subtilis trp* operon is controlled by regulated transcription termination.

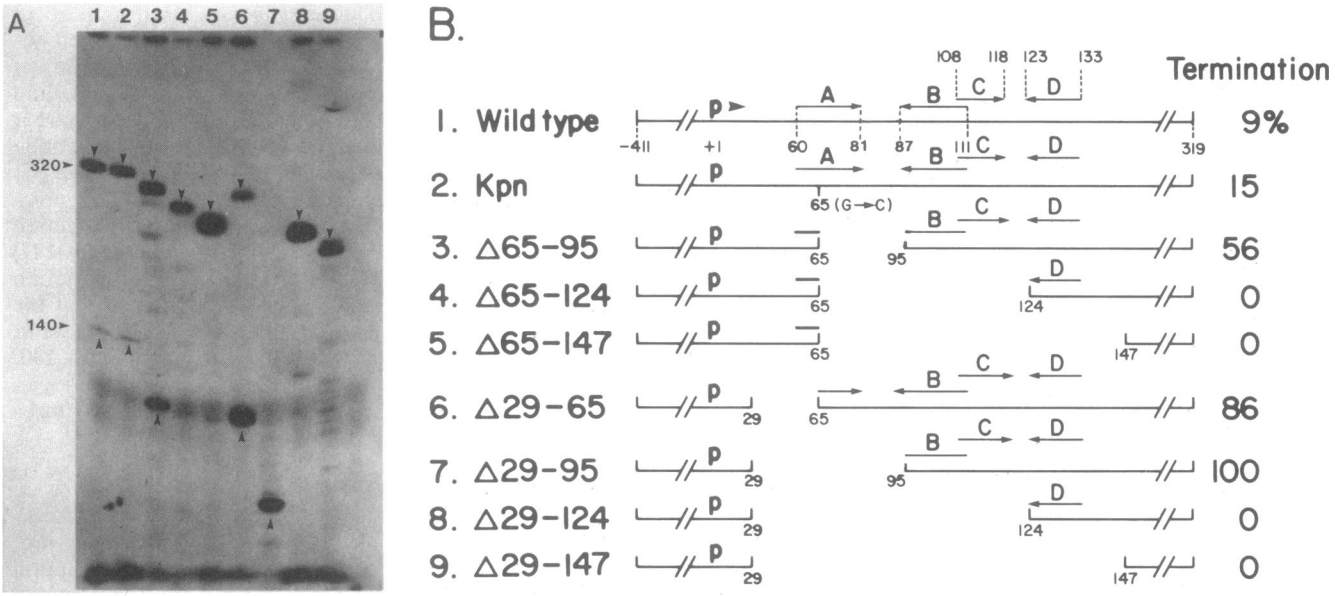

FIG. 7. Gel electrophoresis of transcription products synthesized from wild-type and mutant DNA templates. (A) Lanes 1 through 9 represent in vitro transcriptions with templates 1 through 9 diagrammed in panel 7B. Downward arrowheads point to readthrough bands, while upward arrowheads point to terminated bands. *Serratia marcescens Hpa*II restriction fragment containing the *trp* promoter and leader region was transcribed with *E. coli* RNA polymerase and used as size standards (250 and 175 nucleotides long). Estimated sizes are indicated at the left. (B) Diagram of templates used for in vitro transcription reactions. **p** and +1 indicate the *trp* promoter and transcription initiation site, respectively. Regions of dyad symmetry that could form potential secondary structure (segments A, B, C, and D) are indicated by arrows above each template. The G → C change at position 65 in the Kpn mutant is indicated below the Kpn template. The name of each deletion template includes the 5′ and 3′ deletion endpoints; these are diagrammed to indicate the presence or absence of segments A, B, C, and D. Deleted regions were replaced with a *Kpn*I linker (5′-GGGTACCC-3′) as described in the text. Insertion of the *Kpn*I linker to deletion endpoints resulted in the reconstruction of wild-type sequences from position 60 to 64 in the case of templates 3, 4, and 5 but not in the remaining templates. The relative molar ratio of terminated to readthrough transcripts is expressed as percent termination at the right of each template.

None of the in vivo RNA mapping experiments show conditions in which most of the transcripts appear to extend past the terminator; the highest level of readthrough transcripts appears to be 30 to 40% of that of the terminated transcripts. Under in vitro conditions, 90% of the transcripts extend past the terminator, and one might expect to approach that number under fully derepressed conditions. However, the ratio of readthrough to terminated transcripts is not necessarily an accurate measure of the percentage of readthrough transcription. These experiments measure the steady-state levels of each transcript, and their abundance is a reflection of their rates of synthesis and degradation. There is some evidence that secondary structure at the 3′ end of an RNA transcript can increase its stability (38), and both the 130- and 180-nucleotide terminated transcripts would end with a hairpin structure. It is also possible that the readthrough transcripts are degraded by a 3′ exonucleolytic processing mechanism, which pauses at the stable hairpin structure. Such a mechanism has been seen at the 3′ end of the *E. coli trp* operon (23). If this is the case, some of the apparent terminated transcripts seen under inducing conditions could in fact be degraded readthrough transcripts.

