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# Attenuation in the *Escherichia coli* tryptophan operon: Role of RNA secondary structure involving the tryptophan codon region

(leader transcript/translational regulation/transcription termination)

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**ABSTRACT** **The secondary structure of the terminated *trp* leader transcript from *Escherichia coli* was analyzed by RNase T1 partial digestion. Base-paired regions were recovered by nondenaturing gel electrophoresis and identified by denaturing gel electrophoresis and fingerprinting. The tandem tryptophan codons in the leader peptide coding region were found to be base paired with a more distal region of the transcript. This and other secondary structures that the *trp* leader RNA can form help explain the physiological response of the operon as well as the behavior of regulatory mutants.**

Transcription of the tryptophan (*trp*) operon of *Escherichia coli* is regulated at two sites, a promoter–operator and an attenuator. At the promoter–operator the rate of transcription initiation is regulated in response to changes in the intracellular level of free tryptophan (1, 2). At the attenuator, a site located within the transcribed 162-base-pair leader region that precedes the structural genes of the operon, transcription is either terminated to give a 140-residue leader transcript or allowed to proceed into the structural genes (3). Termination at the attenuator is regulated by the levels of charged and uncharged $tRNA^{Trp}$ (4, 5). Charged $tRNA^{Trp}$ presumably is required for translation (6) of the segment of the leader transcript that codes for a 14-residue peptide containing adjacent Trp residues (7). The 3′ half of the terminated *trp* leader transcript exhibits extensive secondary structure *in vitro* (8). Studies of *trp* leader mutants indicate that the capacity to form this secondary structure is essential for normal regulation of transcription termination at the attenuator (6, 9, 10). In the present study we demonstrate that the RNA region containing the adjacent Trp codons also participates in secondary structure. The various secondary structures that *trp* leader RNA can form help explain the physiological response of the operon as well as the behavior of the regulatory mutants we have studied.

## MATERIALS AND METHODS

**Preparation of [$\alpha$-$^{32}$P]GTP-Labeled Terminated *trp* Leader RNA.** *In vitro* transcription reactions contained, in 25 $\mu$l: 20 mM Tris acetate (pH 7.9), 0.1 M KCl, 0.1 mM $Na_2$ EDTA, 0.1 mM dithiothreitol or 0.5 mM 2-mercaptoethanol, 0.15 mM each of unlabeled ATP, CTP, and UTP, 20 $\mu$M unlabeled GTP, $\alpha$-$^{32}$P-labeled GTP, 0.4–1 $\mu$g of *E. coli* RNA polymerase, and 0.1–0.5 $\mu$g of *E. coli trp pol Hpa* II 570 restriction fragment (8) or 1 $\mu$g of *E. coli trp polE* plasmid (pPS21, unpublished) DNA. The reactions were stopped after 20 min at 37°C by the addition of 25 $\mu$l of 0.025% xylene cyanol/0.025% bromphenol blue/0.1% sodium dodecyl sulfate/14 M urea and heating for 2 min at 95°C. Leader RNA was purified by electrophoresis in 6% polyacrylamide slab gels (24 × 0.1 cm) containing 7 M urea in 0.09 M Tris borate (pH 8.3)/2.5 mM EDTA (TBE buffer) at 400 V for 4 hr. Leader RNA was detected by autoradiography, cut from the gel, mashed, and incubated at 37°C with shaking in 0.5 M ammonium acetate/10 mM magnesium acetate/0.1% sodium dodecyl sulfate/0.1 mM EDTA with 40 $\mu$g of carrier RNA. After removal of polyacrylamide by filtration through glass wool, the RNA was precipitated by the addition of 3 vol of ethanol. The RNA was reprecipitated from 0.3 M sodium acetate, washed with ethanol, and dried under reduced pressure.

