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A Hairpin Structure Upstream of the Terminator Hairpin Required for Ribosomal Protein L4-Mediated Attenuation Control of the S10 Operon of *Escherichia coli*

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Ribosomal protein L4 of *Escherichia coli* regulates transcription of the 11-gene S10 operon by promoting premature termination of transcription (attenuation) at a specific site within the 172-base untranslated leader. We have analyzed the roles of various domains of the leader RNA in this transcription control. Our results indicate that the first 60 bases of the leader, forming the three proximal hairpin structures, are not essential for in vivo L4-mediated attenuation control. However, a deletion removing the fourth hairpin, which is immediately upstream of the terminator hairpin, eliminates L4's effect on transcription. Base changes disrupting complementarity in the 6-bp stem of this hairpin also abolish L4 control, but compensatory base changes that restore complementarity also restore L4's effect. In vitro transcription studies confirm that this hairpin structure is necessary for L4's role in stimulating transcription termination by RNA polymerase.

In *Escherichia coli*, the synthesis of most ribosomal proteins (r-proteins) is under autogenous control; i.e., one of the proteins encoded by an operon is not just an r-protein but is also an operon-specific repressor (16). When the regulatory protein is synthesized in excess of its binding site on nascent rRNA, the free regulatory protein reduces the output of proteins from its operon, thus reestablishing the balance between rRNA and r-protein synthesis. In most r-protein operons for which there are data, this regulation occurs at the level of mRNA translation (16). However, in the S10 operon, the regulatory r-protein L4 regulates both transcription and translation (5, 14, 15). Furthermore, the two regulatory effects can be separated genetically, indicating that L4 control results from two simultaneous but independent processes (1, 13).

Transcription regulation of the S10 operon results from L4-stimulated termination of transcription (attenuation) in the untranslated leader about 140 bases from the transcription start (5, 14, 15). Our in vitro studies suggest that this process involves pausing of the RNA polymerase at the attenuator, followed by sequential stabilization of the paused transcription complex by the general transcription fidelity factor NusA and then r-protein L4, and finally release of the attenuated transcript and RNA polymerase from the template (7, 8, 15).

The secondary structure of the S10 leader consists of six hairpins connected by short single-stranded regions (Fig. 1) (10). In previously published experiments, we showed that the largest hairpin, called "HE," containing the site of L4-mediated transcription termination, is essential for both transcription and translation control (1). In vitro transcription experiments employing antisense oligonucleotide competition techniques also implicated the more proximal hairpin HD (Fig. 1) in L4-mediated transcription control (8). However, the role of HD had not been analyzed in vivo, and the contributions of the most proximal three hairpins, HA, HB, and HC, had not been studied at all. Here, we report that deletions removing hairpins HA, HB, and HC, individually or collectively, have little or no effect on L4 regulation of tran-

