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Transcription termination at the *trp* operon attenuators of *Escherichia coli* and *Salmonella typhimurium*: RNA secondary structure and regulation of termination

(transcription regulation/restriction fragments/RNase T_1-resistant sequences)

FRANK LEE AND CHARLES YANOFSKY

Department of Biological Sciences, Stanford University, Stanford, California 94305

Contributed by Charles Yanofsky, August 8, 1977

ABSTRACT **Transcription termination at the attenuators of the *trp* operons of *Escherichia coli* and *Salmonella typhimurium* was studied *in vitro* using DNA restriction fragments as templates. Readthrough transcription beyond the terminators occurred with 5 and 30% efficiency, respectively, in *E. coli* and *S. typhimurium*. This difference is correlated with the stability of proposed secondary structures of the respective *trp* leader transcripts. Secondary structure analyses of the two leader transcripts revealed a well-conserved pattern of RNA base pairing. This and the possibility that *trp* leader RNA is translated suggest a model for regulation of transcription termination that is based on ribosome movement along the RNA and a shift between alternative RNA base-pairing configurations.**

The tryptophan (*trp*) operon of *Escherichia coli* has two transcription control sites, a promoter-operator (1–3) and an attenuator (4). At the attenuator, a site located in the transcribed leader segment of the operon, transcription is either terminated or allowed to continue into the structural genes of the operon (4). Transcription termination at the attenuator appears to be regulated in response to changes in the extent of charging of $tRNA^{Trp}$ (5, 6). *Salmonella typhimurium* has a *trp* attenuator that functions much like the one in *E. coli* (F. Lee and C. Yanofsky, unpublished). The sequences of the leader-attenuator regions of the *trp* operons of both species have been determined (F. Lee, K. Bertrand, G. Bennett, and C. Yanofsky, unpublished.

In this paper we report the results of *in vitro* transcription studies with restriction fragments of the *trp* operons of both organisms. We show that the *trp* leader transcripts have appreciable secondary structure. We suggest how this structure may play a role in regulating transcription termination at the attenuator.

MATERIALS AND METHODS

Restriction fragments of the *E. coli* and *S. typhimurium trp* operons were derived from plasmids pVH153 (7) and pKB5 (ref. 8; G. Bennett, K. D. Brown, and C. Yanofsky, unpublished), respectively. In transcription experiments, fragments were added (final concentration 0.1–0.5 μg/ml) to reaction mixtures containing in 25 μl: 20 mM Tris acetate, pH 7.9; 0.1 mM dithiothreitol; 0.1 mM EDTA; 4.0 mM Mg acetate; 0.1 M KCl; three unlabeled nucleoside triphosphates at 0.15 mM each and one α-^{32}P-labeled triphosphate at 0.01–0.04 mM; and 30–50 μg of RNA polymerase per ml (9). Incubations were for 30 min at 37°. After phenol extraction and ethanol precipitation with 50 μg of carrier tRNA, samples were resuspended in 20 μl of 0.025% xylene cyanol and bromphenol blue containing 8 M urea. ^{32}P-Labeled RNA was analyzed by electrophoresis on 10 or 12% denaturing polyacrylamide gels in Tris/borate/EDTA at pH 8.3 containing 7 M urea (10). RNA samples to be subjected to limited RNase T_1 digestion were dissolved in 10 mM Tris, pH 7.9/10 mM $MgCl_2$/0.1 mM EDTA, and RNase T_1 was added to a final concentration of 10 units/ml. Incubations were carried out at 20°. Samples were withdrawn at various times, chilled, and then diluted with the same buffer. Diethylpyrocarbonate was added to 1% before the samples were precipitated with ethanol in the presence of carrier tRNA. They were then dissolved in urea/dye solution and loaded on denaturing gels. RNA fragments were eluted from polyacrylamide gels, digested to completion with RNase T_1, and fingerprinted (11).

All procedures using recombinant DNA were performed in accordance with the National Institutes of Health Guidelines.