The existence of a second transcript originating upstream of −50 was unexpected. The in vitro transcription of a DNA fragment extending from −411 to +319 did not show a second transcript, suggesting an origin upstream of −411, although a promoter not recognized by the σ43 polymerase used in these experiments would not have been detected. Our current hypothesis is that this transcript extends from some upstream operon, perhaps the nearby *aroFBH* cluster (13). An open reading frame extends from −411 to +5, making it likely that another gene overlaps this region with no intervening transcription terminator.

The significance of this second transcript to the expression of the *trp* operon is not clear. The existence of a 370-nucleotide transcript seen in some of the RNA mapping experiments could correspond to an upstream transcript extending into the *trp* structural genes. Deletion of a region of the chromosome from −48 to −2, which removes the previously defined *trp* promoter, does not confer a Trp$^-$ phenotype on the cell (D. Henner, unpublished data), indicating that the upstream promoter can transcribe the *trp* operon. However, the same deletion in a strain carrying the *mtr-264* allele abolishes the methyltryptophan resistance phenotype, suggesting that the upstream transcript cannot produce enough of the *trp* enzymes to confer methyltryptophan resistance. Whatever the origin of this upstream transcript, it appears to be regulated by the same transcription termination in the *trp* leader region as the transcript from the *trp* promoter.

Alternative RNA secondary structures. Examination of the sequence of the *trp* leader region led to the prediction that alternative secondary structures could form in the transcript of this region (Fig. 6 and 8). To determine whether these structures play a role in regulation of *trp* operon transcription, a series of leader mutations and deletions were produced which should affect the stability of and abolish the formation of these alternative RNA secondary structures. Deletion of the termination structure [Δ(nucleotide 65–147)] caused constitutive synthesis of β-galatosidase in strains with the *trpE-lacZ* fusion. Correspondingly, in vitro transcription analyses gave 100% readthrough with the

