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Alternative secondary structures of leader RNAs and the regulation of the *trp, phe, his, thr,* and *leu* operons

(attenuation/transcription termination/transcription read-through/enteric bacteria)

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ABSTRACT **The *trp, phe, his, thr,* and *leu* operons of enteric bacteria are regulated by a transcriptional attenuation mechanism. Under conditions of amino acid sufficiency, transcription terminates at an attenuator site after a leader of about 150 nucleotides has been synthesized. Under conditions of limitation of a controlling amino acid, transcription continues past the attenuator into adjacent structural genes. As demonstrated by others, each of the five leader RNAs contains two regions of potential secondary structure which are partially overlapping. One of these regions occurs at the 3′ terminus of the leader and is named the "terminator." The other region, which potentially can preclude the formation of the terminator, is named the "preemptor." Conditions that allow the preemptor to form result in derepression. We report here that the five published leader RNA sequences contain an additional potential region of secondary structure, which we call the "protector." The protector partially overlaps the preemptor in such a way that pairing of the former precludes pairing of the latter. For derepression to occur, a ribosome that is translating the leader must block the protector without blocking the preemptor, a condition that is met when the ribosome is arrested at the 3′ end of a set of control codons. Including the protector in the model for attenuation explains why derepression of the operon does not result from the arrest of a ribosome at a codon preceding the control set. It also explains why termination is the outcome when transcription occurs in the absence of ribosomes. Finally, termination is the predicted outcome when unfettered translation of the leader RNA occurs, resulting in release of the ribosome at the translational stop signal.**

Evidence that genes may be controlled by a transcription termination mechanism was first obtained by Roberts (1) in studies with λ phage. Transcription initiated at promoters P_L and P_R is doomed to early termination unless a phage-encoded protein allows transcription to continue (i.e., "readthrough") into adjacent regions. A similar regulatory mechanism, termed "attenuation," has recently been found in the case of the *his* (2–5), *trp* (6–10), *phe* (11), *thr* (12), and *leu* (13) operons of enteric bacteria. The following are basic features of this attenuation mechanism.

(*i*) Most transcription initiated at the relevant promoter terminates before the structural genes are reached, resulting in the synthesis of a leader RNA of about 150 nucleotides (6–8, 11, 13).

(*ii*) The site at which termination occurs ("attenuator") is similar to previously identified transcription termination sites (for a review, see ref. 14). It is a palindromic G·C-rich region followed by a series of adenosines on the coding strand (4, 5, 8, 11–13). The corresponding region of the leader RNA, which has a potential stem-and-loop structure followed by a series of uridines, is called the "terminator." A point mutation in the palindromic sequence of the attenuator which would reduce the potential self-pairing of the DNA chains and of the terminator of the leader RNA reduces the frequency of transcription termination, thus resulting in derepression of the operon (refs. 12 and 15; unpublished data).

(*iii*) Each of the five leader RNAs contains a second potential stem-and-loop structure proximal to the terminator and overlapping it in such a way that pairing of one region precludes pairing of the other (5, 8, 11–13). Lee and Yanofsky (8) postulated that formation of the proximal stem and loop allows derepression by preventing the formation of the terminator. In support of this suggestion, Zurawski *et al.* (10) showed that a mutant in which the proximal stem and loop is destabilized does not respond to specific amino acid starvation.

(*iv*) Within each leader RNA, translational start and stop signals are positioned so that a peptide of 14–28 amino acids might be synthesized (4, 5, 9, 11–13). In case of the *trp* operon, there is indirect evidence that the leader is translated (16, 17).

(*v*) Each leader peptide has a tandem set of codons for the specific amino acid(s) regulating the operon (4, 5, 9, 11–13).

(*vi*) Extensive transcriptional read-through requires positive control factors not present in a purified *in vitro* transcription system (2, 3, 6, 7, 18). Transcriptional read-through requires that a ribosome initiate the synthesis of the leader peptide (10), which means that a ribosome may be considered as a positive control factor (5). In fact, the whole complex of factors required for protein synthesis cooperates to give positive control (3).

