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REPORT

Surprising flexibility of leader RNA determinants for r-protein L4-mediated transcription termination in the *Escherichia coli* S10 operon

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ABSTRACT

***Escherichia coli* ribosomal protein L4 autogenously regulates transcription of the S10 operon, which encodes L4 and 10 other ribosomal proteins. Regulation results from L4-stimulated premature transcription termination at a U-rich site in the untranslated leader. The process requires transcription factor NusA. Here we report a detailed analysis of the RNA requirements for NusA-dependent, L4-mediated transcription control. We found that efficient regulation requires multiple features of the S10 leader, including two hairpins, called HD and upper HE, a connecting tether, and a U-rich sequence at the distal side of HE. As expected, regulation was optimal when all 7 Us were maintained in the U_4CGU_3 sequence at the termination site. However, despite the apparent specificity of L4 action on only the S10 operon, there is surprising flexibility at the primary sequence level for the HD-tether-HE region. Changes in the sequence of non-base-paired nucleotides flanking the HD hairpin or an A at the second position of the HD loop reduced L4 regulation, but other changes had little or no effect. Furthermore, generic hairpins from other RNAs could replace the natural HD and upper HE hairpins with little or no reduction of L4 control, suggesting that the secondary structure elements are also relatively generic. The lack of specific sequence requirements suggests that L4 may recognize multiple elements within this region of the nascent leader.**

Keywords: attenuation; autogenous control; ribosomal protein; RNA structure

INTRODUCTION

Ribosomal proteins (r-proteins) in a wide range of organisms regulate their own synthesis. A well-studied example of such autogenous control is the regulation of the 11-gene *Escherichia coli* S10 operon by r-protein L4, the product of the third gene of the operon (Lindahl & Zengel, 1979; Yates & Nomura, 1980; Zengel et al., 1980; Zengel & Lindahl, 1994). Only free L4 protein regulates: As long as the synthesis of the protein's normal target, 23S rRNA, is balanced with the production of L4, newly synthesized L4 is quickly sequestered and assembled into ribosomes, and expression of the S10 operon proceeds. However, if L4 accumulates in excess of 23S rRNA, then the unbound protein feedback inhibits synthesis of the 11 r-proteins encoded by the S10 operon (Zengel et al., 1980).

Whereas other regulatory r-proteins in *E. coli* work at the level of translation initiation (Zengel & Lindahl, 1994; Keener & Nomura, 1996), L4 inhibits both transcription and translation (Yates & Nomura, 1980; Lindahl et al., 1983; Freedman et al., 1987; Zengel & Lindahl, 1990a). Genetic studies showed that these processes work independently and require overlapping but distinct determinants within the 172-nt S10 leader mRNA (Lindahl et al., 1983; Freedman et al., 1987; Zengel & Lindahl, 1990a; Sha et al., 1995a). We have focused on transcription control, which results from L4-stimulated premature termination of transcription (attenuation) at a specific site in the leader, about 30 nt upstream of the first gene of the operon (Zengel & Lindahl, 1990a, 1990b).

In vitro transcription experiments identified several distinct steps in L4-stimulated transcription attenuation (Zengel & Lindahl, 1992; Sha et al., 1995b). First, RNA polymerase pauses spontaneously at the termination (attenuation) site. Second, NusA stabilizes the paused transcription complex and sensitizes the complex to the regulatory effect of L4. Third, L4 interacts with the paused transcription elongation complex, presumably through binding to the nascent S10 leader transcript,

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and creates a "superpaused" complex that leads to termination of transcription. Each step in the attenuation process requires a distinct element in the S10 leader, located within the 3′ half of the leader that forms the hairpins HD and HE (Fig. 1). A cluster of Us at the pause site is necessary for the spontaneous RNA polymerase pause and an intact upper HE hairpin is required for NusA stimulation of the pause (Sha et al., 1995a). These two elements form the transcription termination region. The upstream hairpin HD and the ascending side of HE are required for L4 stimulation of termination (Sha et al., 1995a; Zengel & Lindahl, 1996). These elements form the L4 response region.

