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Transcription termination *in vitro* at the tryptophan operon attenuator is controlled by secondary structures in the leader transcript

(alternative RNA secondary structure/transcription regulation)

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ABSTRACT **The role of alternative RNA secondary structures in regulating transcription termination at the attenuator of the tryptophan (*trp*) operon of *Serratia marcescens* was examined *in vitro* by transcribing mutant DNA templates having deletions of different segments of the *trp* leader region. Deletions that removed sequences corresponding to successive segments of postulated RNA secondary structures either increased or decreased transcription termination at the attenuator. The results obtained are consistent with the hypothesis that transcription termination results from RNA polymerase recognition of a particular RNA secondary structure, the terminator. This structure forms only in the absence of an alternative, preceding, RNA secondary structure, the antiterminator.**

Expression of the tryptophan (*trp*) operon of many enterobacterial species is subject to attenuation as well as repression control (1). Operons concerned with the biosynthesis of histidine, phenylalanine, threonine, leucine, isoleucine, and valine are also regulated by attenuation (2–10). In each case, transcription termination control is coupled to translation of a short peptide coding region located in the leader segment of the transcript of the operon. The position of the translating ribosome on the leader segment of the transcript is believed to be communicated to the transcribing polymerase through the formation of alternative secondary structures in the transcript (1–14). One secondary structure is thought to act as a transcription termination signal, while the alternative structure prevents formation of this termination signal. The alternative RNA structures currently believed to mediate control of attenuation in the *trp* operon of *Serratia marcescens* are shown in Fig. 1. On the basis of *in vivo* studies with *trp* leader deletion mutants that can form only RNA structure 3:4 (14, 15) and earlier *in vivo* and *in vitro* studies of attenuation in the *trp* operons of *Escherichia coli* and *S. marcescens* (19–21), it was proposed that the hairpin secondary structure 3:4 is recognized by the transcribing RNA polymerase as the transcription termination signal. This secondary structure, designated the "terminator," is assumed to form at high frequency whenever cells have an adequate supply of tryptophan. It was also proposed that, when cells are starved of tryptophan, the ribosome translating the leader peptide coding region stalls over either of the adjacent tryptophan codons, thereby masking RNA segment 1 (Fig. 1*b*). Under these conditions, hairpin secondary structure 2:3, the "antiterminator," would form prior to the synthesis of RNA segment 4 and thereby preclude formation of the terminator. The RNA polymerase molecule engaged in transcribing the operon would then continue transcription beyond the attenuator. In cells that are incapable of initiating translation at the leader peptide start codon, secondary structure 1:2 would form and prevent 2:3 formation, thereby facilitating 3:4 formation. In accord with the latter expectation, *trp* leader mutants of *S. marcescens* and *E. coli* that are defective in translation initiation show *increased* transcription termination at their respective attenuators *in vivo* (12, 15).

Secondary structure 1:2 (Fig. 1*a*) is the alternative 1:2 structure we considered previously (14). We favored a theoretically less stable 1:2 structure at the time but the key deletion mutant, no. 432, that led us to select this less stable alternative, has been found to have a much larger deletion than previously supposed. We assume that a second deletion occurred in the handling of plasmid 432 to give what we now find as the extent of this deletion. Preliminary nuclease cleavage analyses suggest that structures 1:2 and 3:4 as drawn in Fig. 1 do form in the spontaneously folded intact leader transcript (unpublished data). Secondary structures analogous to those pictured in Fig. 1*a* have been found in studies with the *E. coli trp* leader transcript (11, 13).

We previously examined the role of the postulated RNA secondary structures in attenuation *in vivo* by analyzing the regulatory behavior of deletion mutants lacking different segments of the leader region of the *trp* operon of *S. marcescens* (14, 15). The results of these studies support the regulatory model we have described that involves the RNA secondary structure alternatives shown in Fig. 1. In this report, we present the results of *in vitro* transcription analyses using DNA restriction fragments having many of the same deletions. The objective of these studies was to determine whether in a minimal transcription system RNA polymerase would respond to alternative RNA secondary structures, as we presume it does *in vivo*. We find that the deletions affect transcription termination at the *trp* attenuator *in vitro* in complete accordance with the RNA secondary structure model of regulation by attenuation.