**Partial Digestion with RNase T1.** The RNA was digested in 25 $\mu$l of 10 mM Tris·HCl (pH 7.9)/10 mM $MgCl_2$/0.1 mM $Na_2$EDTA (TME buffer) with 0.5 unit of RNase T1 (Sankyo Corp.) for 13 min at 20°C. Digestion was stopped by the addition of 0.395 ml of TME, with 0.3 M sodium acetate, 4 $\mu$l of diethylpyrocarbonate, and 40 $\mu$g of carrier RNA. The RNA was precipitated with ethanol, washed with ethanol, and dried under reduced pressure. For denaturing gel electrophoresis the sample was taken up in TBE with 7 M urea/0.05% sodium dodecyl sulfate/0.01% xylene cyanol/0.01% bromphenol blue, applied to a 12% polyacrylamide/7 M urea/TBE gel, and electrophoresed at room temperature for 8 hr at 200 V. For native (nondenaturing) gel electrophoresis the sample was taken up in TME with 25% (vol/vol) glycerol/0.01% xylene cyanol/0.01% bromphenol blue, applied to a 9% polyacrylamide/0.01 M Tris borate (pH 8.3)/5 mM $MgCl_2$ gel and electrophoresed at 4°C for 18 hr at 150 V.

**Fingerprint Analysis of RNase T1 Oligonucleotides.** Two-dimensional fingerprints of RNase T1 digests were prepared as described (11).

## RESULTS

The existence of extensive secondary structure in the 3′ half of the *E. coli* terminated *trp* leader transcript is inferred from the resistance of this region to RNase T1 digestion (8). Analysis of the oligonucleotide products of RNase T1 partial digestion of *trp* leader RNA on denaturing gels suggested that the secondary structure consisted of two alternative stem and loop structures (8). To analyze further the extent and nature of the secondary structure of the terminated *trp* leader transcript we used native gel electrophoresis to recover base-paired RNA fragments. We were particularly interested in determining the portion of the transcript that pairs with the Trp codon region, a segment known to be protected from RNase T1 attack (8). Fig. 1 shows an autoradiograph of RNase T1 partial digests of terminated

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Abbreviations: TBE, Tris/borate/EDTA; TME, Tris/$MgCl_2$/EDTA.

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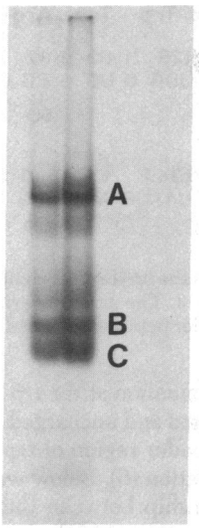


FIG. 1. Native gel electrophoresis of RNase T1 partial digests of *E. coli* terminated *trp* leader RNA. The two lanes show duplicate samples.

*trp* leader transcript separated by native gel electrophoresis. Three major bands (A, B, and C) are typically observed. The labeled fragments from bands A, B, and C were eluted and subjected to electrophoresis on denaturing gels (Fig. 2). The RNA bands on these gels were located by autoradiography, eluted, digested to completion with RNase T1, and identified by fingerprint analysis (Fig. 3).

Band A was the slowest migrating band on native gels (Fig. 1). Digestion of terminated *trp* leader RNA for various times with RNase T1 established that band A RNA is the most labile partial digestion product (data not shown). Denaturing gels resolved band A into three oligonucleotides (A1, A2, A3; Fig. 2, lane A). Fingerprint analysis revealed that band A1 is intact terminated *trp* leader RNA (Fig. 3*A1*; Fig. 4). Band A2 is the 3′ 70 residues of leader RNA, and band A3 is the 5′ 70 residues of terminated *trp* leader RNA. Because polyacrylamide gel electrophoresis separates oligonucleotides primarily on the basis of size, the comigration of A2 and A3 with A1 suggests that the

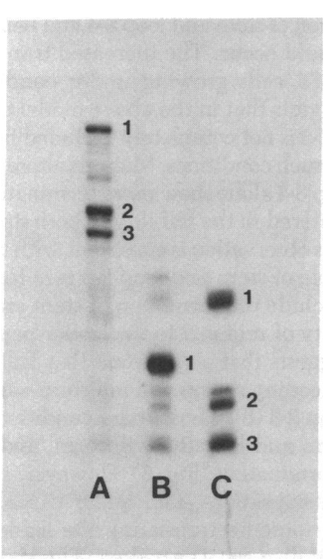


FIG. 2. Denaturing gel electrophoresis of major RNA bands from native gel electrophoresis of RNase T1 partial digests of *E. coli* terminated *trp* leader RNA. Lane letters correspond to the bands shown in Fig. 1.