RESULTS

Transcription Termination on *trp* Operon Restriction Fragments. Previous studies *in vitro* established that when purified RNA polymerase transcribes the *trp* operon of *E. coli*, it generally terminates transcription after synthesizing the first 140 residues of *trp* RNA (12). The 3′-OH termini of the *in vitro* RNA transcripts are at about the same position as the termini of the *in vivo* transcripts (13). Thus, *in vitro*, RNA polymerase must recognize the region of the *trp* operon that signals transcription termination *in vivo*. We have quantitated the frequency of transcription termination *in vitro* by using as templates restriction fragments of the *E. coli* and *S. typhimurium trp* operons on which the *trp* promoter is the only promoter (Fig. 1). The *E. coli* restriction fragment is 570 base pairs long and has approximately 260 base pairs following the site of transcription initiation, while the *S. typhimurium* DNA fragment is approximately 500 base pairs long and contains approximately 280 base pairs following the transcription start site (F. Lee, K. Bertrand, G. Bennett, and C. Yanofsky, unpublished). When each of these fragments is transcribed and the products are analyzed by electrophoresis on polyacrylamide gels, two major RNA bands are observed (Fig. 2). A prominent RNA species appears at the position expected for attenuator-terminated RNA 140 residues in length. In addition, a fainter band appears at a position corresponding to a molecule approximately twice this length. When these two bands are eluted and analyzed by two-dimensional fingerprinting after digestion with RNase T_1, the fingerprints of the shorter bands correspond to the first 140 nucleotides of *trp* RNA, while the longer bands correspond to the first 260 (*E. coli*) or 280 (*S. typhimurium*) nucleotides of *trp* RNA (Fig. 3). These longer bands must therefore represent readthrough message, resulting from transcription to the ends of the fragments. When the radioac-

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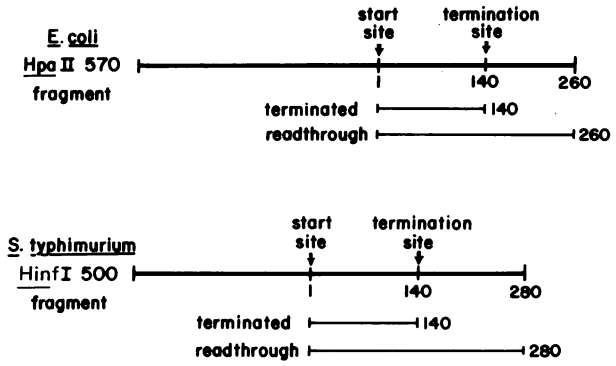

FIG. 1. Transcription of DNA restriction fragments containing *trpPOL* from *E. coli* and *S. typhimurium*. Shown are the expected lengths in nucleotides of the RNA transcripts arising from either transcription termination at the *trp* attenuator or readthrough transcription to the end of the DNA fragment.

tivity in each of these species is determined, we calculate that on a molar basis there is 5 and 30% readthrough, respectively, on the templates from *E. coli* and *S. typhimurium*.

The oligonucleotide map of the short *S. typhimurium* transcript (Fig. 3*C*), when related to the DNA sequence of the leader region, allows the site of transcription initiation to be assigned to approximately the same base pair as in *E. coli*. Fingerprints of the short *S. typhimurium* RNA transcript labeled with [α-^{32}P]UTP (not shown) reveal uridylate-rich 3′-oligonucleotides analogous to those found on the *E. coli* terminated transcript, indicating that termination of transcription within the *S. typhimurium* leader region occurs at approximately the same position as in *E. coli*. In *S. typhimurium*, termination is within the DNA segment corresponding to the T_1