MATERIALS AND METHODS

***In Vitro* Transcription.** *Hpa* II restriction fragments containing the *trp* promoter and leader regions of *S. marcescens* were used as templates. The restriction fragments were derived from the *trp* leader deletion plasmids described previously (14, 15). DNA concentrations were estimated by spotting DNA solutions on agarose containing ethidium bromide (22). [α ^{32}P]GTP was purchased from Amersham. RNA polymerase holoenzyme, prepared by a modification of the procedures of Burgess and Jendrisak (23) and Gonzales *et al.* (24), was generously provided by R. Fisher. Transcription analyses were carried out under two conditions: (*i*) low concentrations of nucleoside triphosphates, RNA polymerase, and DNA template

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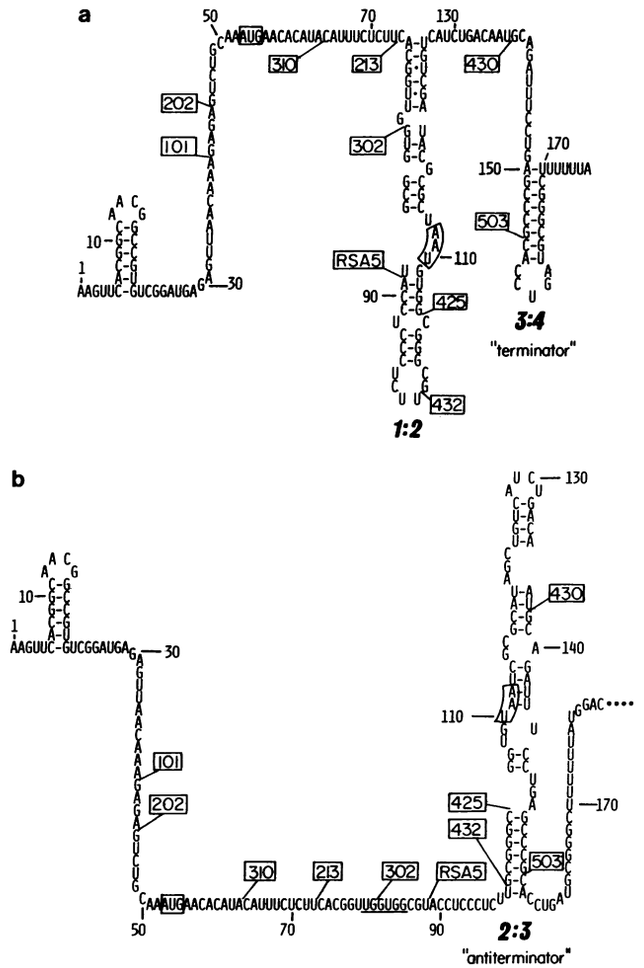

FIG. 1. The alternative leader RNA structures believed to mediate control of attenuation in the *trp* operon of *S. marcescens in vivo*. Numbering is from the presumed 5′ nucleotide of the transcript. The leader peptide initiation and termination codons are boxed, and tryptophan codons are indicated by underlining in *b*. The numbered RNA secondary structures are those postulated to either cause or prevent transcription termination at the attenuator. (*a*) Structure 1:2 is thought to form *in vivo* when ribosomes do not initiate synthesis of the leader peptide (15); under these conditions hairpin loop 3:4, the terminator, is believed to form and be recognized as the transcription termination signal. (*b*) Structure 2:3, the antiterminator, is believed to form in cells starved of histidine, glycine, tryptophan, or arginine because the translating ribosome sterically masks the critical portion of RNA segment 1 when it stalls over a relevant codon. Formation of the antiterminator presumably precludes formation of the terminator and thus allows read-through into the structural genes of the operon. The various structures have the following calculated free energies of formation (16–18): 1:2, $\Delta G = -19$ kcal/mol (1 cal = 4.18 J); 2:3, $\Delta G = -30$ kcal/mol; 3:4, $\Delta G = -19$ kcal/mol. The calculated free energy of formation of the hairpin loop formed by pairing of nucleotides 6–11 with nucleotides 16–21 is −10.4 kcal/mol. Secondary structures other than those pictured can theoretically form, as noted previously (14, 19). Numbers in boxes indicate the right-hand end points of the deletions used.

