**Communication**

Transcription Analyses with Heteroduplex trp Attenuator Templates Indicate That the Transcript Stem and Loop Structure Serves as the Termination Signal*  

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DNA sequences that control transcriptional termination by prokaryotic RNA polymerases normally contain an inverted repeat, or self-complementary sequence, about 10 base pairs upstream from the site of RNA chain termination. Point mutations that interrupt this self-complementarity can reduce or eliminate RNA chain termination. We have constructed heteroduplex DNA templates using wild type and mutant attenuators for the _Escherichia coli_ trp operon to probe the relative contributions of the two DNA strands in the termination process. Transcription analyses show that only the sequences in the transcribed DNA strand determine whether or not a heteroduplex terminator can function. This result strongly supports a model in which the formation of a stem and loop structure in the nascent RNA transcript is the signal for transcriptional termination.

Transcriptional termination sites are important regulatory loci in both prokaryotic and eukaryotic genomes (1-4). Terminator sites most often are used to restrict transcription to defined genetic units. However, many such sites can also act conditionally to allow either termination or transcriptional readthrough and are termed attenuator sites (3, 4). The biochemical mechanisms by which DNA sequences at termination sites act to stop transcription or to permit readthrough are not well understood. Bacterial termination sites show certain sequence similarities 30-40 base pairs upstream from the base that determines the 3′OH terminus of the completed transcript (defined here as base −1). In particular, the RNA sequences from −1 to −9 are often rich in uridine residues, especially for strong terminators that are effective in _vitro_ even in the absence of accessory termination factors. Additionally, the DNA sequences from about −10 to −40 normally contain an inverted repeat sequence from 3 to 12 bases in length (1-4). Since these sequences are self-complementary in the RNA transcript, they can interact to form a duplex stem-loop structure in either the RNA or either DNA strand. The inverted repeat sequences at a terminator play an essential role in termination since point mutations in either arm of the repeat which interrupt the self-complementarity of the sequences can reduce or eliminate termination at that site (2, 3). Similarly, mutations that alter the AT-rich sequence proximal to the termination site can also reduce or eliminate termination (5, 6).

Many workers have adopted the _ad hoc_ hypothesis that the critical step in termination involves formation of a stem-loop structure in the nascent RNA transcript which acts somehow to prevent further RNA chain elongation by the RNA polymerase in the DNA-RNA complex, and indirect evidence from several kinds of experiments is consistent with this view (3-5). However, there is no direct evidence that an RNA duplex structure is involved. Elongation by RNA polymerase is unaffected by addition of single-stranded or duplex RNA (7, 8) or polynucleotide analogues (9), and termination is not eliminated in high salt concentrations which would be expected to eliminate binding of a duplex structure to most protein binding sites (10). Furthermore, models can be designed in which elongation is blocked due to formation of a stem-loop structure in the nontranscribed DNA strand, or through formation of a DNA-RNA complementary duplex between the nascent RNA and the complementary region of the nontranscribed DNA strand (11). These latter two models can be distinguished from the RNA duplex model since they require a role for the DNA sequences in the nontranscribed DNA strand, while the RNA duplex model does not.

We have devised an experiment to test the relative role of sequences in the two DNA strands in transcriptional chain termination, using point mutations in the _Escherichia coli_ trp attenuator sequence that disrupt the complementarity of the inverted repeat sequence, and permit transcriptional readthrough in _vitro_ (3). We construct heteroduplex templates in which the mutant terminator sequence is in one strand and the wild type terminator sequence is in the other (see diagram in Fig. 1). Transcription of both of the two possible heteroduplex templates should reveal whether one of the two DNA strands plays a dominant role in the process and, if so, which one.

We have employed three _E. coli_ trp attenuator mutants, isolated and generously provided to us by Charles Yanofsky and his co-workers (12, 13). _trp_ L153 is a GC → AT transition at position 132 in the _trp_ leader which alters the arm of the repeat sequence nearest to the termination site (Table I). It reduces termination in _vitro_ from 95 to 26%. _trp_ L29 and L75 are two mutations earlier in the leader sequence which have no effect on _in vitro_ termination. They were chosen as controls for the possible effects of heteroduplex sites on transcription.