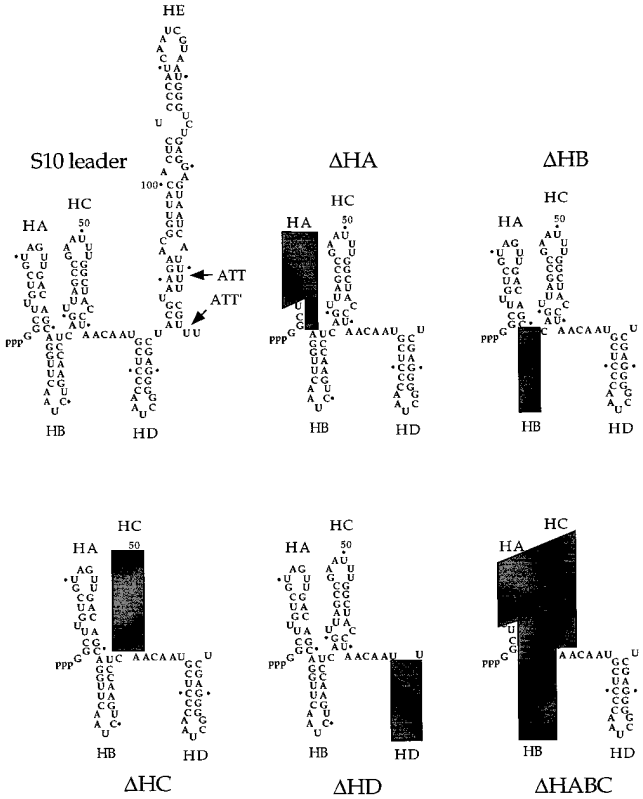

FIG. 1. Hairpin deletions in the S10 leader. The secondary structure of the S10 leader was determined by Shen et al. (10). Bases beyond the attenuation site, including the initiation codon of the S10 gene (nucleotides 173 to 175), are not shown. The sites of in vivo (ATT) and in vitro (ATT and ATT′) termination are indicated. Bases deleted in the various hairpin deletion plasmids are indicated by the shaded areas. Bases in hairpin HE were left intact in all of the mutants.

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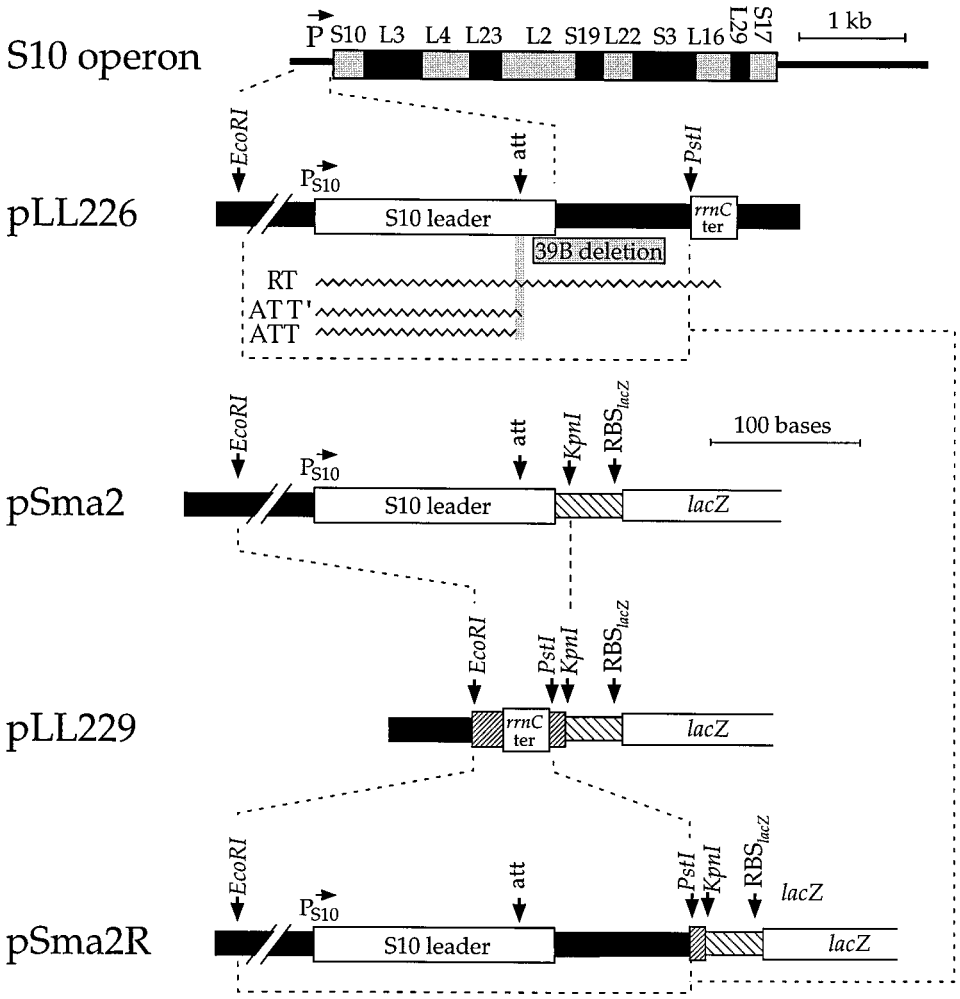

FIG. 2. Map of the S10 r-protein operon and plasmids used in this study. Plasmid pLL226 was used as a substrate for site-directed mutagenesis. Transcripts initiated at the S10 promoter are illustrated by the wavy lines. The bar below the pLL226 map indicates the region deleted in pLL226-39B. These two plasmids and their hairpin deletion derivatives were used for in vitro transcription. Plasmid pLL229 was used as the intermediate for subcloning mutated leader fragments from pLL226 to create analogs of pSma2. The resulting plasmids, pSma2R (with the wild-type leader sequence) and leader mutant derivatives, were used to monitor in vivo transcription.

scription in vivo or in vitro. In contrast, the HD hairpin structure is essential for L4-mediated transcription control.