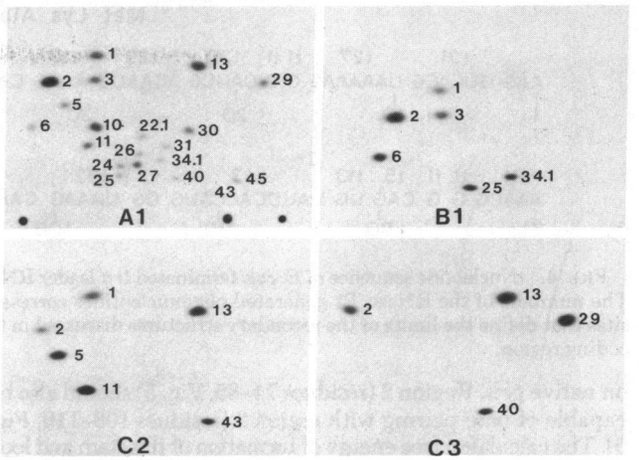


FIG. 3. Fingerprints of RNase T1-digested RNA isolated from the denaturing gel shown in Fig. 2. Cellulose acetate electrophoresis was from left to right and homochromatography on polyethyleneimine plates was upward. Panel numbers correspond to the bands shown in Fig. 2. Panel *A1* is the complete terminated leader RNA fingerprint. The spot numbers correspond to those previously assigned (ref. 11; Fig. 4).

separate 5′ and 3′ halves of terminated *trp* leader RNA are physically paired and move as a unit on native gels. The nature of this base pairing is evident from the analysis of RNA fragments more resistant to RNase T1 digestion than those present in band A.

The labeled RNA in band B from native gels moved as a single major species (B1) on denaturing gels (Fig. 2, lane B). Fingerprint analysis revealed that band B consists of oligonucleotides from residue 108 to the 3′ end of terminated *trp* leader RNA (Figs. 3, 4, and 5).

Band C from native gels resolved into three bands (C1, C2, C3) on denaturing gels (Fig. 2, lane C). Fingerprint analysis revealed that band C1 contains the oligonucleotides from residues 51 or 52 to 95 (Fig. 5). Band C2 RNA contains residues 71 to 95 (Figs. 3*C2* and 5). Band C3 RNA contains the oligonucleotides from residues 51 or 52 to 70 (Figs. 3*C3* and 5). The comigration of the 43-residue band C1 RNA with the 24-residue band C2 RNA and the 18-residue band C3 RNA suggests that on native gels C2 RNA and C3 RNA are base-paired to yield a structure similar to band C1 RNA. This was confirmed by demonstrating that the labeled RNA in bands C3 and C4, when applied separately to native gels, migrated differently from each other and from band C1 RNA (data not shown).

## DISCUSSION

We have shown that certain regions of terminated *trp* leader RNA form stable base-paired structures *in vitro*. Fig. 5 illustrates the most likely secondary structures that form in terminated *trp* leader RNA *in vitro* based on our analysis of regions of the transcript that show resistance to RNase T1 digestion and the base-pairing rules established by studies of defined oligonucleotides (12, 13). Four regions of base-pairing, capable of forming three stem and loop structures, are proposed. Region 1, which includes the tandem Trp codons and the leader peptide translation stop codon (residues 54–68, Fig. 5), can base-pair with region 2, which is immediately distal to the translation stop codon (residues 76–91, Fig. 5). The calculated free energy formation of the resulting stem and loop (called stem and loop 1·2) is $\Delta G = -11$ kcal/mol (12, 13). Base pairing between regions 1 and 2 is sufficiently stable to allow *trp* leader RNA cut at residue 70 (i.e., in the loop of stem and loop 1·2, Fig. 5) to travel at the same position as intact terminated *trp* leader RNA

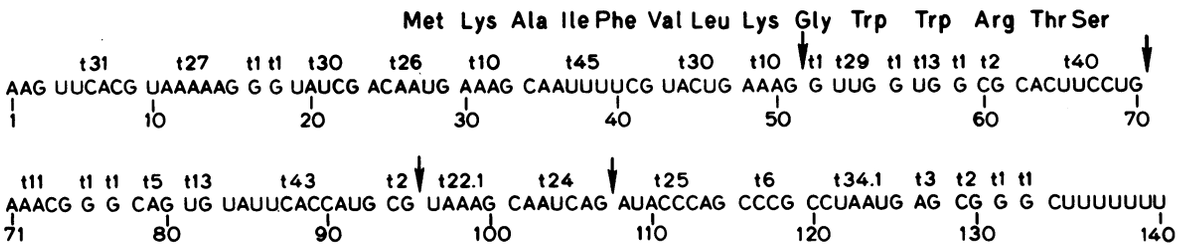


FIG. 4. Nucleotide sequence of *E. coli* terminated *trp* leader RNA. The sequence is presented as the products of complete RNase T1 digestion. The numbers of the RNase T1-generated oligonucleotides correspond to those presented in Fig. 3. The arrows mark the RNase T1 cleavage sites that define the limits of the secondary structures discussed in the text. The predicted *trp* leader peptide is aligned above the corresponding coding region.