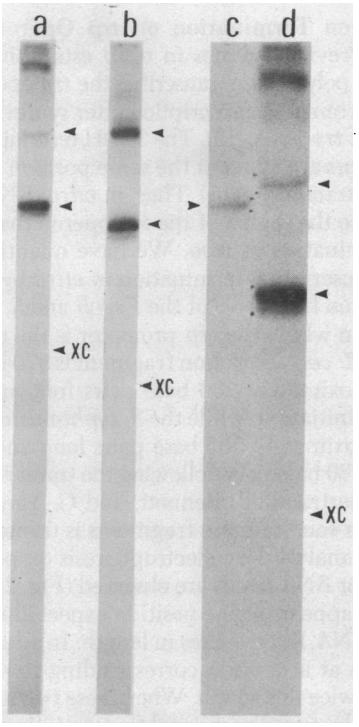

FIG. 2. RNA transcribed from either (a) the *E. coli Hpa* II 570 fragment, or (b) the *S. typhimurium Hin*fI 500 DNA fragment, resolved on 10% polyacrylamide Tris/borate/EDTA gels containing 7 M urea. Slots c and d show RNA transcribed from the *E. coli Hpa* II 570 fragment under normal conditions (d) or with ITP (150 μM) replacing GTP (c). XC designates the location of the xylene cyanol dye marker.

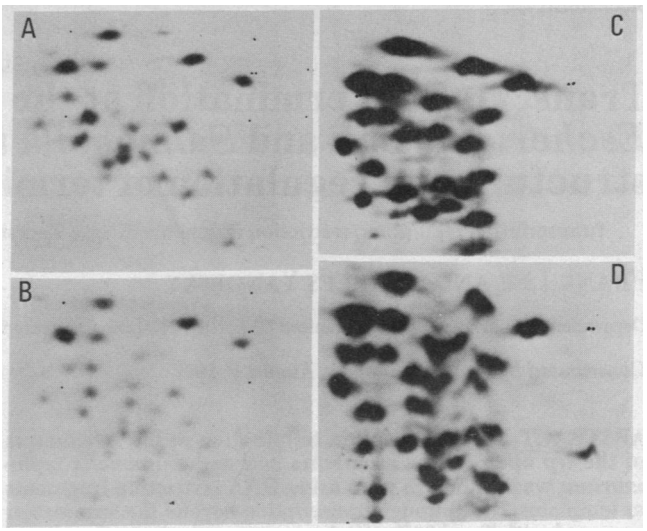

FIG. 3. RNase T_1 fingerprints of GTP-labeled terminated and readthrough transcripts synthesized on *E. coli* and *S. typhimurium* restriction fragments. All RNA samples were isolated from polyacrylamide gels such as those shown in Fig. 2. Electrophoresis was from left to right; homochromatography was from bottom to top. (*A*) Terminated RNA from *E. coli*; (*B*) readthrough RNA from *E. coli*; (*C*) terminated RNA from *S. typhimurium*; (*D*) readthrough RNA from *S. typhimurium*.

oligonucleotide U-U-U-U-U-U-G (F. Lee, unpublished). Estimates of the length of the two uridylate-rich 3′-oligonucleotides suggest that termination occurs at the end of the fifth and sixth uridylate residues.

Secondary Structures within Leader RNA. The DNA nucleotide sequences of the transcription termination regions in *E. coli* and *S. typhimurium* have many similarities (F. Lee, K. Bertrand, G. Bennett, and C. Yanofsky, unpublished). Among these are G-C-rich and A-T-rich sequence blocks preceding and including the termination site, respectively. The RNA sequences corresponding to these regions could form stable "stem and loop" secondary structures (F. Lee, K. Bertrand, G. Bennett, and C. Yanofsky, unpublished). To determine if such secondary structures exist, we performed RNase T_1 partial hydrolysis experiments with purified leader RNA under conditions where ribonuclease exhibits a preference for unpaired regions of RNA (14). The partial products were separated on denaturing polyacrylamide gels and digested to completion with RNase T_1; their component T_1 oligonucleotides were identified by two-dimensional fingerprinting.

When the 140-residue-long *E. coli* leader RNA is subjected to RNase T_1 digestion under conditions favoring base pairing, four prominent RNA species with different sensitivities to the enzyme are observed (Fig. 4 *left*). They are approximately 70, 40, 25, and 10–20 residues in length. The longest band is the most labile. Each of the bands was eluted from the gel and digested to completion with RNase T_1 and fingerprinted. Identification of the T_1 oligonucleotides from GTP- and UTP-labeled RNA and knowledge of the length of each partial product allow us to assign each partial product to its position in the overall sequence. Band 1 extends from residue 71 to the 3′ end of the molecule; band 2 extends from residue 101 to the 3′ end of the molecule; band 3 includes residues 71–93; and band 4 is a mixture of oligonucleotides from different regions of leader RNA (including the T_1 oligonucleotide from residues 81 to 93 and oligonucleotides in the vicinity of residue 50). Thus, specific regions of the leader RNA molecule are relatively insensitive to ribonuclease attack and therefore presumably are base paired to other regions.