To learn more about the features in the S10 leader necessary for L4 regulation, we have analyzed the effects of mutations in the HD-HE region. Because L4 specifically regulates only the S10 operon, we expected very stringent criteria, particularly for the L4 response region. However, our detailed structure analysis suggests that the requirements of the S10 leader region are very flexible. Only limited primary sequence requirements in the S10 leader were uncovered. Moreover, the requisite secondary structures are not very constrained, as generic HD and upper HE hairpins suffice for L4 regulation.

RESULTS AND DISCUSSION

System for monitoring L4-mediated control

To study L4-mediated attenuation control, we used strains carrying two plasmids: a "target plasmid" with the wild-type or mutant S10 leader followed by a *lacZ* reporter gene, and a "source plasmid" with an L4 gene under control of the *ara* promoter. Because the target plasmid lacks determinants necessary for L4-mediated translation control, the *lacZ* reporter gene is subject to only transcription control. To measure the ability of mutant leaders to respond to L4 oversynthesis, strains were first induced with IPTG to activate transcription of the S10 leader and reporter gene. Arabinose was then added to part of the culture to turn on oversynthesis of L4. Ten minutes later, samples of the two parallel cultures were pulse-labeled with ^{35}S-methionine. Whole-cell extracts were fractionated by SDS-polyacrylamide gel electrophoresis and the fraction of the radioactivity in a given lane found in the β-galactosidase band was used as a measure of the rate of synthesis of the reporter protein. The effect of L4 on reporter gene expression was calculated by dividing the synthesis rate of β-galactosidase in the presence of arabinose (+L4) by the synthesis rate in the absence of arabinose (−L4).

S10 leader secondary structure

Figure 1 illustrates two possible secondary structures of the HE stem in the S10 leader. In one state (Fig. 1A), hairpin HE has a long, bulged stem as determined by solution structure probing of the free, full-length leader RNA (Shen et al., 1988). In the other state (Fig. 1B), there is no pairing between nucleotides below helix 1 of the "upper-HE" hairpin (nt 106–123). Although the bulges are conserved in species utilizing L4 control of the S10 operon (Allen et al., 1999), the latter form is more likely

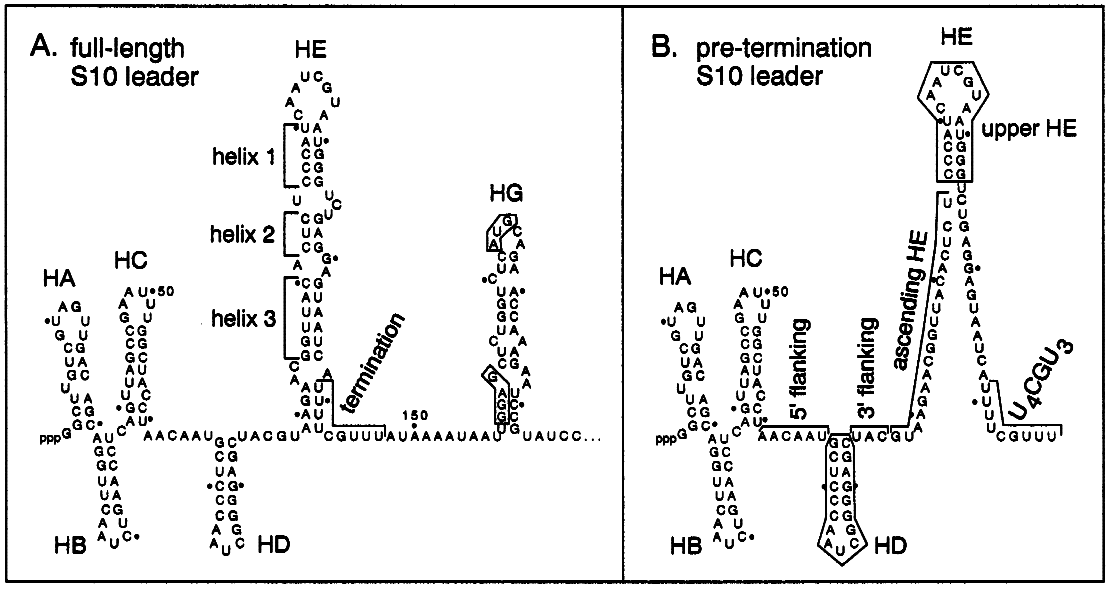

FIGURE 1. Secondary structures of the S10 leader RNA. The secondary structure in **A** was determined by enzymatic and chemical probing of the full-length S10 leader (Shen et al., 1988). The structure in **B** illustrates the prediction that the lower two-thirds of the HE hairpin is not base paired in the nascent leader, when RNA polymerase is paused at the cluster of Us. The elements of the secondary structure characterized in this report are indicated in **B**.