MATERIALS AND METHODS

Bacterial strains and plasmids. All experiments were performed with *E. coli* K-12 strain LL308 [F′ *pro lacI*^{q}Z$^{\Delta M15}$/Δ(*lac-pro*) *recA*] (6) carrying the indicated plasmids. Maps of pertinent regions of plasmids used in this study are shown in Fig. 2. Plasmids pLL226 and pLL226-39B (13) carry both ColE1 and M13 origins of replication. Single-stranded DNA from these plasmids was prepared (11) and used for site-directed mutagenesis (3). Plasmid pSma2R (with wild-type or mutant leaders) was constructed, via pLL229, from the pSma2 plasmid described previously (13) (Fig. 2). Both pSma2 and pSma2R are subject to L4-mediated attenuation control upon induction of L4 oversynthesis. Plasmid pLL125 (6) carries genes for r-proteins L4, L23, and L2 expressed from the *lac* promoter.

Protein synthesis. The rate of β-galactosidase synthesis was monitored essentially as described previously (2). In brief, aliquots of cultures growing exponentially in glucose minimal medium were labeled for 1 to 2 min with 25 μCi of [^{35}S]methionine per ml (approximately 1,000 μCi/mmol) immediately before and 10 min after induction of L4 oversynthesis by addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were then lysed in sodium dodecyl sulfate (SDS) gel sample buffer (4) at 95°C, and the resulting lysates were fractionated by gel electrophoresis on SDS-polyacrylamide (7.5% [wt/vol]) gels as described previously (4), except that the ratio of acrylamide to bisacrylamide was reduced to 30:0.2 in order to increase resolution of high-molecular-weight proteins.

In vitro RNA synthesis. Standard 40-μl transcription reaction mixtures contained 20 mM Tris-acetate (pH 7.9), 4 mM Mg-acetate, 0.1 mM EDTA, 100 mM K-glutamate, 20 nM RNA polymerase, and 20 to 25 nM supercoiled plasmid DNA. Where indicated, NusA was added to 40 nM, and L4 or S7 was added to 120 to 160 nM. In early experiments (14), these reaction components minus plasmid DNA were mixed at 37°C with GTP, CTP, and ATP (to 500 μM each); UTP (to 100 μM); and 20 μCi of ^{35}S-UTP. Elongation was started by addition of DNA and terminated after 15 min by addition of 40 μl of 50 mM EDTA containing 10 μg of yeast carrier RNA, 40 μl of phenol, and 40 μl of chloroform-isoamyl alcohol (24:1). The samples were then processed and analyzed as described previously (14). Reaction products were quantitated by scintillation counting of the radioactivity in gel bands corresponding to the indicated RNA transcripts (14). In later experiments (15), all reaction components except nucleotides were mixed together and then incubated at 37°C for 10 min with CTP and GTP (500 μM each) to allow formation of the initiation complex and incorporation of the proximal three nucleotides (pppGGC). A single round of transcription elongation was then started by addition of ATP (to 500 μM), UTP (to 100 μM), 5 to 10 μCi of [^{32}P]UTP, and rifampin (to 10 μg/ml) and terminated at the indicated times as described above.