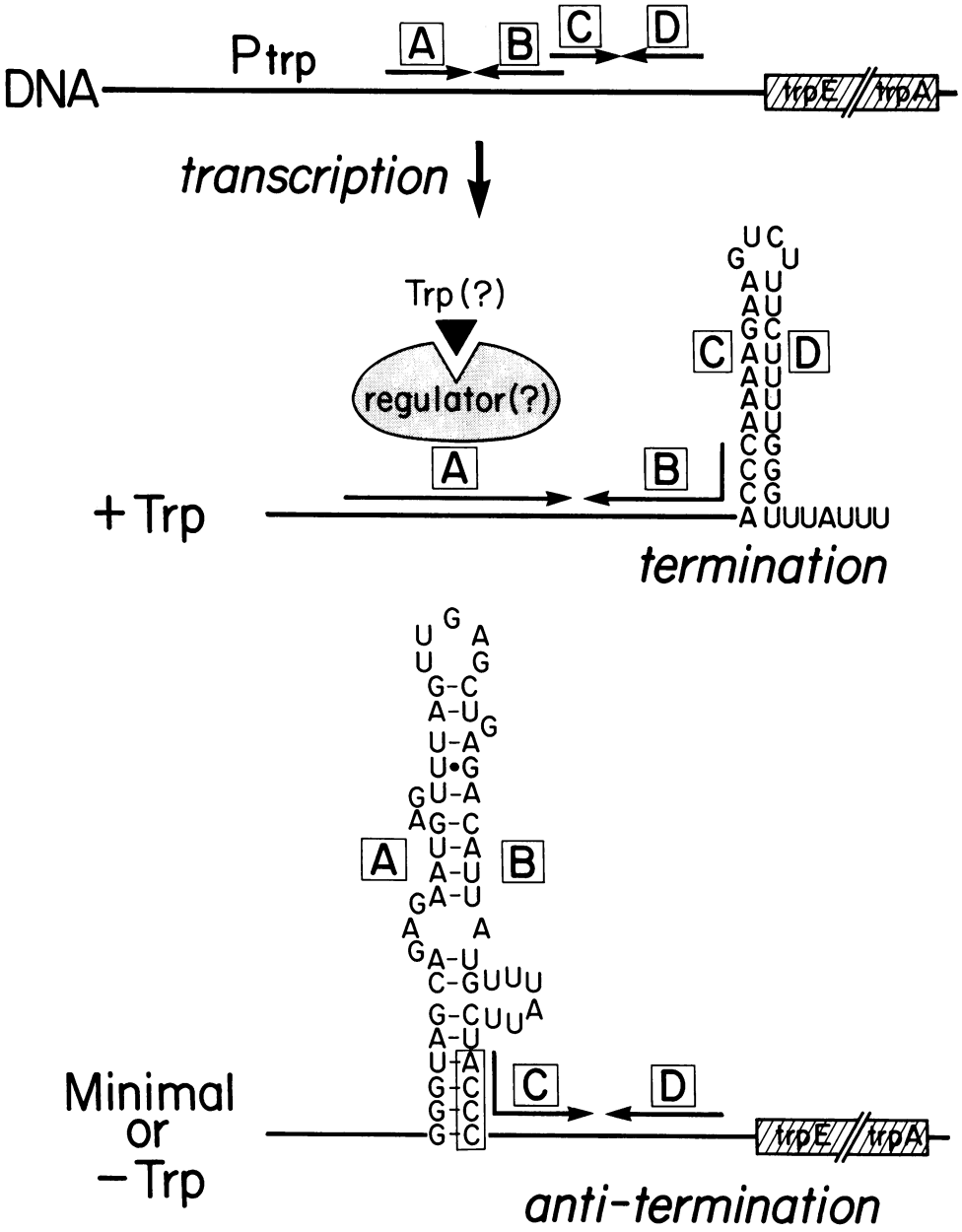

FIG. 8. Schematic representation of regulated transcription termination model of the *B. subtilis trp* operon. Boxed letters indicate segments. Boxed nucleotides indicate the overlap of segments B and C.

Δ(nucleotide 65–147) template. Since the levels of β-galactosidase with the Δ(nucleotide 65–147) deletion are approximately the same in the presence of tryptophan or in minimal-CH medium, it is likely that transcription termination is the major regulatory mechanism employed to control transcription of the *B. subtilis trp* operon. Deletion Δ(nucleotide 29–95), which removes portions of the putative antitermination structure (segment A and a part of segment B, Fig. 6), shuts off production of β-galactosidase. Again, in vivo data agree with in vitro findings, as the Δ(nucleotide 29–95) template gives complete termination at the C-D hairpin structure. These results establish that segment A is necessary to prevent formation of the terminator. The results of in vitro transcription experiments with the other deletion templates are entirely consistent with the alternative secondary structure model; removal of portions of the antiterminator led to increased termination, and removal of the terminator leads to readthrough.

The single-base mutations have more subtle effects. The Kpn mutation should lower the stability of the antitermination structure, leading to increased formation of the termination structure. Both in vivo and in vitro data support this expectation; β-galactosidase levels decreased threefold in minimal-CH medium, and there was a twofold increase in termination in vitro. When a complementary change was introduced which should restore the stability of the antiterminator (Kpn-C), β-galactosidase activity (in the absence of tryptophan) was restored to the wild-type level. These results implicate interaction between RNA segment A and B. The Kpn-C mutation also results in partially consti-

tutive expression in the presence of tryptophan. As discussed below, the Kpn mutation is in a region which may be recognized by a regulatory factor, and therefore the mutation might affect both RNA secondary structure and recognition by a regulatory factor.

How the alternative secondary structures are modulated. Previously we reported that the presence of the *trp* promoter and leader region on a high-copy plasmid confers resistance to the tryptophan analogue 5-methyl tryptophan (5-MeTrp). We presume that the high copy number titrates out a *trp* regulatory factor, thereby causing constitutive expression of the chromosomal *trp* operon (32). We postulated that the act of transcription, rather than presence of the DNA segments surrounding the promoter, was responsible for 5-MeTrp resistance. Thus, plasmids lacking part of the −35 region or containing single-base changes which abolished promoter activity did not confer 5-MeTrp resistance. In addition, the *trp* operon promoter was not necessary to confer resistance, as completely unrelated promoters placed adjacent to the *trp* leader region were also effective (32). These observations suggested that the regulatory molecule might bind the nascent transcript itself rather than DNA.

The minimal segment of the *trp* leader region necessary to confer 5-MeTrp resistance was determined by deletion analysis (32). This segment contained a series of short direct repeat sequences which overlap segment A of the antiterminator. Presumably the postulated active regulatory factor binds the RNA at or near segment A. A strong candidate for the *trp* regulatory molecule is the product of the 5-MeTrp resistance locus (14). Mutations at this locus, designated *mtr*, result in constitutive synthesis of the enzymes of the *trp* operon (15). As shown in this work, *mtr* mutations cause transcription readthrough into the *trp* structural genes. Existing evidence is insufficient to indicate whether the product of the *mtr* locus senses the level of tryptophan or of charged $tRNA^{Trp}$.

The model we have proposed for the regulation of transcription of the *trp* operon of *B. subtilis* is very different from the model of attenuation control of the *trp* operon of *E. coli* and other enterobacteria. In enterobacteria, synthesis of a tryptophan-containing leader peptide modulates formation of the alternative RNA secondary structures that regulate transcription termination (25, 40, 41, 46). In *B. subtilis* there is no coding region for a tryptophan-rich leader peptide, nor is there one in the related organism, *Bacillis pumilus* (M. Kuroda, unpublished data). In *E. coli*, transcription of the *trp* operon is also regulated by repression (27, 28, 35, 42); in *B. subtilis* there is no evidence for transcription initiation control of *trp* operon expression.

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