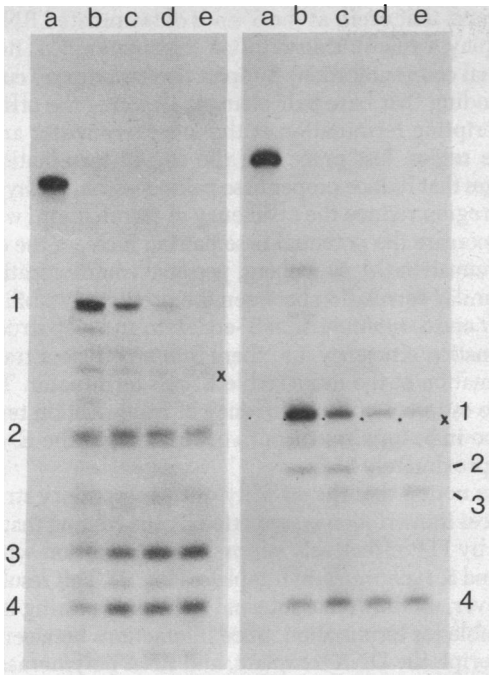

FIG. 4. RNA fragments resulting from partial RNase T_1 digestion of isolated, terminated leader RNA transcripts. (*Left*) *E. coli*. The undigested sample is in slot a; b–e are samples digested for 5, 15, 30, and 60 min, respectively, at 20°. (*Right*) *S. typhimurium*. The undigested sample is in slot a; b–e are samples digested for 5–60 min as for *E. coli*. All RNA products were resolved on 12% polyacrylamide Tris/borate/EDTA gels containing 7 M urea. ×, Location of the xylene cyanol marker dye.

On the basis of the partial digestion data and the known nucleotide sequence of the transcript, a structure such as the one pictured in Fig. 5 may be proposed. The features of this structure include significant base pairing between residues 74–85 and 108–119; these two regions are joined by a non-base-paired loop 22 residues long. As shown in Fig. 5, a region composed entirely of guanylate and cytidylate residues, from positions 115 to 119, is capable of base pairing with two different regions of the molecule, i.e., with nucleotides 74–78 or 129–133. The fact that in the partial product in band 1 all of the guanylate residues in these regions appear to be relatively resistant to RNase T_1 attack suggests that either a triple helical structure (15) exists or tertiary interactions occur. Tertiary interactions may also account for the RNase T_1 resistance of the guanylate residues at positions 93, 95, and 100 of the unpaired loop. As noted above, however, the longest RNA species, band 1, is the most labile of the partial products, suggesting that the loop region is moderately sensitive to RNase attack. Bands 2 and 3 correspond to products generated by digestion at the guanylate residues located at 93 and 100 within the loop. To evaluate the stability of the proposed structure, we considered separately the two possible stem and loop structures using the base pairing rules of Tinoco *et al.* (16, 17). For the region from residue 74 to residue 119, we calculate a $\Delta G \cong -10$ kcal (-42 kJ)/mol, while for the second stem and loop region, extending from residues 114 to 134, the $\Delta G \cong -20$ kcal (-84 kJ)/mol. Thus each of these regions is expected to be quite stable under physiological conditions.

A similar partial RNase T_1 hydrolysis analysis was carried out with isolated leader RNA from *S. typhimurium*, with similar results. The pattern of RNase T_1 resistant bands is shown in Fig. 4 *right*. Two major bands are seen (bands 1 and 4); the largest is approximately 60 residues in length and the smallest is about 10–15 residues long. Each of these bands from GTP- and UTP-labeled RNA was analyzed by complete RNase T_1 digestion and fingerprinting, and the component T_1 oligonucleotides were identified by relating them to the corresponding DNA sequence (F. Lee, K. Bertrand, G. Bennett, and C. Yanofsky, unpublished). Band 1 extends from approximately residue 77 to residue 132 (because of the series of guanylate residues around position 77, we are uncertain of the precise endpoint). This band is similar in location to the longest band from *E. coli* leader RNA, but is shorter and does not include the 3′ end of the molecule. Band 4 is analogous but not identical to the shortest species arising from *E. coli* leader RNA. It is composed of a mixture of oligonucleotides from the region 50 to 60. The other two bands, 2 and 3, are generally present in lower yield and are observed less reproducibly. These bands (Fig. 4 *right*) include the regions from approximately residues 42 to 74 (band 2) and residues 96 to 125 (band 3). Band 3 appears to be comparable in location to band 2 from *E. coli*.