on native gels. Region 2 (residues 74–85, Fig. 5) should also be capable of base pairing with region 3 (residues 108–119, Fig. 5). The calculated free energy of formation of this stem and loop (stem and loop 2·3) is $\Delta G = -12$ kcal/mol. Stem and loop 2·3 has not been observed *in vitro*, presumably because stem and loop 3·4 and stem and loop 1·2 form preferentially. Genetic evidence indicating that stem and loop 2·3 does exist and functions *in vivo* is presented below. Region 3 (residues 114–121, Fig. 5) can also base-pair with region 4 (residues 126–134, Fig. 5). The calculated free energy of formation of this stem and loop (stem and loop 3·4) is $\Delta G = -20$ kcal/mol. The existence of this stem and loop is inferred from the relative resistance of the G+C-rich region from residue 107 to the 3′ end of the transcript to RNase T1 digestion (*Results* and ref. 8).

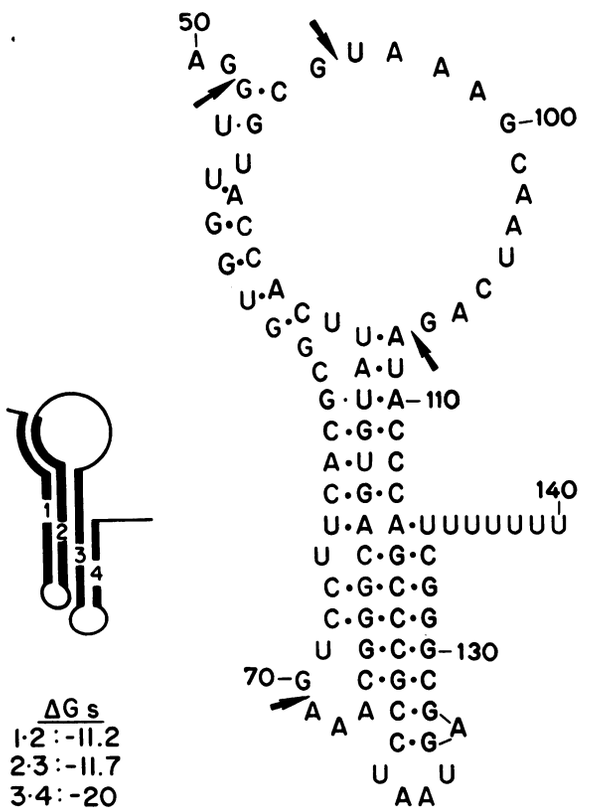


FIG. 5. Proposed secondary structures in *E. coli* terminated *trp* leader RNA. Four regions can base pair to form three stem and loop structures. The arrows mark the RNase T1 cleavage sites discussed in the text. Fingerprints of RNase T1 digests of RNA bands C1 and C3 (Fig. 2) showed reduced yields of oligonucleotide t2 (Figs. 3*C3* and 4). This suggests that C1 and C3 contain a minor amount of RNA containing residues 51 or 52 to 93, and residues 71 to 93, respectively. The calculated free energies of formation of the stem and loop structures [in kcal/mol (1 kcal = 4.184 kJ)] are shown.

Transcription termination at the *trp* attenuator is regulated by the levels of charged and uncharged $tRNA^{Trp}$ in the cell (5). Translation of the leader region of *trp* mRNA is an essential feature of this regulation (6). Below we discuss evidence that implicates a relationship between this translalation and the extensive secondary structure of the leader RNA in the control of transcription termination at the *trp* attenuator.

**Role of Stem and Loop 3·4.** Mutations in the *trp* leader region that relieve transcription termination at the *trp* attenuator *in vivo* and *in vitro* also lower the stability of stem and loop 3·4 (10). Thus the stability of stem and loop 3·4 (the stem and loop most proximal to the 3′ end of the terminated transcript) is an essential feature of the signal for RNA polymerase to terminate transcription.