**EXPERIMENTAL PROCEDURES**

The following plasmids were provided by Dr. Charles Yanofsky (Stanford University, Stanford, CA). They each contain an RI segment of the _E. coli_ trp operon (see text and Refs. 12 and 13) and _trp_ attenuator mutations, pPD58 (L75), pD27 (L29), L153 (L153), and pHV153 (trp*), if noted.

*M13mp2* phage and the host strain _E. coli_ 71-18 were obtained from Dr. Joachim Messing (University of Minnesota, Minneapolis, MN). Restriction endonucleases EcoRI, HpaII, and HinfI were obtained from Bethesda Research Laboratories (Gaithersburg, MD), and digestion conditions were those described by Bethesda Research Laboratories. Bacteriophage T4 gene 32 protein in large quantities was generously provided by Dr. Junko Hosoda (University of Califor-
TABLE I

| Mutation | Alteration | Effect in vitro | Reference |
|----------|------------|----------------|-----------|
| trp·        | Wild type sequence | 95% termination | 12 |
| L153      | GC → AT at position 132 in 3:4 stem | 26% termination | 12 |
| L29      | GC → AT at position 29 in start codon | 95% termination | 13 |
| L75      | GC → AT at position 75 in 3:2 stem | 95% termination | 13 |

E. coli trp leader mutations employed in heteroduplex studies

The sequence shown above is that of the wild type trp leader transcript immediately prior to the termination site at the attenuator at position 1014 (see Ref. 3). The positions of the self-complementary sequences are underlined with arrows.

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Although single-stranded DNA containing molecules alone are expected to be retained on these filters, we consistently observed that a substantial fraction of M13-trp heteroduplex complexes passed through the filter in the absence of T4 gene 32 protein. The filter was then washed with 2 ml of 10 mg/ml of bovine serum albumin and the filter was placed upper surface down in a solution containing endonuclease HpaII. After 3 h at 37 °C, the excised restriction fragments were precipitated with 0.5 M NaCl and 3 volumes of ethanol.

The trp HpaII 570 fragment was separated by agarose gel electrophoresis and was isolated by adsorption to glass beads as for Method A.

Transcription of trp HpaII 570 fragments was carried out in a reaction (50 μl) containing 40 mM Tris, pH 8, 4 mM MgCl₂, 4 mM spermidine, 10 mM mercaptoethanol, 100 mM KCl, 2.5 mM ATP, 1.4 mM UTP, 1.1 mM GTP, 0.4 mM [α-32P]CTP (specific activity, 20 cpn/pmol), 0.01-0.05 μg of HpaII 570 fragment, and 0.3 μg of E. coli RNA polymerase holozyme (about 30% active holozyme molecules by quantitative assay with T7 DNA) (19, 20). Transcription was carried out for 30 min at 37 °C. Rifampicin (40 μg/ml) was added, and after 10 min further synthesis, the products were extracted with phenol and ethanol and precipitated with ethanol after addition of 100 μl of a solution containing 1.5 M ammonium acetate, 37.5 mM EDTA, and 45 μg/ml of E. coli tRNA. Transcripts were dissolved in washing buffer (0.2% sodium dodecyl sulfate, 0.3 M sodium acetate) and repurified and stored in 0.5 volumes of ethanol at −20 °C.

The remainder of the sample (3000 to 5000 cpn) was heated to 90 °C for 1 min and then loaded onto an 8% denaturing polyacrylamide gel in tris/borate/EDTA buffer at pH 8.3 containing 7 mM urea. Gels were pre-electrophoresed for 2 h before use. Electrophoresis was continued until the xylene cyanol dye had reached the bottom of the gel.

Gels were analyzed by autoradiography and also by counting slices from each track to measure the amount of radioactivity in each RNA species quantitatively.