(*vii*) In addition to positive factors, extensive transcriptional read-through requires a lower-than-normal level of a specific aminoacyl-tRNA (2, 19–21).

Lee and Yanofsky (8) made a major contribution to our understanding of this complex regulatory mechanism when they developed the arrested-ribosome model to explain the regulation of the *trp* operon. In this model, control of attenuation is a consequence of the secondary structure of the leader RNA which in turn is determined by the progress of the ribosome translating the RNA. When the cell is deficient in tryptophan, a ribosome translating the nascent *trp* leader RNA becomes arrested at one of the tandem tryptophan codons. In this situation, the proximal alternative stem-and-loop structure forms and prevents the formation of the terminator, thus allowing transcription read-through. When the cell has sufficient tryptophan, the ribosome proceeds past the tryptophan codons and, in so doing, prevents the proximal stem and loop from forming. The result is that the terminator forms and transcription is terminated.

The elegant experiments of Yanofsky and coworkers have provided support for this model, which has been adopted to explain regulation of the *phe* operon (11) and, with some modifications, the *his* (5), *thr* (12), and *leu* (13) operons. However, the model does not provide answers to several questions. For example, why does derepression not occur during limitation for amino acids other than the control ones? How is it prevented when the ribosome is released from the stop codon

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before the polymerase reaches the attenuator? This question is significant for those cases such as the *his* leader in which a long sequence occurs between the stop codon and the terminator (5). Why does termination at the attenuator occur at high frequency in a purified *in vitro* transcription system in the absence of protein synthesis (2, 6, 8, 11, 13)?

An analysis of the secondary structure of the five leader RNAs, given below, uncovered features that are remarkably similar in the five RNAs and help to answer the questions posed above. These common features together with those previously noted provide the basis for a slightly modified arrested-ribosome model presented in preliminary form by Gemmill *et al.* (13) and in more complete form here.

Predicting the alternative secondary structures of the five leader RNAs

A complete and systematic search was made for stable pairing regions in each of the leader RNAs to locate significant potential structures in addition to the ones published with the sequences. The search used a stringent program (to be published elsewhere by E.B.K.) that requires all primary pairing regions to have a free energy of −7 kcal/mol (1 cal = 4.184 joules) or a more negative value when the helix is evaluated by the rules of Borer *et al.* (22) and Tinoco *et al.* (23). Weaker pairing regions, termed "secondary regions," are allowed only under a set of stringent conditions. A secondary region must be adjacent to a primary region—i.e., separated from it by no more than a few mismatched bases or a single bulged-out base in one strand. Furthermore, formation of an acceptable secondary region requires at least two consecutive base pairs if they are both G·C pairs or otherwise at least three base pairs. G·U pairs are treated as mismatched bases.

Use of these stringent rules permitted detection of a number of pairing regions that are similar in the five leader RNAs and would have sufficient stability to direct the folding of the leader RNA during synthesis. The new regions together with the previously published regions in the five leader RNAs are presented in Fig. 1. Each potential stem and loop structure is shown