It is possible to draw a structure for the *S. typhimurium*

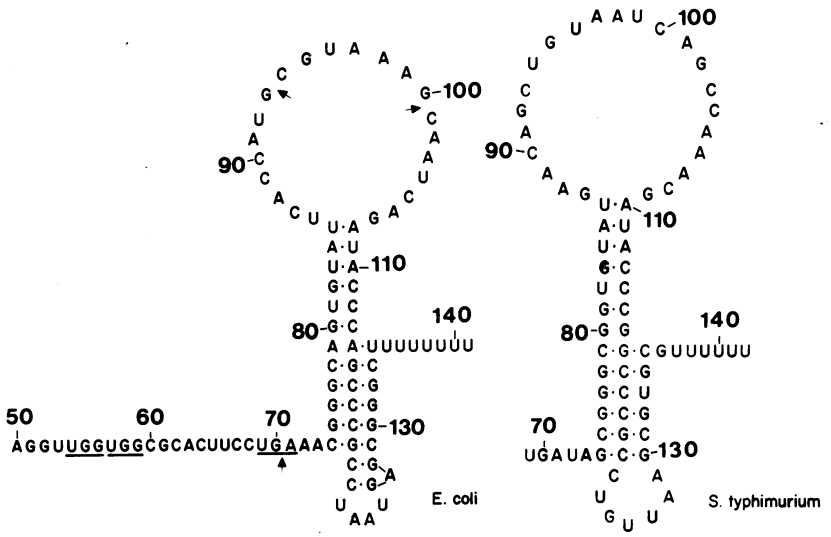

FIG. 5. The proposed secondary structure for the last half of the terminated leader RNA transcripts from *E. coli* and *S. typhimurium*. For the *E. coli* transcript the locations of the tandem tryptophan codons and the stop codon in phase with the tryptophan codons are indicated by underlining; the cleavage sites for the RNase T_1 partials are marked by arrowheads.

transcript that is quite similar to that proposed for *E. coli* (Fig. 5). Base pairing between two regions of RNA, residues 74–85 and 110–122, would explain the relative RNase resistance of the guanylate residues between residues 71 and 132 (band 1). The 3′ end of the RNA is not included in any of the bands, suggesting that the G-U bond at 136 is cleaved and the potential base pairs beyond residue 132 may not be as stable in *S. typhimurium* as in *E. coli*. In both cases, guanylate residues within the long unpaired loop are somewhat resistant to hydrolysis. The possibility of dual base pairing of one region of the RNA molecule (residues 117–122) also exists in the *S. typhimurium* structure and involves G-C base pairs exclusively. The nucleotide sequence of the region from residue 74 to residue 135 leads us to predict slightly different 3′ stem and loop structures for *S. typhimurium* than for *E. coli* (Fig. 5). If we consider the two possible stem and loop structures within the *S. typhimurium* leader RNA separately (15, 16), we arrive at $\Delta G \cong -15$ kcal/mol for the region from residues 74 to 122 and $\Delta G \cong -6$ kcal/mol for the region from 117 to 135. The lower expected stability for the 3′ stem and loop structure in *S. typhimurium* compared to *E. coli* (−6 compared to −20 kcal/mol) may account for the observed differences in the 3′ endpoints of some of the RNase-resistant bands, as well as the greater readthrough *in vitro* with the *S. typhimurium trp* operon fragment.