RESULTS

We had originally planned to clone the trp· attenuator sequence as well as selected trp attenuator mutants into the vector M13mp2. Isolation of trp inserts in both of the two possible orientations for each allele would provide M13mp2 phage bearing single-stranded DNAs with trp inserts complementary to each of the two DNA strands. The trp· attenuator as well as the attenuator mutants of choice were available cloned on an EcoRI fragment of 7100 base pairs (15) bearing the trp promoter, attenuator, trp genes E, D, and part of trpC (trp POLED'C). Combination of appropriate complementary strands would allow direct construction of both possible heteroduplex templates for each trp attenuator mutation.

The trp· fragment was cloned and M13mp2 trpA· (+) and M13mp2 trpA· (-) phage were identified bearing the transcribed and nontranscribed strands of the trp· POLED'C RI fragment, respectively. However, no stable clones of trp· attenuator mutant L153 were obtained, possibly because insertion of a strong unregulated promoter into a multicopy vector is toxic for the host cell (21). Consequently, we sought other methods for construction of the needed heteroduplexes.

If DNA of a plasmid bearing a trp attenuator mutation is cut with EcoRI, denatured with alkali, and annealed with excess M13trpA· (+) or M13trpA· (-) DNA, the trp·/trpA· homoduplexes are formed in the two respective reactions, together with small amounts of the original trp·/trp· homoduplex (Fig. 1). The amount of the latter can be estimated by gel electrophoresis of the hybridization products. Cleavage of the products allows isolation of a trp HpaII 570 DNA fragment bearing the trp promoter/attenuator region. Transcription of this fragment gives RNA products of characteristic size arising from the trp promoter and terminating at the attenuator (140 bases) or reading through to the end of the fragment (260 bases; see Ref. 22). Inspection of

Schleicher and Schuell) presoaked in the same buffer. This leads to preferential retention of heteroduplex DNA molecules while renatured parental homoduplex molecules pass through the filter. (A-
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When transcription takes place in the presence of purified trp repressor, repression depends on added cryptophan. Transcription of trp HpaII 570 fragments from trpAM plasmids bearing the L153 mutation gives predominantly the 260-base transcript (Fig. 2, track A) and quantitative analysis shows about 75–80% readthrough of the trp attenuator (Table II, part II). Finally, transcription of heteroduplexes prepared with trpA mutations L29 (Fig. 2, tracks E and F) or L75, which affect readthrough in vivo but not in vitro, gave low readthrough and gave normal amounts of the trp 140-base transcript, confirming that the presence of a heteroduplex base mispair earlier in the trp leader does not by itself induce readthrough in vitro.

While the results of Fig. 2 are qualitatively convincing, the presence of contaminating homoduplex template introduces the need for a substantial correction, especially in the case of the trpA+/trpA* template. This correction depends on our ability to measure accurately the amount of homoduplex that reforms. This is difficult since the M13mp2trpA+/trpA* heteroduplexes do not migrate as sharp bands on agarose gels, but as diffuse smears. Consequently, the error in our estimation of the per cent readthrough for the trpL153/trpA* heteroduplex is on the order of 100% (i.e. 7 ± 7%). Accordingly, we have used a second method to prepare heteroduplex templates in which the M13mp2trpA+/trpA* heteroduplexes are separated from refolded homoduplex DNAs by nitrocellulose filter binding (Method B, see “Experimental Methods”). This method gave lower recoveries of heteroduplex products, but allowed isolation of such templates free of homoduplex con-