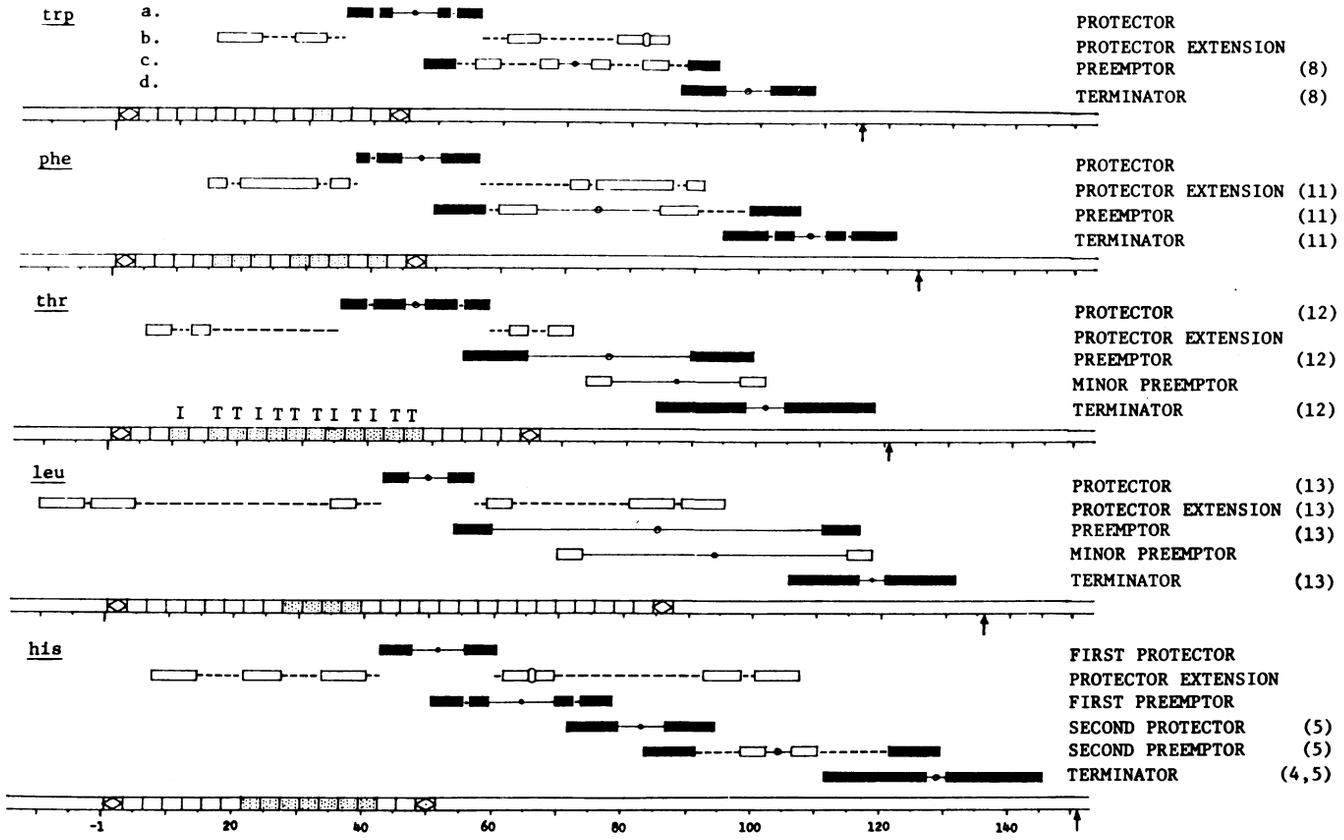

FIG. 1. Potential pairing regions in the leader RNAs of the *trp* (9), *phe* (11), and *thr* (12) operons of *Escherichia coli* and the *his* (5) and *leu* (13) operons of *Salmonella typhimurium*. Potential stem-and-loop structures are presented in the order in which they would form during synthesis, with the first one to form being on the top line. Each potential structure is presented on a single line except in the case of the protector regions which are separated into protectors and protector extensions. The complementary sequences that form a pairing region are represented by two equal rectangles; the unpaired bases forming the hairpin loop are represented by a line joining them. When a stem is formed by more than one region, the rectangles are joined by dashes representing the bases of the interior loops (23). Filled rectangles are pairing regions that are indispensable for the attenuation control mechanism. Open rectangles are auxillary pairing regions that play a secondary role. When a pairing region has a single bulged-out base in one strand, this base is represented by a bulge in one of the half-regions (see line b). When a pairing region has been described, the reference is given at the right. The leader RNA is represented by a double line below the pairing regions. Numbering starts with the known or presumptive initiating AUG. Translational start and stop signals are shown as diamonds on the RNA strands, and the tandem control codons are stippled. In the case of the *thr* leader, these are labeled T for threonine and I for isoleucine. The message portion of the leader is divided to indicate the location of the codons. Arrows point to the approximate position on the RNAs where termination occurs.