**Role of Stem and Loop 2·3.** The *trpL75* mutation, which destabilizes only stem and loop 2·3 (from $\Delta G = -12$ kcal/mol to $\Delta G = -2$ kcal/mol), has two effects on attenuation (6). First, *trpL75* cells do not respond to tryptophan starvation by fully relieving transcription termination at the attenuator. Second, when grown with excess tryptophan, *trpL75* cells terminate transcription more efficiently than wild-type cells. A model in which stem and loop 2·3 normally prevents the formation of stem and loop 3·4 (note that the two are mutually exclusive, Fig. 5) can account for the phenotype of *trpL75*. Thus, when wild-type cells are starved of tryptophan, if stem and loop 2·3 is formed before region 4 is synthesized, the formation of stem and loop 3·4 (required to signal transcription termination) would be temporarily excluded. Alternately, if, under conditions of tryptophan excess, stem and loop 2·3 could not be formed, formation of stem and loop 3·4 and hence transcription termination would occur. The increased transcription termination in *trpL75* cells growing under conditions of excess tryptophan suggests that in the above model the formation of stem and loop 2·3 is not completely excluded in wild-type cells growing under such conditions. Mutants altered in the stability of stem and loop 3·4 alone show more termination relief *in vivo* than mutants altered in the stability of both stem and loops 2·3 and 3·4 (8). This observation is consistent with the above model in which the role of stem and loop 2·3 is to form initially and temporarily exclude the formation of stem and loop 3·4.

The proximity of region 2 to the leader peptide translation stop codon suggests that a ribosome that fully translates the leader peptide coding region will interfere with the formation of stem and loop 2·3 (8). Thus, under conditions of tryptophan excess only stem and loop 3·4 will form, and this will signal transcription termination (Fig. 6). However, under conditions of tryptophan starvation—i.e., when $tRNA^{Trp}$ is not fully charged—the ribosome translating the leader region of the transcript will stall at the Trp codons. This should permit stem and loop 2·3 to form, thereby excluding the formation of stem and loop 3·4 and allowing relief of transcription termination (ref. 8, Fig. 6).

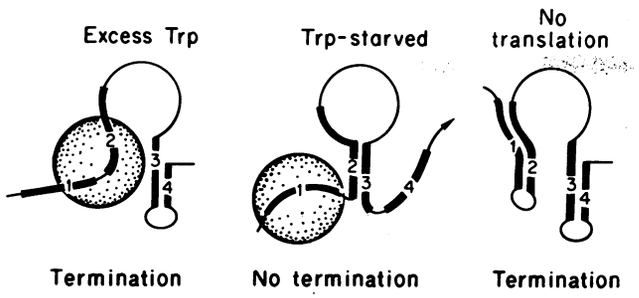


FIG. 6. Model for attenuation in the *E. coli trp* operon. Under conditions of excess tryptophan the ribosome (the shaded circle) translating the newly transcribed leader RNA will synthesize the complete leader peptide. During this synthesis the ribosome will mask regions 1 and 2 of the RNA and prevent the formation of stem and loop 1·2 or 2·3. Stem and loop 3·4 will be free to form and signal the RNA polymerase molecule (not shown) transcribing the leader region to terminate transcription. Under conditions of tryptophan starvation, charged $tRNA^{Trp}$ will be limiting and the ribosome will stall at the adjacent Trp codons in the leader peptide coding region. Because only region 1 is masked, stem and loop 2·3 will be free to form as regions 2 and 3 are synthesized. Formation of stem and loop 2·3 will exclude the formation of stem and loop 3·4, which is required as the signal for transcription termination. Therefore, RNA polymerase will continue transcription into the structural genes. Under conditions in which the leader peptide is not translated (i.e., *trpL29 in vivo*, transcription *in vitro*, or starvation for amino acids occurring early in the leader peptide), stem and loop 1·2 will be free to form as regions 1 and 2 are synthesized. Formation of stem and loop 1·2 will prevent the formation of stem and loop 2·3 and thereby permit the formation of stem and loop 3·4. This will signal transcription termination.