to represent the nascent leader when RNA polymerase is paused at the cluster of Us, as at least 13 nt at the 3′ end of the nascent RNA chain are still located in the transcription bubble and the RNA exit channel of RNA polymerase (Korzheva et al., 2000) and therefore are not available for intramolecular base pairing (Monforte et al., 1990). In this state, the ascending side of hairpin HE, containing nt 89–105, can be considered a "tether" between HD and upper HE.

The transcription terminator hairpin HE

Transcription termination at rho-independent terminators involves pausing of the transcription elongation complex at the termination site in response to the formation of a hairpin in the nascent RNA chain (Farnham & Platt, 1981; Artsimovitch & Landick, 2000; Toulokhonov et al., 2001). Recent experiments with the *his* pause site suggest that NusA may stimulate pausing/termination by stabilizing interaction between the RNA hairpin and the RNA polymerase flap domain (Toulokhonov et al., 2001). Because the upper HE hairpin is very similar to the *his* pause hairpin (Artsimovitch & Landick, 2000), we were interested in identifying specific features of the upper HE hairpin important for regulation of the S10 operon.

Our earlier studies showed that the upper HE hairpin (Fig. 1) is a determinant for NusA-mediated stabilization of the intrinsic pause (Zengel & Lindahl, 1992; Sha et al., 1995a). An intact helix in the HE hairpin is required, but several nucleotide changes in the 8-base loop have no effect (Freedman et al., 1987; Zengel & Lindahl, 1992; Sha et al., 1995a; Fig. 2A, mutants 1–5). To analyze in more detail the important structural features of this hairpin, we constructed additional mutations altering the upper HE helix and/or loop. Changes in the sequence or length of the HE loop had little or no effect on L4 regulation (Fig. 2A, mutants 9 and 11–16), indicating that no specific determinants in the loop are required. Replacement of the entire upper HE hairpin (helix 1 in Fig. 1) with similar natural hairpins also maintained L4 control of transcription, although the regulation was reduced (Fig. 2A, mutants 8 and 10). The reduced regulation of the hairpin substitution mutants likely reflects the changed helix, as a change in the helix alone also resulted in reduced regulation (Fig. 2A, mutant 7). Because none of these helix changes are predicted to cause rearrangements of the HE hairpin or significantly different ΔG values (based on RNA folding predictions using the Zuker mfold program; Mathews et al., 1999), the reduced activity may reflect small changes in the NusA response.

The HE hairpin differs from typical rho-independent terminators by having several bulges (Fig. 1A) that are highly conserved among bacteria whose S10 leaders can respond to L4-mediated autogenous control (Allen et al., 1999). To investigate if the internal bulges are necessary for L4 control, we introduced mutations that we predicted would strengthen or weaken the propensity for formation of a long HE stem. By changing sequences on one or the other side of the HE hairpin, we made two mutants predicted to form a bulgeless "perfect" hairpin. In either case, the resulting leader was no longer regulated by L4 (Fig. 2C, mutant 27; data not shown). Moreover, the constitutive low level expression of the *lacZ* reporter gene from these perfect hairpin leaders suggests that these mutant hairpins may now function as strong terminators even in the absence of L4.

In typical rho-independent transcription termination, base pairing in the region upstream of the termination site appears to facilitate release of the transcript (Yarnell & Roberts, 1999). However, when we changed the sequence on the ascending side to eliminate base pairing of either helix 2 or helix 3 (Fig. 1), regulation persisted (data not shown), suggesting that formation of an extended stem is not required for L4-mediated transcription control. Together with the perfect hairpin mutants, the helix 2 and helix 3 disrupting mutants suggest that the bulges per se are not required for L4 regulation. Because the HE hairpin is also required for L4-mediated translation control (Freedman et al., 1987), these features may contribute to this other level of autogenous control.