The pairing regions that have not been described previously are given here in order from top to bottom for each leader. Regions presented on a single line in the figure are grouped together. Numbering starts with the adenosine of the initiating AUG. *trp* leader: 36–39 and 41–42 with 50–51 and 53–56; 16–22 and 28–32 with 61–65 and 78–81 and 83–85; 66–68 with 74–76. *phe* leader: 38–39 and 41–44 with 51–56; 60–65 with 85–90. *thr* leader: 6–9 and 13–15 with 62–64 and 68–71; 73–76 with 98–101. *leu* leader: 34–37 with 59–62; 72–75 with 105–108. *his* leader: 43–47 with 56–60; 8–14 and 22–27 and 34–40 with 62–65 and 67–69 and 93–98 and 101–107; 51–55 and 57–59 with 70–72 and 74–78; 99–102 with 107–110.

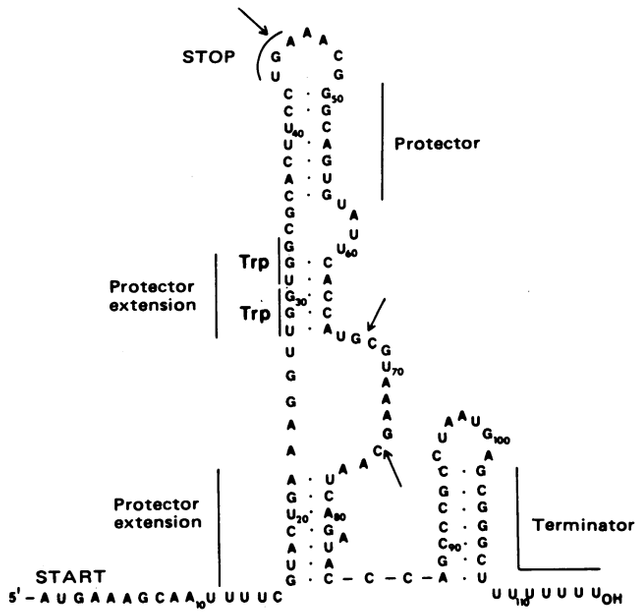

FIG. 2. A secondary structure predicted for the *trp* leader RNA synthesized in the absence of ribosomes. The arrows point to the linkages in the *in vitro* synthesized product that are most susceptible to RNase T1 hydrolysis (8). The 5′ noncoding region is not shown. Numbering starts with the initiating AUG.

on a separate line. In some cases the stem of a structure is formed by two or three neighboring paired regions presented together on a line.

The pairing regions are presented in a fashion that stresses the order in which they are synthesized. The first regions that may pair, necessarily at the 5′ end of the leader, are placed on the top line. The second regions that may pair (assuming no interference from preceding pairing regions) are identified on the second line, and so on. The bottom line depicts the terminator pairing region, always at the 3′ terminus of the leader. Because in many cases pairing regions overlap each other, the complete structure of a RNA is found by starting with a pairing region near the 5′ end and, proceeding downward, finding the next structure that is distal to it and that can coexist with it in the same molecule, and so on to the 3′ end. The structure of the RNA is thus determined by its biosynthetic pathway and is not necessarily the same as the structure achieved at thermodynamic equilibrium (24). As soon as the distal half of a pairing region is released from the polymerase, the region will fold up very rapidly [the time required for an intramolecular helix to develop is less than 50 μsec (25)]. The time scale over which attenuation operates is in the range of 1 to 5 sec [polymerase travels at 45 nucleotides per sec at 37°C (26)], whereas the half-time for disruption of the paired regions considered here is in the range of minutes (27, 28). Therefore we believe the important consideration is which pairing region forms first and not which pairing region is strongest.

To facilitate discussion of the multiple pairing regions in Fig. 1, they have been given names descriptive of their proposed functions. The transcription termination stem and loop (4, 5, 8, 11–13) is called the "terminator." The alternative stem and loop that would preclude the formation of the terminator (5, 8, 11–13) is called the "terminator preemptor" or simply the "preemptor." The stem and loop that precludes the formation of the preemptor and thus allows the terminator to form is called the "terminator protector," or "protector" for short. Finally, a pairing region that extends the length of the protector stem but is not essential for the protector function is termed a "protector extension."