RESULTS

Construction of plasmids and strains. To analyze transcriptional regulation in the absence of translation control, we used the operon fusion plasmid pSma2R carrying the S10 promoter and proximal 165 bases of the S10 leader upstream of a *lacZ* gene complete with its own ribosome binding site (Fig. 2). We

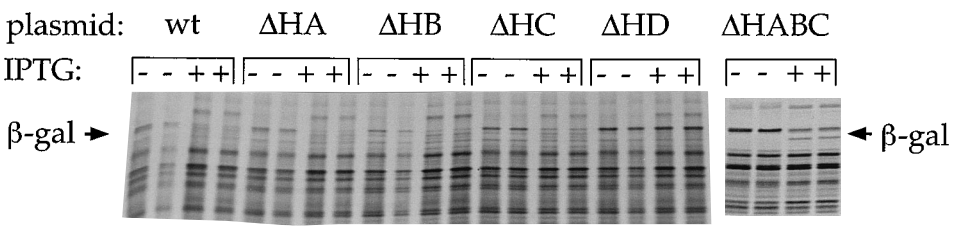

FIG. 3. Effects of hairpin deletions on in vivo regulation of transcription. Strains containing pSma2R or the indicated deletion derivatives as well as the P_{lac}-L4 plasmid were grown exponentially in glucose minimal medium. At a density of about 10^8 cells per ml, aliquots of the culture were pulse-labeled with [^{35}S]methionine immediately before (−) and 10 min after (+) induction of L4 oversynthesis by addition of IPTG (1 mM). Extracts prepared in SDS sample buffer were analyzed by gel electrophoresis. A photograph of the resulting autoradiogram is shown. The band corresponding to β-galactosidase (β-gal) is indicated. The bands above and below the β-galactosidase band whose intensities increase after IPTG induction are not expressed from the S10 promoter and are not related to L4 control. wt, wild type.

have previously shown that L4-mediated transcription control requires only the first 149 bases of the 172-base leader (13). The rate of β-galactosidase synthesis from pSma2R is repressed fivefold in response to induction of L4 oversynthesis, the same degree of repression that L4 has on mRNA synthesis from the structural genes of the S10 r-protein operon (13). The role of various leader hairpins was determined by constructing analogs of pSma2R lacking individual hairpins.

We first introduced the mutations into the S10 leader carried on plasmid pLL226 (Fig. 2). To conveniently subclone these mutations into the transcription fusion environment, we generated an intermediate vector, pLL229 (Fig. 2), which carries a transcription terminator upstream of the *lacZ* gene. Subcloning of *Eco*RI-*Pst*I fragments from mutagenized pLL226 into pLL229 cut with *Eco*RI and *Pst*I generated variants of pSma2R carrying the mutated leader sequence. Each of the pLL229-type hairpin deletion plasmids was then transformed into a strain already carrying a second compatible plasmid containing an L4 gene under control of the *lac* promoter and operator.

Effect of hairpin deletions on transcription regulation in vivo. The effect of the hairpin deletions on L4-mediated regulation of transcription was determined by pulse-labeling of cells with [^{35}S]methionine before and after IPTG induction of L4 oversynthesis and monitoring the effect of induction on the incorporation of radioactivity into β-galactosidase. Autoradiograms of SDS-polyacrylamide gels with extracts from cells containing pSma2R plasmids with hairpin deletions of HA, HB, HC, or HD are shown in Fig. 3. It can be seen that the synthesis of β-galactosidase is inhibited by L4 to about the same extent with the HA, HB, or HC deletion constructs as with the wild-type leader plasmid. To be certain that these three hairpins do not perform redundant functions, we constructed another deletion simultaneously removing all three hairpins (Fig. 1). This construct also exhibited essentially the wild-type level of L4 control (Fig. 3). We conclude that the three promoter proximal hairpins are not essential for L4-mediated attenuation control of the S10 operon (although we cannot exclude a minor role in L4 regulation).

In contrast to the deletions of the three proximal hairpins, the deletion of the fourth hairpin, HD, completely abolished the L4 inhibition of β-galactosidase synthesis (Fig. 3). This result indicates that hairpin HD is essential for L4-mediated autogenous control. The synthesis of β-galactosidase in the absence of IPTG was also increased in the HD deletion strain, suggesting that the absence of this hairpin results in increased constitutive transcription of the *lacZ* gene. Although we cannot rule out effects on translation efficiency or mRNA stability, this observation is consistent with previous studies suggesting that during exponential growth, only one in two or three transcripts is elongated past the S10 attenuator (12).