and (*ii*) high concentrations of nucleoside triphosphates, RNA polymerase, and DNA template in the presence of spermidine.

Low nucleoside triphosphates. Reactions were carried out for 30 min at 30°C in 150 mM KCl/4 mM $MgCl_2$/36 mM Tris acetate, pH 7.8/0.1 mM Na_2EDTA/5% glycerol/0.1 mM dithiothreitol/bovine serum albumin (20 μg/ml)/150 μM ATP/150 μM CTP/150 μM UTP/20 μM unlabeled GTP/6 μCi of [α-^{32}P]GTP/4 nM *Hpa* II restriction fragment/10 nM RNA polymerase holoenzyme. The final volume of each reaction mixture was 25 μl.

High nucleoside triphosphates. Reactions were carried out for 2 hr at 30°C in 120 mM KCl/4 mM $MgCl_2$/40 mM Tris·HCl, pH 8/10 mM 2-mercaptoethanol/4 mM spermidine/15% glycerol/2.7 mM ATP/1.1 mM GTP/1 μCi of [α-^{32}P]GTP/1.4 mM UTP/0.7 mM CTP/35 nM *Hpa* II restriction fragment/350 nM RNA polymerase holoenzyme (25). The final volume of each reaction mixture was 5 μl.

Under both sets of conditions, transcription reactions were stopped by the addition of an equal volume of stop solution (13). Each entire reaction mixture was loaded on a polyacrylamide/7 M urea gel (13) and electrophoresed. Transcription of wild-type and mutant 101, 202, 310, and 213 templates was analyzed on 6% polyacrylamide/urea gels and mutant 302, RSA5, 432, 425, 430, and 503 templates, on 10% polyacrylamide/urea gels.

Secondary Structure Analysis. Possible RNA secondary structures were examined using the computer program of Zuker and Stiegler (16) as modified by R. Feldmann (personal communication). Calculations of the theoretical stabilities of predicted structures using this program are based on published values of stacking and destabilizing energies as compiled by Salser (17).

RESULTS

Transcription Studies with Restriction Fragments Having Deletions within the *S. marcescens trp* Leader Region. The nucleotide sequences of the *trp* operon deletion mutant templates used in this study have been determined (14, 15). The templates, *Hpa* II restriction fragments, are 240–400 base pairs long and contain a functional wild-type *trp* promoter, an intact or deletion-containing *trp* leader region, and *ca.* 75 base pairs beyond the normal site of transcription termination. The deletions (Fig. 1) remove different segments of the leader region and hence the corresponding segments of the leader transcript that are thought to form secondary structures that control attenuation *in vivo* (14, 15). Transcription studies were carried out *in vitro* under two different conditions. One set of reactions was carried out with the minimal transcription system used previously (26) consisting of an *Hpa* II restriction fragment as template, low concentrations of the four ribonucleoside triphosphates, one of which was labeled, and *E. coli* RNA polymerase holoenzyme. Reactions were also carried out with high concentrations of the triphosphates, RNA polymerase holoenzyme, and DNA template in the presence of spermidine (25). Under the latter conditions, the molar yield of transcripts is increased approximately 1000-fold and the polymerization rate more closely approaches the *in vivo* rate. In either case, the two products of transcription, the terminated transcript and the read-through transcript, were separated on acrylamide/urea gels and located by autoradiography. The relevant RNA-containing gel bands were excised and quantified by determining their radioactivity in a scintillation counter. Representative results are shown in Fig. 2.