A predicted structure of the *trp* leader RNA synthesized in the absence of ribosomes

The foregoing principles are first applied to the situation in which the *trp* leader RNA is synthesized in the absence of ribosomes. The secondary structure would start to form with the folding of the pair of protector regions shown on line a of Fig. 1 and would be extended by the bonding of the two protector extension regions on line b. At this point, the preemptor on line c cannot form because it is blocked but, as the chain grows, the terminator on line d can form. Under these conditions of synthesis, the predicted initial structure (Fig. 2) also happens to be the most thermodynamically stable structure predicted for the isolated leader RNA. Support for this structure comes from an analysis of the isolated *trp* leader by Lee and Yanofsky (8), who found the RNA to be largely resistant to RNase T1 digestion and concluded that it has extensive secondary structure. The few points that they found to be susceptible to RNase T1 are indicated by arrows in Fig. 2. The fact that these points are all in single-stranded sequences in the predicted structure provides support for the model and for the proposal that the protector regions on lines a and b of Fig. 1 are stable regions.

Protectors are found in all five operons and all have similar features

Fig. 1 shows the existence of protector structures in all five leader RNAs and that these structures are similar in the five leaders. Each protector has a stem made up of one or two short helices with a loop of no more than eight unpaired bases. Loops of this size have been shown to be optimal for rapid loop closure and for stability of stem-and-loop structures (23). In each case the protector overlaps the outermost helix of the preemptor. Because it is only the outermost helix of the preemptor that overlaps the terminator (Fig. 1), this is the region that must be blocked for termination to occur. In order to make these relationships clear, the crucial regions in each RNA—i.e., the protector, the outermost helix of the preemptor, and the terminator—are shown as black boxes in Fig. 1. The inner helices of the preemptors are shown as open boxes to indicate that their role in regulation is not decisive. There are no inner preemptor helices in the case of *thr* (12) or *leu* (13) leaders (Fig. 1). Where they do occur in the *trp*, *phe*, and *his* leaders (Fig. 1) their prior folding would increase the probability of folding of the outermost helix. In this way their presence could have an indirect influence on the extent of derepression.

Protectors ensure attenuation when translation is unfettered

Under conditions of amino acid sufficiency, a ribosome is expected to move past the control codons at a normal rate. Without the postulated protector function, one must make an assumption in order to explain termination—namely, that the ribosome pauses at the translational stop codon long enough for the polymerase to transcribe the terminator region. In the most extreme case, the *his* operon, the pause of the ribosome would have to be longer than 2 sec if the RNA polymerase travels 45 nucleotides per sec. (26). If, instead of pausing, the ribosome were released and if there were no protector region, the preemptor would form and read-through would be the result of unfettered translation. The existence of protectors in each of the leaders explains why this does not occur. If the ribosome were released from the leader immediately upon reaching the stop codon and if, at this moment, the second half of the outermost region of the preemptor were not yet synthesized, the protector will pair and then, as the terminator is synthesized, the latter will pair and result in termination. In the case of the

trp leader the structure at termination could be the one in Fig. 2. The structure postulated for the *leu* leader at termination under these conditions is shown in Fig. 3A.

Protectors explain in part the specificity of derepression

Operons involved in amino acid biosynthesis are derepressed upon limitation for one (or at most a few) amino acids. Part of this specificity may be explained by considering the effect of a ribosome's stalling at a leader codon that precedes the control codons. For example, the ribosome in Fig. 3B is stalled at an arginine codon (13) in response to a severe limitation in the intracellular concentration of arginine. The positioning of the protector is such that a ribosome stalled before the control codons does not interfere with protector formation. Again, the consequence of protector region pairing is termination.

All five leaders show several potential pairing regions, called "protector extensions" (Fig. 1) because they can extend the length of the protector stem. These are placed on a separate line from the protector and shown as open boxes because they are not essential for blocking of the preemptor. There is some variability in the position of these protector extensions but in no case do they extend so far distally as to overlap the terminator. Except in the case of the *thr* leader, if a ribosome were arrested on one of the early noncontrolling codons, one or more of the protector extension regions could form in addition to the protector. A situation of this type is illustrated in Fig. 3B.