Effect of hairpin HD stem mutations on in vivo transcription regulation. Having shown that bases contained within hairpin HD are required for L4-mediated attenuation control, we wanted to examine the roles of primary sequence and secondary structure of the HD RNA in this regulation. We were interested, for example, in determining the role of the GGG...CCC sequence in HD, since these bases could potentially interact with the CCC...GGG sequence at the top of hairpin HE (Fig. 1), thereby generating an alternative secondary structure. Interestingly, all of these CG base pairs are preserved in five different enterobacterial species (9).

We first made single-base changes on either side of the stem of the HD hairpin, disrupting the CG base pair involving nucleotides 70 and 80 (Fig. 4a). However, these mutations had a relatively modest effect on L4-mediated regulation (data not shown), suggesting that a single-base change in the HD stem does not sufficiently destabilize the structure to elicit a detectable effect on L4 control. Therefore, we restored to double-base changes involving two of the three CG base pairs (Fig. 4a). We found that double-base changes on either side of the HD hairpin eliminated the L4-mediated regulation and in-

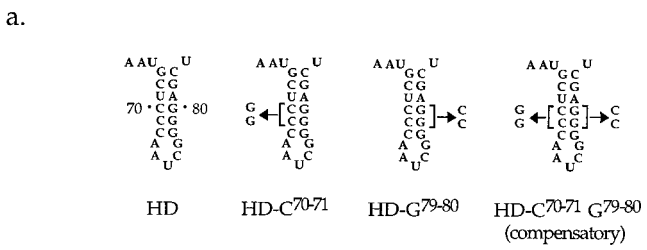

FIG. 4. Analysis of the role of hairpin HD in L4 control. (a) Base substitutions in hairpin HD. (b) Effects of hairpin HD base changes on in vivo transcription. Cells were labeled and analyzed as described in the legend to Fig. 3. wt, wild type; β-gal, β-galactosidase.