When the wild-type template was transcribed, RNA polymerase molecules either terminated transcription at the attenuator to produce a 176-nucleotide terminated leader transcript or continued to the end of the restriction fragment to form a 250-nucleotide read-through transcript (19). With most deletion-mutant templates, terminated and read-through transcripts were also observed (Fig. 2); however, their size varied in accordance with the length of the deletion. The relative molar amounts of read-through transcript and terminated transcript were determined with each DNA template. The read-through percentages are presented in Fig. 3. With all the templates exhibiting low or moderate levels of read-through (except those of wild type and deletion mutants 101 and 202) higher read-through values were obtained under conditions allowing increased transcription. The explanation for the read-through

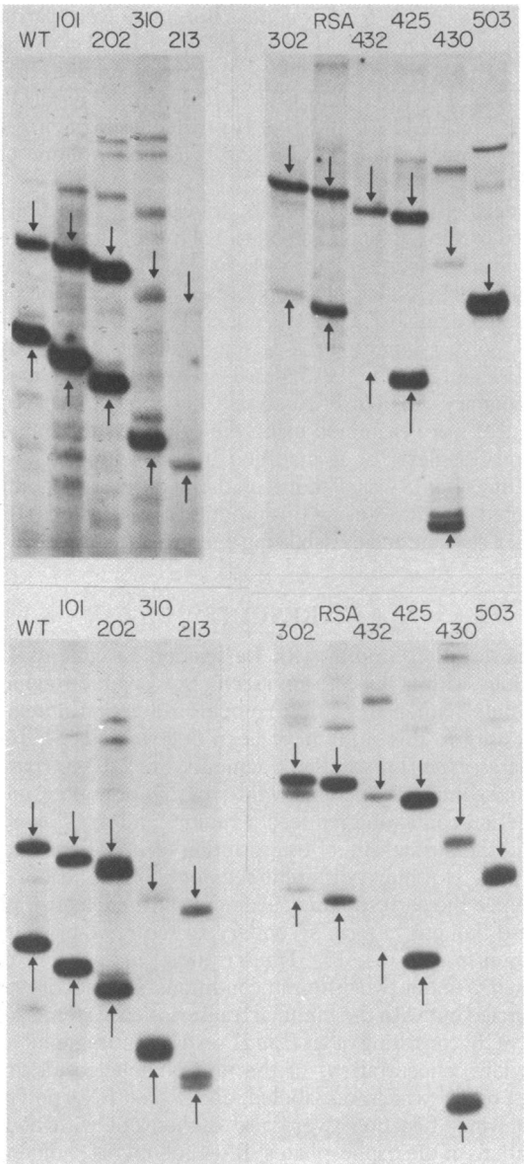

FIG. 2. Gel electrophoresis of transcription products synthesized from wild-type (WT) and deletion mutant DNAs. *S. marcescens Hpa* II restriction fragments containing the *trp* promoter and leader regions were used as templates. The restriction fragments were derived from the *trp* leader deletion plasmids described previously (14, 15). Transcription analyses were carried out under two conditions—low (*Upper*) and high (*Lower*) concentrations of nucleoside triphosphates. Downward arrows point to read-through bands while upward arrows point to terminated bands.

differences under the two conditions is not known; however, that pattern was consistent whichever condition was used. Under either condition used, about 30% of the RNA polymerase molecules that transcribed the wild-type template continued transcription beyond the attenuator. This result is identical to the *in vitro* value estimated previously (19). The data in Fig. 3 show that, as the right-hand deletion end point extends further into the leader region (Fig. 1) and removes more distal segments of the transcript, read-through is initially unaffected but then it decreases, increases, decreases again, and finally increases to 100%.