**Role of Stem and Loop 1·2.** Although *trp* leader mutants that destabilize stem and loop 1·2 have not yet been isolated, the phenotype of the *trpL29* mutant is particularly relevant to the role of stem and loop 1·2. The *trpL29* mutation, which changes the AUG translation start codon for the *trp* leader peptide to AUA (6), causes increased transcription termination *in vivo* and an inability to relieve transcription termination fully in response to tryptophan starvation. We suspect that, when the wild-type operon is transcribed *in vitro*, stem and loop 1·2, which is synthesized first, forms and excludes formation of stem and loop 2·3. The exclusion of stem and loop 2·3 then facilitates formation of stem and loop 3·4 and thereby causes transcription termination. We would expect, therefore, that in *trpL29* cells *in vivo* the absence of translation of leader RNA should permit stem and loop 1·2 to form. This should prevent formation of stem and loop 2·3, allowing stem and loop 3·4 to form and signal transcription termination (Fig. 6). The increased transcription termination in *trpL29* and *trpL75* cells growing in excess tryptophan suggests that the mechanism that signals for transcription termination in wild-type cells growing under such conditions is not acting to its full potential. This could be explained if in wild-type cells the ribosome that fully translates the leader region occasionally is released before the signal for transcription termination is given. In such a case we believe that stem and loop 2·3 would form first and thereby temporarily prevent formation of stem and loop 3·4.

Although it is likely that stem and loop 1·2 influences *in vitro* transcription termination, *in vivo* a ribosome that has reached or passed the Trp codons of leader RNA should prevent stem and loop 1·2 from forming (the Trp codons are residues 54–59, Fig. 5). A ribosome is believed to mask about 3 codons 3′ to the codon that is being read (14). Thus, a ribosome stalled in region 1 at the adjacent Trp codons, or at the Arg codon immediately 3′ to the Trp codons, would completely mask region 1 and prevent formation of stem and loop 1·2 (Fig. 5). However, a ribosome stalled 4 or more codons 5′ to the Trp codons in the leader peptide coding region should not interfere with the formation of stem and loop 1·2. A ribosome stalled at the Gly codon immediately 5′ to the Trp codons would only mask about half of region 1, presumably allowing part of stem and loop 1·2 to form (Fig. 5). A ribosome stalled at the Thr or Ser codons immediately 3′ to the Trp-Trp-Arg codons should mask region 1 and part of region 2, thereby preventing the formation of stem and loops 1·2 and 2·3 (Fig. 5). Thus, it is possible that the position of stem and loop 1·2 within leader RNA may account for the observation that starvation for amino acids occurring prior to the Trp residues in the *trp* leader peptide fails to elicit relief of transcription termination (6). According to this explanation, relief of transcription termination will occur only when the ribosome translating leader RNA stalls at a codon that prevents the formation of stem and loop 1·2 but allows the formation of stem and loop 2·3 (Fig. 6). The codons that fulfill this condition (Trp-Trp-Arg) specify those amino acids that when depleted in the cell elicit relief of transcription termination (6).

Analysis of RNase T1 partial digestion products of the terminated *trp* leader RNA of *Salmonella typhimurium* by denaturing gel electrophoresis suggests the existence of stem and loop structures analogous to those we have described for the *trp* leader RNA of *E. coli* (8). Table 1 shows the expected stabilities of the three stem and loop structures of the terminated *trp* leader transcript of *Salmonella typhimurium* and the equivalent structures for the *trp* transcript of *Serratia marcescens*. The terminated leader transcripts of the *E. coli* threonine, histidine, and phenylalanine operons are also capable of forming analogous competing stem and loop structures (Table 1). In particular, each leader transcript described in Table 1 has the following features in common with the *E. coli trp* operon leader transcript: a stem and loop 3·4 immediately proximal to the presumed transcription termination site; a stem and loop 1·2 that includes all, or the last two to four, presumed specific leader peptide codons and, except for the *thr* leader transcript, the leader peptide translation stop codon; and a stem and loop

Table 1. Estimated stability of secondary structures in bacterial operon leader transcripts

| Bacterium | Operon | Calculated ΔG values 1·2 | 2·3 | 3·4 |
|---|---|---|---|---|
| *E. coli* | *trp* | −11 | −12 | −20 |
| *Salmonella typhimurium* | *trp* | −12 | −25 | − 5 |
| *Serratia marcescens* | *trp* | −16 | −30 | −17 |
| *E. coli* | *his* | − 9 | −12 | −37 |
| *E. coli* | *thr* | −12 | −24 | −29 |
| *E. coli* | *phe* | −33 | −35 | −24 |