The site of transcription termination

Like a classical rho-independent transcription terminator, the HE hairpin is followed by a U-rich sequence (U_4CGU_3; Fig. 1). In an in vitro system, we observe

FIGURE 2. Summary of the response of S10 leader mutations to oversynthesis of r-protein L4. Mutant leader sequences for the indicated regions of the S10 leader are given, with differences from the wild-type (w.t.) sequence indicated in red. Elements from other RNAs that were substituted into the S10 leader sequence are indicated by underlining; the sources are indicated in the rightmost column and described in detail in Materials and Methods. The effect of L4 induction on synthesis was determined by gel analysis of extracts from [^{35}S]methionine-labeled cells. The repression of reporter protein synthesis in response to L4 induction is indicated. Each value represents the average of four or more independent labelings with the standard deviations also given. For easier inspection, the values for the L4-mediated expression were divided into arbitrary intervals and color-coded: red indicates little or no L4 regulation (± L4 Quant. equal to or greater than 0.65); blue indicates clear L4 regulation (± L4 Quant. equal to or less than 0.39); black indicates intermediate responses (± L4 Quant. between 0.42 and 0.56).

A. Mutations in the upper HE hairpin

#	Name	Sequence of upper HE	β-gal Synth. - L4 + L4	+/- L4 Quant.	Source
	no leader			0.95 ± 0.06	
	w.t.	CCCAU CAAUCGUA AUGGG		0.18 ± 0.01	
1	HE stem1	UGCAU CAAUCGUA AUGGG		0.56 ± 0.04	
2	HE stem2	CCCAU CAAUCGUA AUGCA		0.69 ± 0.08	
3	HE comp	UGCAU CAAUCGUA AUGCA		0.25 ± 0.03	
4	HE loop1	CCCAU CAGACGUA AUGGG		0.35 ± 0.01	
5	HE loop2	CCCAU CAAUCGGC AUGGG		0.23 ± 0.03	
6	HE1	GGGUA CAAUCGUA UACCC		0.33 ± 0.04	
7	HE3	ACAGG CAAUCGUA CCUGU		0.48 ± 0.06	23S
8	HE2	ACAGG UUAAUAUU CCUGU		0.39 ± 0.04	23S
9	HE5	CCCAU CUAGUCUU AUGGG		0.26 ± 0.01	*his* att
10	HE4	CCUGA CUAGUCUU UCAGG		0.42 ± 0.02	*his* att
11	AD1	CCCAU --GAAA-- AUGGG		0.30 ± 0.02	
12	AD1-G>A	CCCAU --AAAA-- AUGGG		0.27	
13	AD2	CCCAU -AAUCG-- AUGGG		0.26 ± 0.02	HD loop
14	AD3	CCCAU GUA--AUA AUGGG		0.21 ± 0.02	23S
15	AD4	CCCAU CUAAUG-A AUGGG		0.22 ± 0.02	*trp* att
16	AD5	CCCAU UUUUGAAGU AUGGG		0.20 ± 0.04	*Hinf* HE

B. Mutations in the descending side of hairpin HE and downstream

#	Name	Sequence of descending HE and downstream	β-gal Synth. - L4 + L4	+/- L4 Quant.
	no leader			0.95 ± 0.06
	w.t.	UCU GAG GA GUAAUC A UUUUCGUUU AUAAAAUAAUUGGAG		0.18 ± 0.01
17	U4>A	UCU GAG GA GUAAUC A AAAACGUUU AUAAAAUAAUUGGAG		0.74 ± 0.01
18	Δ8	UCU GAG GA GUAAUC A UUUUCGUUU A--------UUGGAG		0.17 ± 0.01
19	U4/U3>A	UCU GAG GA GUAAUC A AAAACGAAA AUAAAAUAAUUGGAG		0.52 ± 0.03