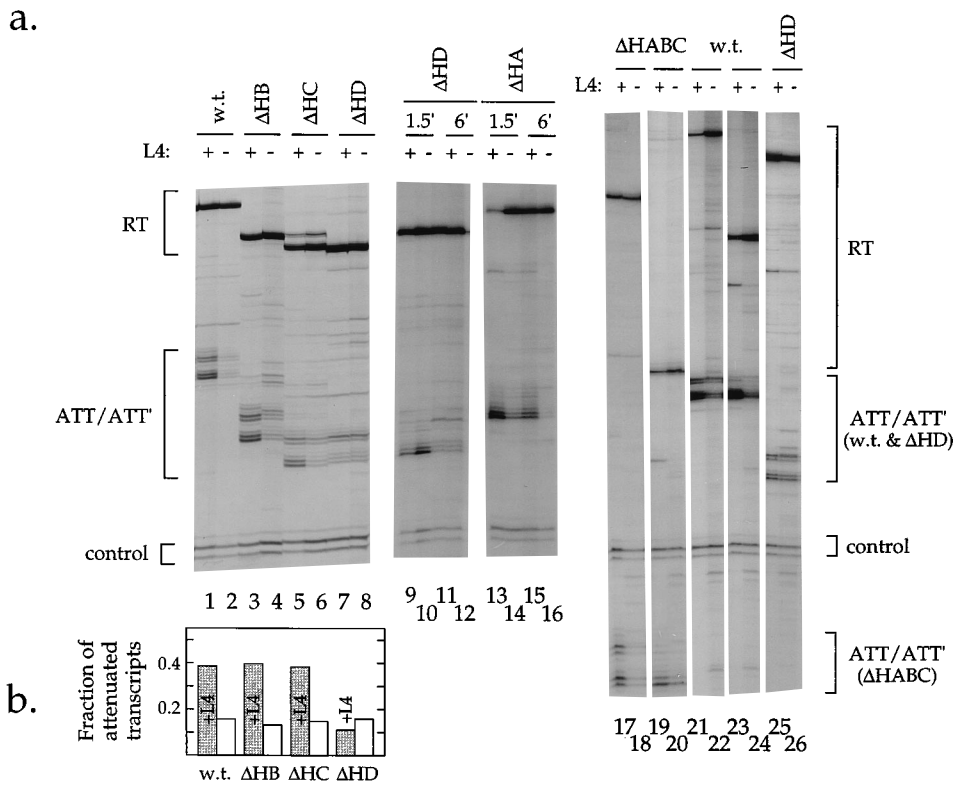

FIG. 5. Effects of hairpin deletions on the regulation of in vitro transcription. Transcription reactions were performed in the presence of NusA and either r-protein L4 (+L4) or, as a control, r-protein S7 (−L4) (14, 15). The templates were wild-type leader (w.t.) plasmids pLL226 and pLL226-39B (Fig. 2) or derivatives containing the indicated hairpin deletions. Note that the deletion in plasmid pLL226-39B has no effect on L4 control of transcription in vivo (13), although there is a subtle effect on the relative distribution of attenuated transcripts ending at ATT versus those ending at ATT′ (15). (a) Autoradiogram of transcripts fractionated on an 8% urea–polyacrylamide gel. The RT and ATT/ATT′ transcripts are indicated (see Fig. 1). The control band represents an unidentified RNA also transcribed from the plasmid whose synthesis is not affected by L4, hence providing an internal reference for each reaction. In the reactions shown in lanes 1 to 8, transcription elongation proceeded for 15 min before being stopped by addition of phenol-EDTA. The reactions shown in lanes 9 to 16 were terminated, as indicated, after 1.5 or 6 min of elongation. The reactions shown in lanes 17 to 26 were terminated after 1.5 min of elongation. The templates for reactions in lanes 1 to 16, 19, 20, 23, and 24 were pLL226-39B and the indicated deletion derivatives. The templates for lanes 17, 18, 21, 22, 25, and 26 were pLL226 and the indicated deletion derivatives. (b) RNA products from the transcription reactions in lanes 1 to 8 were quantified by scintillation counting of the radioactivity in the bands of the gel shown in panel a. The radioactivity in each band was corrected for the number of U residues in the transcript and then used to calculate the fraction of transcripts that initiated from the S10 promoter and that are paused or terminated at the attenuator.

creased the basal rate of β-galactosidase synthesis (Fig. 4b), similar to the effect of deleting the entire HD hairpin (Fig. 3). Moreover, when we combined the two double mutations to create compensatory base changes that restore the hairpin structure but replace two of the CG base pairs with GC base pairs (Fig. 4a), both L4 control and the normal basal level of β-galactosidase synthesis were restored (Fig. 4b). We conclude that the secondary structure of HD is important for L4-mediated attenuation of the S10 operon. In addition, these results make it unlikely that an alternative structure involving base pairing of GGG or CCC in HD with CCC or GGG, respectively, in HE is involved in L4 control of transcription.

Effect of hairpin deletions on in vitro transcription regulation. Earlier in vitro studies (7, 15) showed that L4-mediated attenuation of transcription in the S10 leader involves sequential stabilization by NusA and L4 of a transcription complex paused at the ATT/ATT′ site in the attenuator (Fig. 1). The L4-dependent step in this pathway cannot occur in the absence of the NusA-dependent step (7, 15). Hence, loss of L4 control of transcription in vivo could reflect a defect either at the level of NusA-dependent pausing or at the subsequent step of L4 stabilization of the paused complex. We previously showed that a deletion removing the proximal four hairpins, which eliminates L4 attenuation control in vivo, specifically abolishes the L4 step in vitro while leaving the NusA-dependent pause intact (15). Since the in vivo studies reported above implicate hairpin HD, but not HA, HB, or HC, in L4 attenuation control, we expected to find a normal NusA-dependent pause in vitro with templates lacking any of the proximal four hairpins but a loss of L4 response in the HD deletion only. We tested this prediction by in vitro transcription studies using pLL226 (Fig. 2) or derivatives containing the various hairpin deletions as templates.