Transcription with ITP in Place of GTP. Substituting the analog ITP for GTP during RNA synthesis should result in the formation of RNA molecules in which G · C base pairs are replaced by weaker I · C base pairs. If RNA strand interactions are important in the termination process, we might expect ITP incorporation to alter the efficiency of transcription termination at the attenuator. When this possibility was tested with ITP and the *Hpa* II 570 fragment from *E. coli*, the terminated leader transcript was absent and a single longer species was produced (Fig. 2*c*). Although this species has a different mobility from that of the control (GTP) readthrough species, it gave an identical oligonucleotide fingerprint pattern. Apparently, then, RNA in which inosinate replaces guanylate has altered electrophoretic mobility. The striking result is that in the presence of ITP the readthrough species is made almost exclusively. Similar results were obtained with ITP and the *S. typhimurium Hin*fI fragment.

DISCUSSION

Secondary Structure of *trp* Leader RNA and Transcription Termination. Both *E. coli* and *S. typhimurium* contain within their *trp* operon leader regions transcription termination sites recognized by *E. coli* RNA polymerase *in vitro*. By using specific restriction fragments from each operon as templates for transcription, the amount of readthrough transcription that occurs at each attenuator can be quantitated. Under identical conditions there is 30 and 5% readthrough, respectively, with *S. typhimurium* and *E. coli* DNA. The nucleotide sequences of the *trp* attenuator regions of the two species, although similar in general structure, have a number of differences in the transcribed regions just preceding the termination sites (F. Lee, K. Bertrand, G. Bennett, and C. Yanofsky, unpublished). These differences probably account for the nonidentical efficiencies of transcription termination since the nucleotide sequences beyond the termination sites are highly conserved in the two species (F. Lee, K. Bertrand, G. Bennett, and C. Yanofsky, unpublished). The DNA sequences beyond the termination sites in phage λ are also thought not to function in transcription termination (18).

It has been suggested that the "stem and loop" secondary structures that occur at the 3′ ends of terminated RNA species may play a role in transcription termination (M. Rosenberg, personal communication). Support for this proposal comes from the finding that base pair changes affecting the efficiency of transcription termination at the λ t_{R1} terminator are located in the region just preceding the site of termination. Those changes that reduce proper base pairing within the hypothetical stem region reduce the efficiency of termination, while those that increase the potential base pairing increase the efficiency of termination (M. Rosenberg, personal communication). There is a similar correlation between the predicted stabilities of the *E. coli* and *S. typhimurium* 3′-end stem and loop structures and termination efficiency, i.e., there is more efficient transcription termination at the more stable *E. coli* terminator. There is as yet no estimate of the efficiency of transcription termination *in vivo* in *S. typhimurium* to compare with the *E. coli* value of approximately 85%.

The notion that the stability of this secondary structure influences transcription is supported by the finding that replacing GTP by ITP effectively suppresses termination with both *E. coli* and *S. typhimurium* templates (Fig. 2). This result does not, however, establish that internal RNA base pairing is solely responsible for termination, since interactions between the RNA transcript, the DNA template, and RNA polymerase may also be involved.

The results of partial RNase T_1 digestion experiments on leader RNAs from *E. coli* and *S. typhimurium* suggest that there is extensive base pairing between certain regions within each of these molecules. Secondary structures that are consistent with our data are pictured in Fig. 5. A comparison of the nucleotide sequences of the two molecules reveals that the regions involved in base pairing are the most highly conserved, implying that the secondary structure may be functionally important. Also present in each RNA is a segment composed primarily of guanylate and cytidylate residues which can base pair with two different regions of the transcript. This raises the possibility that the RNA molecule can exist in alternative secondary structures (Fig. 5). In *E. coli*, if the first stem and loop were formed, it would extend from residue 74 to residue 119; in this form, residues 115–119 would be base paired to residues 74–78. In the other form, the 3′ stem and loop would exist; in this form, residues 115–119 would be base paired to residues 129–133. Analogous structures are possible for *S. typhimurium*. If these alternative stem and loop structures can form independently, and if each has a different effect on transcription termination, regulation could involve shifting from one form to the other under different physiological conditions. Interestingly, Weidner *et al.* (19) have recently proposed a functional role for a possible shift between alternative base-pairing configurations for 5S ribosomal RNA.