C. Mutations in the ascending side of hairpin HE

#	Name	Sequence of ascending HE (tether)	β-gal Synth. - L4 + L4	+/- L4 Quant.	Source
	no leader			0.95 ± 0.06	
	w.t.	UACGUAAGA AC GGUUAC A CUC U		0.18 ± 0.01	
20	Δ2	UAC--AAGA AC GGUUAC A CUC U		0.31 ± 0.02	
21	Δ5	UAC-----A AC GGUUAC A CUC U		0.68 ± 0.11	
22	Δ8	UAC------ -- GGUUAC A CUC U		0.70 ± 0.16	
23	Δ12	UAC------ -- ----AC A CUC U		0.73 ± 0.19	
24	Ins7	UACGAGCUACGUAAGA AC GGUUAC A CUC U		0.24 ± 0.02	
25	Ins14	UACAAUCGGGGAGCUACGUAAGA AC GGUUAC A CUC U		0.80 ± 0.00	
26	*his* asc.	UACACCAUC AU CACCAU C AUC U		0.84 ± 0.22	*his* att
27	perfect #1	UACGUAAGA U- GGUUAC UCCUCAGA		0.93 ± 0.06	

D. Mutations in hairpin HD and its flanking sequences

#	Name	Sequence of HD and flanking regions	β-gal Synth. - L4 + L4	+/- L4 Quant.	Source
	no leader			0.95 ± 0.06	
	w.t.	AACAAU GCUCCC AAUCG GGGAGC UAC		0.18 ± 0.01	
28	ΔHD	AACAAU ------ ----- ------ UAC		0.79 ± 0.09	
29	ΔHD+flank	------ ------ ----- ------ --C		0.85 ± 0.11	
30	G79/80	AACAAU GCUCCC AAUCG GCCAGC UAC		0.78 ± 0.04	
31	HD8	------ GCUCCC AAUCG GGGAGC UAC		0.65 ± 0.15	
32	HD9	UUGUUA GCUCCC AAUCG GGGAGC UAC		0.81 ± 0.05	
33	HD10	AACAAU GCUCCC AAUCG GGGAGC AUC		0.48 ± 0.01	
34	HD11	UUGUUA GCUCCC AAUCG GGGAGC AUC		0.54 ± 0.04	
35	HD1	AACAAU UUCCGG CAAAU CCGGAA UAC		0.26 ± 0.02	23S
36	HD4	AACAAU GCUCCC GAAUA GGGAGC UAC		0.19 ± 0.07	23S
37	HD3	AACAAU GCUCCC CUCGC GGGAGC UAC		0.54 ± 0.07	23S
38	HD5	AACAAU GCUCCC CACGC GGGAGC UAC		0.17 ± 0.01	23S
39	HD7	AACAAU GCUCGC GUAAUA GCGAGC UAC		0.72 ± 0.02	23S
40	HD12	AACAAU GCUCGC GAAAUA GCGAGC UAC		0.27 ± 0.05	23S
41	HD13	AACAAU GCUCCC AUUCG GGGAGC UAC		0.34 ± 0.03	
42	HD2	AACAAU GC-CCU GAAAA AGG-GC UAC		0.50 ± 0.10	λBoxB
43	HD6	AACAAU GCUGGG GAAA- CCCAGC UAC		0.80 ± 0.03	23S
44	HD-Mmorg	AACAAU AUCCCC GAUAU GGGGAU UAC		0.16 ± 0.00	*Mmorg* HD
45	HD-Vchol	AACAAU GCGUCC UAAUCUU GGAUGC UAC		0.19 ± 0.00	*Vchol* HD

FIGURE 2. *See caption on facing page.*

transcripts corresponding to termination at both clusters of Us (Zengel & Lindahl, 1990b, 1992). In vivo attenuated transcripts contain 3′ ends corresponding to only the proximal cluster (Zengel & Lindahl, 1990a), possibly because in vivo transcripts terminating at the more distal cluster are trimmed back by a nuclease. Because Us are thought to be required for efficient termination, it is not surprising that replacement of the proximal U cluster with As almost completely abrogates L4 regulation (mutant 17 in Fig. 2B), nor is it surprising that a deletion removing 8 bases downstream of the U_4CGU_3 motif has no effect on L4 control (mutant 18). However, an unexpected result was the residual regulation observed when we changed all 7 Us in the U_4CGU_3 motif to As (mutant 19). This mutant exhibited more regulation than the U_4 to A_4 mutant (mutant 18). We know from in vitro studies that the strength of RNA polymerase pausing at the U_4CGU_3 site is very sensitive to the concentration of UTP (Sha et al., 1995b). Because upstream clusters of Us are ignored even at very low UTP concentration, RNA polymerase appears to be potentiated for pausing at this specific position. We assume that this sensitivity is involved in L4-mediated termination. Perhaps the 7 As confer similar sensitivity to the concentration of ATP, thereby allowing L4 stimulation of termination (or very stable pausing) in the absence of Us.