The stability of the above leader RNA secondary structures were estimated according to the rules described in refs. 12 and 13. In *E. coli* terminated *trp* leader RNA the stem and loops are between the following residues: 1·2, 54–67 pairs with 76–91; 2·3, 74–85 pairs with 108–119; 3·4, 114–121 pairs with 126–134. The adjacent Trp codons are residues 54–59 (Fig. 4). *Salmonella typhimurium trp* leader RNA: 1·2, 39–70 pairs with 75–107; 2·3, 73–95 pairs with 100–122; 3·4, 117–122 pairs with 130–135. The adjacent Trp codons are residues 54–59 (8). *Serratia marcescens trp* leader RNA: 1·2, 83–95 pairs with 102–119; 2·3, 94–127 pairs with 132–156; 3·4, 150–156 pairs with 162–168. The adjacent Trp codons are residues 80–85 (15). *E. coli his* leader RNA: 1·2, −50 to −37 pairs with −28 to −15; 2·3, −32 to −7 pairs with 3 to 44; 3·4, 30–43 pairs with 47–62. The His codons are residues −62 to −42 (16). The *Salmonella typhimurium his* leader sequence is virtually identical to the *E. coli his* leader sequence (17). *E. coli thr* leader RNA: 1·2, −112 to −103 pairs with −101 to −90; 2·3, −93 to −84 pairs with −58 to −49; 3·4, −63 to −50 pairs with −43 to −30. The Ile and Thr codons are residues −138 to −100 (18). *E. coli phe* leader RNA: 1·2, 36–65 pairs with 72–112; 2·3, 63–86 pairs with 106–135; 3·4, 116–126 pairs with 132–142. The Phe codons are residues 37–63 (19).

2·3 that can form instead of stems and loops 1·2 and 3·4. In addition, full translation of the leader peptide coding region of each leader transcript is expected to disrupt, or prevent, the formation of stem and loops 1·2 and 2·3 and to allow the formation of stem and loop 3·4. Similarly, a ribosome stalled one, or in some cases two, codons past the last specific codon in the leader peptide coding region is expected to disrupt enough of stem and loop 2·3 to allow stem and loop 3·4 to begin to form. However, a ribosome stalled at the last specific codon in the leader peptide coding region is expected to disrupt the formation of stem and loop 1·2, but to allow most, or all, of stem and loop 2·3 to form. Although in all the leader regions the specific codons are located towards the 3′ end of the leader peptide coding region, the number of specific codons varies between 3 (Trp-Trp-Arg) for the *trp* operons to 12 (4 Ile, 8 Thr) for the *E. coli thr* operon (Table 1). The location of the specific codons within the leader region is such that a ribosome stalled prior to the first specific codon is expected to permit the formation of stem and loop 1·2 and therefore facilitate transcription termination (Fig. 5). In the model we have described above only a ribosome stalled such that stem and loop 1·2 is sufficiently disrupted to allow stem and loop 2·3 to form will elicit relief of transcription termination (Fig. 5). This model therefore predicts that a ribosome stalled at the first few specific codons of those leader regions with many specific codons (i.e., *phe, thr, his*) will not elicit relief of transcription termination. Clearly, data are needed on the effects of ribosome stalling at different codons in the leader peptide coding regions of the various leader transcripts. A further feature of the various leader transcripts is the variation in the relative calculated stabilities of the suggested stem and loop structures (Table 1). It is likely that the different relative stabilities are important in setting the sensitivity and range of relief of transcription termination in response to amino acid starvation. It should be emphasized that in the model that we have presented the relative stabilities of the various stem and loop structures are of less importance than their ability to form in a transient and sequential manner as the leader transcript is synthesized by RNA polymerase and translated by a ribosome.

David Pribnow (personal communication) has deduced many of the RNA region interactions described above as well as their likely effects on attenuation in the *trp* operon. Eisenberg *et al.* (20) have satisfactorily accounted for the physiological effects of a variety of tRNA alterations in terms of the effects of translation of the leader transcript on the formation of the RNA secondary structures mentioned above. It should be noted that it is possible that tertiary interactions may also contribute to the structure of leader RNA. The manner in which such tertiary interactions augment or modify the function of the structure of leader RNA in attenuation awaits elucidation.

**Note Added in Proof.** Essentially identical models have recently been developed by Gemmill *et al.* (21) and by Johnston *et al.* (22) to explain attenuation in the leucine and histidine operons, respectively, of *Salmonella typhimurium*.

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