Translation of Leader RNA and Transcription Termination. In considering mechanisms for the regulation of transcription termination at the *trp* attenuator, we must entertain the possibility that a segment of leader RNA is translated. A ribosome binding site early in the *E. coli trp* leader RNA has been identified (20), and this site can be used to initiate translation *in vivo* and *in vitro* (ref. 21; G. Miozzari and C. Yanofsky, unpublished). The similarity in nucleotide sequence of the corresponding region of *S. typhimurium* suggests that it contains a similar site. In each case a 14-amino-acid peptide may be coded by the RNA segment extending from the AUG codon at residues 27–29 to the translation stop codon around residue 70. The amino acid sequences predicted for the two peptides are identical for the last six residues and include tandem tryptophan residues, at positions 10 and 11 (F. Lee, K. Bertrand, G.

Bennett, and C. Yanofsky, unpublished). The unusual occurrence of tandem tryptophan residues at the same positions in both hypothetical peptides leads us to suspect that they are functionally important; in particular, we propose that translation of the two tryptophan codons may regulate transcription termination at the attenuator. We would expect that *in vivo*, when tryptophan is abundant, ribosomes would proceed beyond the tandem tryptophan codons in leader RNA to the in-phase translation stop codon at about position 70 (Fig. 5). A ribosome at this position would interact with or mask 10 or more residues downstream from the translation stop codon (22) and would be expected to interfere with the formation of the first stem and loop structure. The RNA segment from approximately residue 110 to 120 residue would then be available to form the 3′ stem and loop, which could signal termination of transcription. Alternatively, when tryptophan is limiting, ribosomes would stall at the tandem tryptophan codons at residues 54–59, allowing formation of the first stem and loop structure. If the existence of this structure either precluded the formation of the 3′ stem and loop or altered the overall tertiary structure of this region, transcription termination could be prevented. Thus, according to this model, translation or nontranslation of the tryptophan codons would affect the secondary structure of the RNA transcript and thereby regulate transcription termination.

Other Considerations. A mechanism such as the one proposed is consistent with much of the evidence concerning regulation of attenuation. For example, it is not the concentration of tryptophan that directly affects termination but the concentration of charged or uncharged $tRNA^{Trp}$ (6). In disagreement with the model as presented are the findings that starvation of *E. coli* for either isoleucine or arginine, two amino acids present in the hypothetical leader peptide of *E. coli*, does not relieve termination at the attenuator (5, 23). Whether it is the tandem tryptophan codons or their unique position that is required for eliciting the proper response or whether there is an additional role for $tRNA^{trp}$ in regulating termination is unknown. It has also been observed that strains carrying the altered *su*$^+$7 $tRNA^{Trp}$, which reads UAG instead of UGG and which becomes charged with glutamine in preference to tryptophan, show appreciable termination even when starved of tryptophan (6). This result suggests that the $tRNA^{Trp}$ molecule plays a regulatory role in addition to its participation in translation. Also of relevance is the fact that termination of transcription at the attenuator occurs efficiently *in vitro* with both *E. coli* and *S. typhimurium* templates, despite the obvious absence of translation. This result may be one of several differences between transcription termination *in vivo* and *in vitro*. For example, termination factor *rho* is not required for efficient termination *in vitro* but apparently is involved *in vivo* (24, 25). It is possible, however, that the effect of *rho* mutations on attenuation is indirect. In addition, a deletion removing the distal four of the series of eight A-T base pairs in the *E. coli* attenuator abolishes termination *in vitro* yet allows significant termination (50%) *in vivo* (13). These results are not explained by the simplest version of the model we have presented.

Despite the apparent inconsistencies mentioned, our current findings force us to consider the possibility that RNA secondary structure and translation play crucial roles in regulation of transcription termination at the *trp* attenuator. Studies on the regulation of the *his* operon of *S. typhimurium*, a gene cluster that may have a similar mechanism of termination control, indicate that translation is a prerequisite for transcription (26), a situation contrary to the one we suggest for the *trp* operon. The model we have proposed, along with the findings with the *his* operon, and recent studies on translational polarity (27, 28), recall previous thoughts on how transcription and translation may be functionally coupled (29).

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