The L4 response region

Genetic and biochemical experiments suggest that hairpin HD is required for L4's stimulation of transcription termination, but is dispensable for NusA-stimulated pausing (Sha et al., 1995a; Zengel & Lindahl, 1996). Deletion of the HD hairpin or disruption of the HD helix largely eliminates regulation (Fig. 2D, mutants 28–30; Zengel & Lindahl, 1992, 1996). To further define critical features of the HD hairpin, we substituted the native hairpin with similar hairpin structures from other natural RNAs. Irrespective of source and loop size, most of these constructs were subject to strong L4 regulation (Fig. 2D). We were surprised by these results because we had expected specific features of the native HD hairpin would be essential for the S10 operon-specific L4 control of transcription. We did observe that foreign hairpins with an A in the second position of the loop (e.g., mutants 35, 36, 44, and 45) exhibited stronger regulation than hairpins with a U (mutants 37 and 39). Regulation with the poorly regulated hairpins improved when the U was replaced with an A (mutants 38 and 40), whereas replacement of the A in the native sequence with a U (mutant 41) slightly reduced L4 regulation. These results suggest that the A at the second position in the loop may play a role in L4 regulation, particularly in the presence of other changes in the HD hairpin. Interestingly, a tetraloop-containing hairpin (mutant 43) conferred very little regulation, and a 5-bp helix structure (mutant 42) showed reduced regulation. These two mutants suggest that there are structural boundaries for HD hairpins capable of bestowing L4 control.

The unpaired sequences flanking the HD hairpin, AACAAU on the 5′ side and UA on the 3′ side, are highly conserved in organisms whose leaders are sensitive to L4 control (Allen et al., 1999). Deletions or sequence changes in these regions significantly reduced L4 regulation (Fig. 2D, mutants 31–34). To some extent, the effect on regulation might reflect changes in the structures of these normally non-base-paired sequences (for example, the changes in only the 5′ sequence or only the 3′ sequence fortuitously results in the potential for an elongated HD stem). Nevertheless, the phenotypes of these mutants collectively indicate that the primary sequence and/or secondary structure of these flanking regions are important for L4 control.

In the paused transcription complex, the nascent RNA would likely form the structure shown in Fig. 1B, with the HD hairpin and upper HE hairpin linked by a tether. We found that the length of this tether has limited flexibility: regulation was maintained in leaders with a 2-base deletion or a 7-base insertion (mutants 20 and 24), but longer deletions or insertions significantly decreased control (mutants 21–23 and mutant 25). Base substitutions within the tether, including changes that disrupt base pairing with the descending side of the HE (helix 2 and helix 3 mutants discussed above), had no significant effect on L4 control, consistent with the notion that nt 89 through 105 form a non-base-paired tether during transcription termination. On the other hand, complete replacement of the tether with the analogous segment of the *his* pause hairpin eliminates regulation (Fig. 2C, mutant 26). One explanation for these results is that L4 and/or the transcription elongation complex has multiple contacts with the tether such that loss of one or a few contacts can be tolerated, but complete loss of all contacts eliminates regulation.

In summary, our genetic dissection of the S10 leader suggests that, in spite of the exquisite specificity of L4-mediated regulation of the S10 operon, there is remarkable flexibility in the features of the 80-nt region required for this response. Elements sufficient for optimal L4 regulation include: (1) an intact upper HE helix (we tested only 5 bp helices) containing a 4–9-nt loop with no apparent sequence requirement; (2) a tether between 20 and 29 nt long, with flexibility in the sequence requirement (although replacement of an 18-base segment with a foreign sequence eliminates control); (3) an intact HD hairpin with a 6-bp helix and a loop between 5 and 7 nt long (a tetraloop was poorly regulated), the loop sequence being somewhat flexible but optimal with an A at the second position; and (4) specific non-base-paired sequences flanking the HD hairpin. Taken together, our results suggest that L4-mediated regulation may depend on redundant determinants, per-

haps reflecting multiple interactions between L4 and the paused transcription complex.