Deletion mutants 101, 202, 310, and 213, with right-hand end points immediately before RNA segment 1 (Fig. 1), result in read-through comparable with or less than that observed with the wild-type template (Fig. 3). Deletion 302, which removes

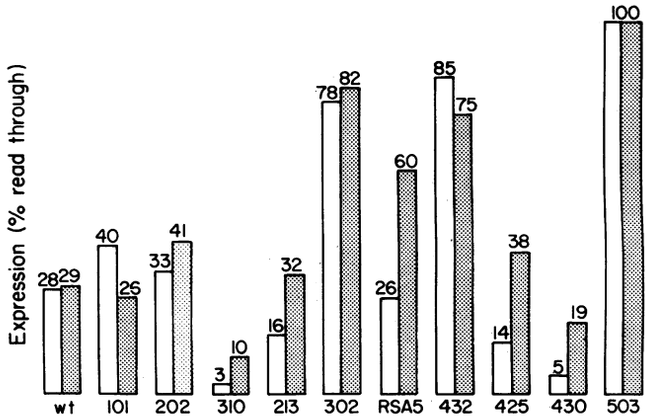

FIG. 3. Quantification of *in vitro* read-through transcription with wild-type (wt) and mutant templates under two transcription conditions. □, Low NTP concentrations; ▨, high NTP concentrations.

part of RNA segment 1, essentially eliminates termination. Deletion mutant RSA5, which lacks somewhat more of RNA segment 1, exhibits high read-through but somewhat less than deletion mutant 302. Deletion 432 removes all of RNA segment 1; it results in complete read-through. Deletions 425 and 430, which remove RNA segments 1 and 2 but leave segments 3 and 4 intact, cause efficient termination. Templates having deletions that remove segments 1, 2, and 3, such as 503, give only a read-through transcript. It is apparent from the summary in Fig. 3 that the extent of termination *in vitro* changes in accordance with the RNA secondary structure model (Fig. 1); termination increases or decreases as the position of the right-hand deletion end point extends further into key segments of the leader region and removes sequences specifying segments of postulated RNA secondary structures.

There are a few results that require additional explanation. We must explain why some deletions with termini in the same segment of the leader region give different read-through values—e.g., nos. 101, 202, and 213 vs. no. 310; nos. 302 and 432 vs. no. RSA5; and no. 425 vs. no. 430 (Fig. 3). These apparent anomalies will be considered in the *Discussion*.

DISCUSSION

The *in vitro* findings summarized in Fig. 3 are in general agreement with the model in which the alternative RNA secondary structures pictured in Fig. 1 either cause transcription termination at the *trp* attenuator or are responsible for its relief (1). These observations therefore reinforce other data consistent with the view that the RNA structure we designate the terminator is the transcription termination signal and that the extent of termination *in vivo* and *in vitro* reflects the frequency of formation of this structure (1). Formation of the terminator would be expected to be influenced by the prior formation of other RNA secondary structures as well as the likelihood that already formed structures rearrange within an appropriate period to assume more stable configurations. It is these latter considerations that we believe can best explain some of the quantitative differences noted in our *in vitro* analyses. In Fig. 4, we postulate the RNA secondary structures that may predominate in the *trp* transcripts produced from each deletion template. We shall consider the transcript structures in the order presented in the figure and attempt to explain our *in vitro* transcription findings on the basis of these structures.

When the wild-type template is transcribed *in vitro*, there is 25–30% read-through (Fig. 3). The secondary structures that presumably form are A, 1:2, and 3:4. An additional secondary structure is possible, involving pairing between segments A and