The *his* leader has a more complex structure than do the other four leaders (Fig. 1). If a ribosome were arrested on one of the proximal noncontrolling codons, attenuation would be achieved by the formation of the first protector, which would preclude the first preemptor. Then the second protector could form and preclude the second preemptor. The result would be termination.

Protectors explain attenuation in the absence of translation

Lee and Yanofsky (8) found that, when *trp* transcripts were formed *in vitro* with purified RNA polymerase, 95% of the chains were terminated at the attenuator. This fact is not readily explained by a model involving only a preemptor and terminator because under the conditions of their experiment the preemptor should form first. On the other hand, it is explained neatly by taking into account that, in the absence of translation, protector regions are the first to pair (Fig. 2) and that, once these have paired, termination is the expected outcome.

In the case of the *his* (2), *phe* (11), and *leu* (13) operons also, transcription in a purified *in vitro* system has been found to be largely terminated at the attenuator. The protector regions found in these leaders (Fig. 1) provide the needed basis for this termination as in the case of the *trp* leader. The structure postulated for the *leu* leader at the moment of termination in the *in vitro* system is the one presented in Fig 3A.

Derepression occurs when the protector is blocked and the preemptor is free to form

According to the arrested ribosome model (8), read-through at the attenuator occurs when a ribosome is arrested at a control codon in situations that allow the preemptor to form. In view of the structures shown in Fig. 1, the situation may be defined more precisely as follows. For derepression to occur, the ribosome must pause or be arrested at a position such that it masks the first half of the protector (so that the protector helix cannot form) and yet does not mask the preemptor. Masking means that the ribosome prevents the RNA from being in a double helix. Masking appears to extend about 10–11 nucleotides downstream from a codon in the A site, because arginine starvation but not threonine starvation derepresses the *trp* operon (10).

The conditions for derepression of the *leu* operon are illustrated in Fig. 3C. The structures are shown at the moment when