Transcription of the leader generates two classes of RNA: read-through (RT) transcripts terminating at the *rrnC* terminator and attenuated (ATT and ATT′ [Fig. 1]) transcripts resulting from RNA polymerases paused at the attenuator (Fig. 2). The addition of L4 to a reaction mixture programmed with wild-type leader DNA causes an increase in the amount of ATT/ATT′ transcripts and a corresponding decrease in the amount of RT transcripts (Fig. 5, lanes 1 and 2 and 21 to 24). In the absence of NusA, RNA polymerase does not pause at the attenuator and does not respond to L4 (7, 15). As expected, templates containing deletions of any or all of the proximal three hairpins showed essentially the same transcription pattern as the wild-type leader template (Fig. 5, lanes 3 to 6, 13 to 16, and 17 to 20). However, with the hairpin HD deletion template, RNA polymerase paused normally at ATT/ATT′ but

showed no response to the addition of L4 (Fig. 5, lanes 7 and 8, 9 to 12, and 25 and 26). These results confirm that hairpin HD is required for L4 stimulation of the attenuation process but is dispensable for the NusA-dependent pause of RNA polymerase at ATT/ATT′.

DISCUSSION

Autogenous control of the S10 r-protein operon in *E. coli* is accomplished in part by an L4-regulated transcription attenuator in the untranslated leader. The attenuation process appears to involve an ordered pathway with at least four steps: (i) spontaneous (factor-independent) pausing of RNA polymerase at the site of attenuation, (ii) stabilization of the paused transcription complex by NusA, (iii) super stabilization of the transcription complex by L4, and (iv) release of the attenuated transcript and RNA polymerase from the template (15). The first three steps have been demonstrated in vitro, but the fourth step has been inferred only from in vivo experiments (7, 8, 15).

Previous in vivo experiments showed that hairpin HE is essential for L4-mediated transcription attenuation (1). Our in vitro transcription studies suggested that different sequences in this hairpin are essential for the different steps in the transcription termination pathway (8). For example, factor-independent pausing of RNA polymerase at ATT/ATT′ is influenced by the sequence around the pause site and, to some degree, by the upper stem-loop in hairpin HE. The NusA-dependent stabilization of the paused complex requires the hairpin structure at the top of HE, while the L4-dependent step is influenced by the ascending 25 bases of hairpin HE (7, 8).

The deletion studies reported here demonstrate that hairpin HD is also essential for in vivo L4-mediated transcription regulation (Fig. 3 and 5). Moreover, the compensatory base substitutions in the helix of HD show that the structure of this hairpin is important (Fig. 4). This conclusion is consistent with earlier in vitro experiments showing that an oligonucleotide complementary to the promoter proximal half of HD (and the sequence between HC and HD) blocked the L4 response without affecting the response to NusA (8). In contrast to hairpin HD, the three promoter proximal hairpins HA, HB, and HC have no significant role in L4-mediated attenuation control.

Together with our previous in vitro results (8, 15), these deletion studies indicate that the L4-dependent step requires a contiguous domain of the leader, including hairpin HD and the promoter proximal bases in HE. The obvious questions now are what specifies the interaction between L4 and this leader domain, and how does L4's interaction with the leader and other components of the transcription complex influence the behavior of RNA polymerase at the termination site? We have not yet been able to demonstrate direct binding of L4 to the leader (reference 15 and unpublished observations), although it seems likely that such a direct interaction defines the specificity of the L4-mediated response. In this connection, we should point out that L4-mediated regulation of translation of the S10 operon can occur in the absence of hairpin HD: substantial translation regulation was observed with a deletion removing hairpins HA, HB, HC, and HD (1). There are two possible explanations for this apparent incongruity: either L4 recognizes a common sequence or structure downstream of hairpin HD (e.g., the ascending bases in hairpin HE) but requires HD to mediate its effect on transcription (not translation) or there are two separate recognition sites for L4 in the leader.

It is not clear why L4 stabilization of pausing requires an RNA structure that is 70 to 80 bases upstream of the site of termination. Since we do not yet know the topology of the paused transcription complex, we can only speculate about potential interactions between L4, hairpin HD, and RNA polymerase. Clearly, an understanding of the structure of this complex will be important not only for understanding L4 regulation of the S10 operon but also for shedding light on the general phenomenon of transcription termination.

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