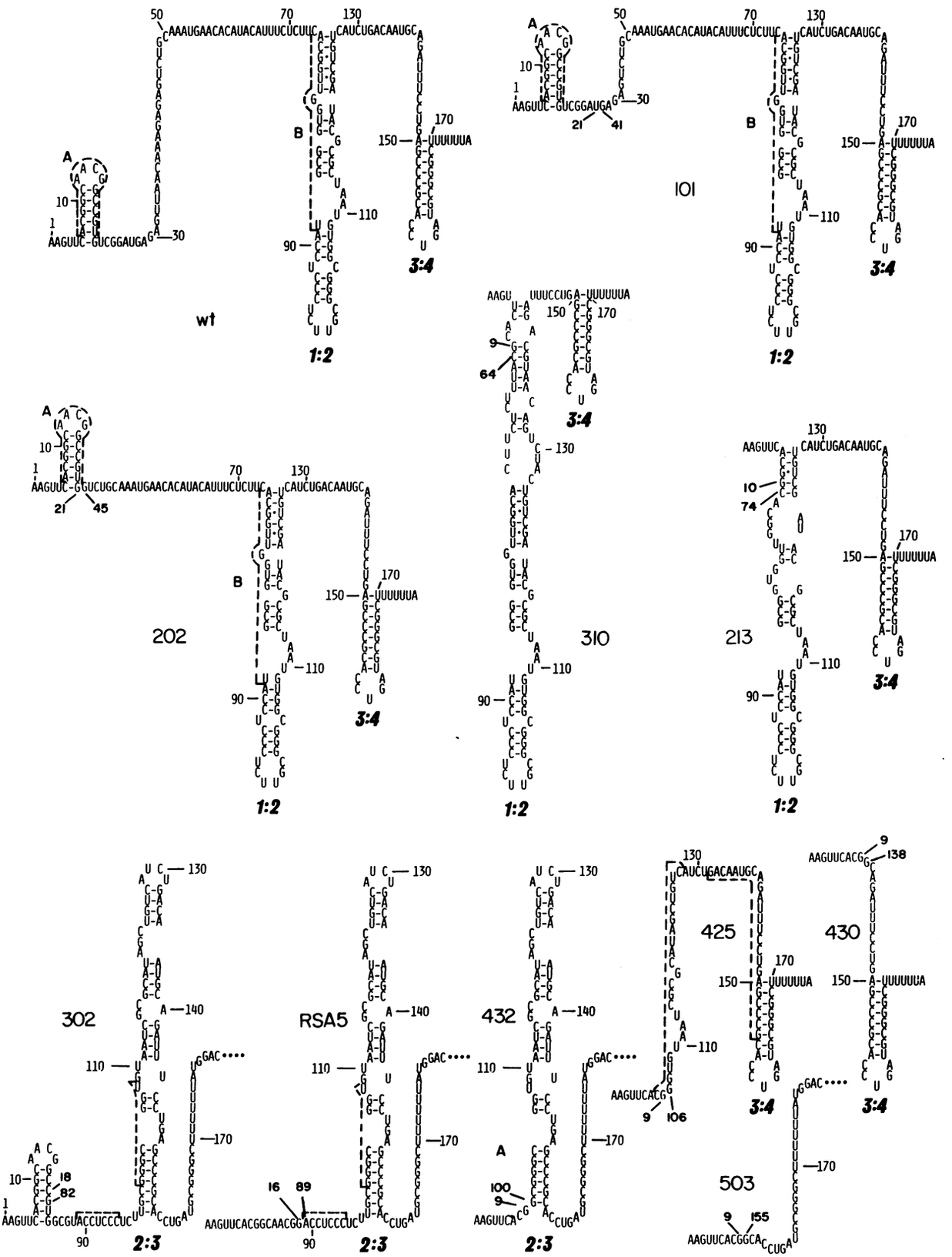

FIG. 4. Potential secondary structures in wild-type (wt) and deletion mutant *trp* leader transcripts. Numbering is from the presumed transcription initiation site. Wild-type numbering is used throughout. Bold numbers indicate nucleotides joined by the deletion. Secondary structures involving segments 1, 2, 3, and 4 are described in Fig. 1. Dashed lines indicate other segments of each transcript that can potentially base pair.

B (Fig. 4 and Table 1). This pairing, if it occurred, would free segment 2 to pair with segment 3, thereby promoting readthrough. We can account for our findings with the wild-type template by assuming that structure 1:2 generally forms before segment 3 is synthesized, permitting 3 to pair with 4 to form the terminator. Occasional pairing of A with B could cause the

moderate read-through that is observed.

If we compare the results obtained with templates from wild type and deletion mutants 101 and 202, we see that they are very similar. As shown in Fig. 4, the transcripts of these deletion templates can form the same secondary structures as wild type.