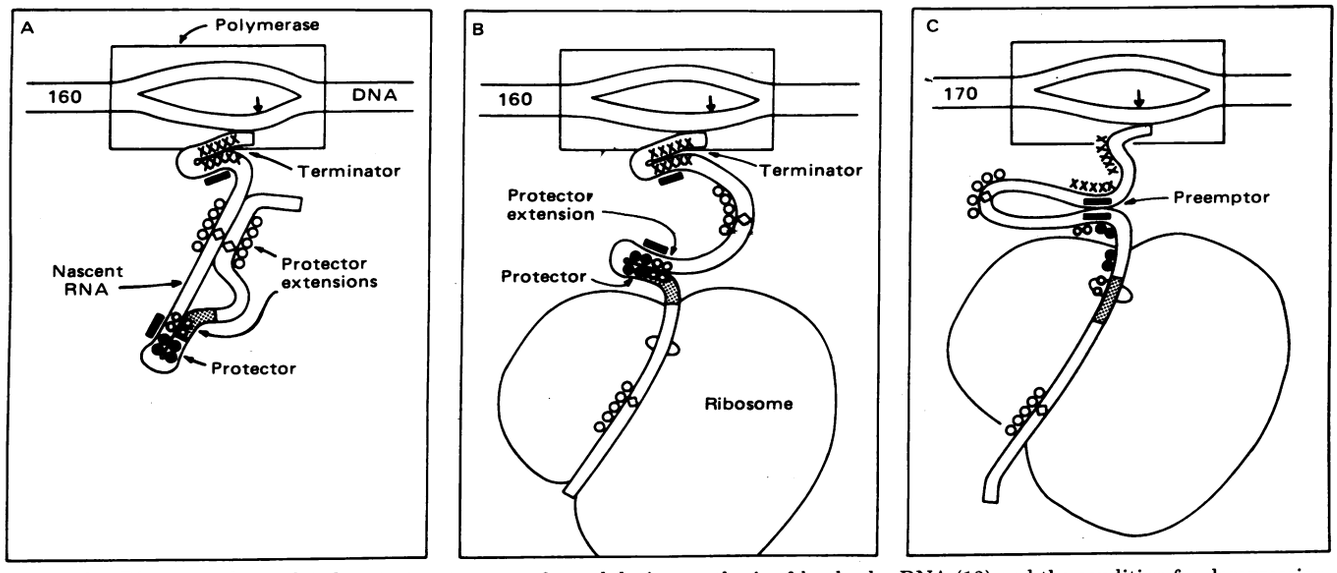

FIG. 3. Diagrams of postulated protector structures formed during synthesis of *leu* leader RNA (13) and the condition for derepression of the operon. (*A*) Transcription nearing termination when the translating ribosome has been rapidly released from the stop codon. (*B*) Transcription nearing termination under conditions such that a ribosome is arrested at a codon proximal to the leucine codon set. (*C*) Transcription read-through under conditions such that a ribosome is arrested at one of the leucine codons. DNA, RNA, RNA polymerase, and ribosomes are drawn approximately to scale but the shapes are schematic. An oval indicates the position of the A site on the ribosomes. The number on the DNA is the number of transcribed nucleotides, assuming that transcription starts 26 nucleotides before the translational initiator AUG (13). The presumed termination point in the coding strand of DNA is indicated by an arrow. Symbols on the nascent RNA are as follows: stippled area, leucine codons; diamonds, translational start and stop codons; XXXX, terminator; ●●●, protector; 000, protector extensions; ■, preemptor.

the ribosome has just reached the third leucine codon and transcription read-through has just occured. The protector is masked and the preemptor is in place.

In the case of the *his*, *phe*, and *thr* leader RNAs, which have long sets of control codons, a ribosome must be arrested at one of the distal codons of the set if it is to mask the first half-region of the protector and cause derepression. This observation leads to the idea that, in these operons, the attenuation mechanism is designed to deal with the early stages of a developing deficiency. A reduction in the concentration of specific aminoacyl-tRNA would decelerate the ribosome at each of the control codons of the long set, so that the ribosome would arrive at a distal control codon and mask the protector at the crucial moment when the polymerase is completing the transcription of the termination signal. With the protector masked, the preemptor would be in place and read-through would result. The particular conjunction of events leading to derepression would not occur if the ribosome progressed more rapidly through the control codon set and masked the preemptor at the crucial moment. It also would not occur if the ribosome proceeded so slowly through the control codon set that it remained lodged at the crucial moment at the proximal end of the set, where it would not mask the protector.

The model for derepression of the *his* operon is more complex than that for the other four operons. Derepression would occur when a ribosome, arrested on a distal histidine codon, masks the first protector (Fig. 1). The first preemptor could then form and block the second protector. Then the second preemptor could form and preclude the formation of the terminator, thus allowing read-through transcription.

Role of the protector in the attenuation mechanism

In general it can be said that a particular secondary structure in a RNA is recognized as being significant when it is consistently found in different examples of that RNA. This has been true for the terminator (4, 5, 8, 10, 12–14) and the preemptor (4, 5, 8, 10, 12, 13), as it was earlier for tRNAs and 5S RNAs. In this paper, potential protector structures have been shown to be present in all the published leader RNAs. The consistent finding of the structures suggests that they have an essential role in the attenuation mechanism. Support for this conclusion comes from the fact that these structures can explain the results of *in vitro* experiments using purified RNA polymerase (termination is the usual outcome) and the results of *in vivo* specificity experiments [derepression occurs upon limitation for only one or a few amino acids (10)]. Additional support has recently been obtained from an analysis of the sequence of the *leu* leader of *E. coli* (unpublished data). When this sequence is compared with that of the *leu* leader of *S. typhimurium* (13) the sequences are seen to differ in many positions along the chain, but the sequences required for the protector, as well as those required for the preemptor and terminator, have been entirely conserved. Further support for a physiological role for the protector could come, as it did in the case of the terminator (refs. 12 and 15; unpublished data) and the preemptor (10), from mutants in which the structure is destabilized. In the case of the protector, such a mutant would be derepressed by a deficiency of amino acids that normally do not regulate the relevant operon.

Note Added in Proof. An analysis of attenuation similar to that presented here has been developed by Oxender *et al.* (29) and Johnston *et al.* (30).

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