Table II

| Template          | Readthrough of the attenuator |
|-------------------|-------------------------------|
| I. Homoduplex templates isolated directly or reconstituted |                               |
| trpA+/trpA*       | 5                             |
| trpL153/trpL153   | 72                            |
| trpL29/trpL29     | 6                             |
| trpL75/trpL75     | 5                             |
| II. Heteroduplex templates prepared by Method A |                               |
| trpL153/trpA*     | 7 (21)                        |
| trpA*/trpL153     | 81 (79)                       |
| trpL75/trpA*      | 5                             |
| trpA*/trpL75      | 5                             |
| trpL29/trpA*      | 6                             |
| trpA*/trpL29      | 6                             |
| III. Heteroduplex templates prepared by Method B |                               |
| trpL153/trpA*     | 22                            |
| trpA*/trpL153     | 84                            |
| trpL75/trpA*      | 6                             |
| trpA*/trpL75      | 6                             |
tamination. Analysis of transcription from these templates (Table II) confirms the finding that transcription termination at the trp terminator is determined by the transcribed DNA strand. In addition, it appears that there is slightly more readthrough of the trp attenuator for both L153 heteroduplexes as compared to the homoduplex templates.

**DISCUSSION**

Our results show that the ability of the trpL153 attenuator mutation to suppress termination depends on its presence in the transcribed DNA strand. This result is the opposite of that predicted if termination were the result of the formation of a stem-loop structure in the nontranscribed DNA strand, or by models in which the nascent RNA chain must complex with the nontranscribed DNA strand to block transcription elongation. Hence, it would seem to rule out these mechanisms at least for strong bacterial terminator sites. The results is that expected if transcriptional termination at this site were determined by formation of a stem-loop structure in the nascent RNA transcript (2–4). Evidence against a major role in termination for the nontranscribed DNA strand has also been presented by Farnham and Platt (23).

One could imagine more complex models for termination in which there are essential base pairing interactions or hairpin structures formed within the transcribed DNA strand, as well as in the RNA product. Such models are made less likely by the demonstration that only 16 base pairs of DNA are open at the elongation site during normal elongation (11). While unwinding measurements have not yet been done for RNA polymerase actually at the terminator site, our results, taken with those of others (23, 24), strongly support a model in which the nascent RNA is displaced from the transcribed strand at a point less than 10 bases from the growing point and hence is free to form an RNA stem-loop structure, which can directly stop chain elongation by RNA polymerase. It has been shown that short self-complementary sequences can cause RNA polymerase to pause during elongation (25) even when termination does not occur, although this may not be true of all such sequences (26).

How does the RNA hairpin structure stop RNA chain elongation? One possibility is that there is a binding site on the enzyme specific for a short RNA duplex. Since RNA chain elongation is insensitive to polyanionic inhibitors (7–9) and since termination is not blocked at high salt concentrations (10), this site cannot be ionic in nature but would have to be directed toward the ribose residues in the RNA, for example. An alternative possibility is that there is no such site on the enzyme, but that formation of the RNA hairpin itself forces a change in the conformation of the ternary DNA-RNA polymerase complex and blocks elongation (4). Resolution of these questions will require detailed mapping of the termination complex, potentially a difficult task since the enzyme appears to be rapidly released (27).

The slight increase in readthrough at the attenuator for both of the trpL153+trpPa+ heteroduplexes is interesting and not predicted by any of the current models for termination. Formation of the RNA stem-loop terminator structure depends on reformation of the DNA duplex and displacement of the nascent RNA chain. This occurs at a site about 10 base pairs from the growing end of the RNA chain, and the L153 mutation is located exactly at that point. Hence, one might speculate that the presence of a mispair in the DNA at this point could increase the probability of readthrough occurring before an effective RNA terminator structure can form, for example by allowing a slightly longer DNA-RNA hybrid to form. It would be quite interesting to study the effects of additional heteroduplex mispairs in the trp attenuator region to explore this phenomenon more closely.

The use of heteroduplex templates allows one to distinguish between several possible models for the action of regulatory sequences that are transcribed into RNA. In addition to prokaryotic terminators, such sites include DNA sequences that induce transcriptional pausing (26, 28) and potential terminator sequences for eukaryotic RNA polymerases I, II, and III (29–32). It will be interesting to use this method to study the mechanism of action of some of these latter sites, especially since in some cases there are no evident RNA stem-loop structures involved (26, 32).

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