Deletion mutant 310 has lost segment A and therefore cannot form the A:B structure of wild type. In addition, it can form a more stable 1:2 secondary structure than wild type (Fig. 4 and Table 1). Either or both of these differences can explain the efficient termination that is observed with the 310 template. Mutant 213 has lost somewhat more of the leader region than mutant 310, including segment A of wild type, but its 1:2 structure is only slightly more stable than the 1:2 structure of the wild type. It gives read-through values comparable with those of wild type, although we expect somewhat greater termination.

Deletion mutants 302 and RSA5 have lost most of RNA segment 1 but can form a normal 2:3 structure. Mutant 302 gives near complete read-through whereas the RSA5 template gives appreciable termination, particularly at low triphosphate concentrations. The transcripts of both deletion templates can form a 1:2 structure of reduced stability (Table 1 and Fig. 4). Therefore this structure cannot explain the reduced read-through obtained with RSA5. However, nucleotides 10–16 and 94–101 of the RSA5 transcript could pair and form an additional 1:2 structure ($\Delta G = -6.6$). This structure could not form in the 302 transcript if the early structure not present in the RSA5 transcript did form. The alternative RSA5 pairing possibility may be responsible for the 302 vs. RSA5 difference. Mutant 432 cannot form a 1:2 structure but can form a near-normal 2:3 structure (Fig. 4 and Table 1). The high read-through observed with this template (Fig. 3) is consistent with these structural features.

Deletion mutant 425 has lost RNA segment 1 and a portion of segment 2. The portion of segment 2 deleted is complementary to the base of the portion of segment 3 that normally pairs with segment 4 (Fig. 4). The 425 transcript therefore can form a normal 3:4 structure and a 2:3 structure of reduced stability and competing ability (Fig. 4 and Table 1). The occasional formation of this abnormal 2:3 structure can account for the moderate read-through observed with this template (Fig. 3). Mutant 430 has lost all of segments 1 and 2 so that *only* a normal 3:4 structure can form. This deletion results in highly efficient termination. Mutant 503 has lost RNA segments 1, 2, and 3 and, as expected, does not form a terminated transcript.

Our *in vitro* studies therefore strongly suggest that, in a minimal transcription system, alternative RNA secondary structures can effectively control transcription termination at the *trp* attenuator. Furthermore, since very similar patterns of read-through are observed with the same deletions *in vivo* (14), it is likely that the same structures participate in attenuation *in vivo*. There are some quantitative differences between our *in vivo* and *in vitro* values, but these may be due to the *in vivo* effects of translation, rate of nucleotide polymerization, and interactions of template and transcript with other cell components. It appears therefore that both *in vivo* and *in vitro* formation of the terminator is the key act that signals transcription termination. All other events implicated in attenuation *in vivo*—e.g., synthesis of the leader peptide, stalling of the translating ribosome, competition between alternative RNA secondary structures, and transcriptional pausing—probably serve no purpose other than to regulate formation of the terminator. It also seems likely that a basic feature of attenuation is that throughout the decision-making period moderately stable secondary structures persist in preference to their more stable alternatives.

Table 1. Estimated stabilities of secondary structures in *S. marcescens* leader transcripts

Mutant	Base pairs deleted	ΔG, kcal/mol 1:2	2:3	3:4	A:B*
wt	—	−19	−29.5	−18.8	−14.1
101	28–40	−19	−29.5	−18.8	−14.1
202	22–44	−19	−29.5	−18.8	−14.1
310	10–63	−25.6	−29.5	−18.8	
213	11–73	−22.1	−29.5	−18.8	
302	19–81	−12.1	−29.5	−18.8	
RSA5	17–88	−12.1	−29.5	−18.8	
432	10–99		−28.7	−18.8	
425	10–105		−17.2	−18.8	
430	10–137			−18.8	
503	10–154				

wt, Wild type.
* A:B pairing involves nucleotides 7–21 and nucleotides 74–88.

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