MATERIALS AND METHODS

Strains and plasmids

Physiological experiments were done in the *E. coli* strain LL308 (Lindahl & Zengel, 1979). DNA manipulations were done using DH5αF′. The L4 source was the high copy number plasmid pBAD-L4 (conferring ampicillin resistance), a derivative of pBAD18 with the L4 gene under control of an arabinose-inducible promoter (Allen et al., 1999). Mutant S10 leaders were constructed in pACYC-*lacZ* (conferring kanamycin resistance), a derivative of pACYC177 (Chang & Cohen, 1978). It contains the IPTG-inducible *trc* promoter from plasmid pTrc99A followed by the S10 leader (nt 1–160, Fig. 1) fused to the natural leader sequence of *lacZ* and intact *lacZ* gene. For mutant leaders containing substitutions equivalent to elements from other RNAs, the sources are as follows: mutant 7: *E. coli* 23S nt 1385–1389 and nt 1398–1402; mutant 8: *E. coli* 23S nt 1385–1402; mutant 9: *E. coli his* attenuator hairpin loop; mutant 10: *E. coli his* attenuator hairpin stem-loop; mutant 13: pentaloop from *E. coli* S10 leader HD hairpin; mutant 14: *E. coli* 23S nt 1093–1098; mutant 15: *E. coli trp* attenuator hairpin loop; mutant 16: *Haemophilus influenzae* S10 leader HE hairpin loop (Allen et al., 1999); mutant 26: ascending side of *E. coli his* attenuator; mutant 35: *E. coli* 23S nt 1486–1503 with deletion of A1490; mutant 36: *E. coli* 23S nt 610–618; mutant 37: *E. coli* 23S nt 1726–1734; mutant 38: *E. coli* 23S nt 1726–1734 with U1729A mutation; mutant 39: *E. coli* 23S nt 1091–1100; mutant 40: *E. coli* 23S nt 1091–1100 with U1094A mutation; mutant 43: *E. coli* 23S nt 121–130; mutant 44: *Morganella morganii* S10 leader HD hairpin; mutant 45: *Vibrio cholerae* S10 leader HD hairpin.

Analysis of L4 regulation of protein synthesis rates

Cultures were grown at 37 °C in AB minimal medium (Clark & Maaløe, 1967) supplemented with 0.5% glycerol, 1 μg/mL thiamine, 20 μg/mL each 19 amino acids (no methionine), 100 μg/mL kanamycin, and 200 μg/mL ampicillin. At OD_{450} = 0.2–0.4 (approximately 5×10^7 cells/mL), two 100-μL aliquots were removed and labeled with [^{35}S]methionine (3 μCi, approximately 1000 Ci/mmol) for 2 min and then lysed by addition of 200 μL SDS lysis buffer and incubation for 2 min at 95 °C. Immediately after (t = 0 min), IPTG was added (to 1 mM) to 1 mL culture. The culture was split in half, and at t = 1 min, arabinose (to 0.25%) was added to one portion. At t = 10 min, two 100-μL aliquots of the IPTG only cells were labeled for 2 min with [^{35}S]methionine; 1 min later, two 100-μL aliquots of the IPTG + arabinose cells were labeled for 2 min.

Total-cell extracts were fractionated by gel electrophoresis on a 7.5% or 12% (wt/vol) SDS polyacrylamide gel (Laemmli, 1970). The 12% gel resolves small proteins like L4 (22 kDa) and allowed us to confirm that the synthesis of L4 was increased following arabinose induction. The 7.5% gel provides better resolution of the β-galactosidase protein. In both cases, the stacking gel contained the standard 30:0.8 ratio of acrylamide to bis-acrylamide, but the running gel contained a reduced 30:0.2 ratio to maximize resolution of larger proteins.

The radioactivity in β-galactosidase protein bands was quantified from the 7.5% gels by using a Molecular Dynamics PhosphorImager. The radioactivity in the β-galactosidase band was first corrected for background by subtracting the signal in a similar sized region of the gel immediately above, and then was normalized to the total amount of radioactivity in the lane. The effect of L4 oversynthesis was then calculated as the ratio between the corrected/normalized β-galactosidase value for a given mutant in the presence of both IPTG and arabinose divided by the β-galactosidase value for the same mutant in the presence of just IPTG.

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