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Mutations That Increase Expression of the *rpoS* Gene and Decrease Its Dependence on *hfq* Function in *Salmonella typhimurium*

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The RpoS transcription factor (also called σ^S or σ^{38}) is required for the expression of a number of stationary-phase and osmotically inducible genes in enteric bacteria. RpoS is also a virulence factor for several pathogenic species, including *Salmonella typhimurium*. The activity of RpoS is regulated in response to many different signals, at the levels of both synthesis and proteolysis. Previous work with *rpoS-lac* protein fusions has suggested that translation of *rpoS* requires *hfq* function. The product of the *hfq* gene, host factor I (HF-I), is a ribosome-associated, site-specific RNA-binding protein originally characterized for its role in replication of the RNA bacteriophage Qβ of *Escherichia coli*. In this study, the role of HF-I was explored by isolating suppressor mutations that map to the region directly upstream of *rpoS*. These mutations increase *rpoS-lac* expression in the absence of HF-I and also confer substantial independence from HF-I. DNA sequence analysis of the mutants suggests a model in which the RNA secondary structure near the ribosome binding site of the *rpoS* mRNA plays an important role in limiting expression in the wild type. Genetic tests of the model confirm its predictions, at least in part. It seems likely that the mutations analyzed here activate a suppression pathway that bypasses the normal HF-I-dependent route of *rpoS* expression; however, it is also possible that some of them identify a sequence element with an inhibitory function that is directly counteracted by HF-I.

The *rpoS* gene encodes a specificity factor for RNA polymerase (44, 46, 59) which is required for the transcription of many genes expressed during the onset of stationary phase. RpoS-dependent adaptations to nutrient limitation and starvation identified so far in *Escherichia coli* include not only shifts in metabolic pathways but also resistance mechanisms protective against life-threatening stresses such as high osmolarity, heat shock, elevated H_2O_2, and UV light (reviewed in references 26 and 37). RpoS is also a virulence factor for *Salmonella typhimurium* (16) and other enteric bacteria. The regulation of this regulatory protein is itself complex: RpoS abundance can be increased by a variety of inducing treatments (37). Control of RpoS can occur both at the level of synthesis and by proteolysis (27, 34, 65); in this respect it is reminiscent of heat shock sigma factor (RpoH) regulation (66). It has also been demonstrated that RpoS abundance is positively regulated by ppGpp (23), and this may provide a unifying mechanism for the multitude of RpoS inducers. An *hns* mutant, lacking the abundant DNA-binding protein H-NS, has an increased level of RpoS in exponential phase, and this effect is remarkable because it occurs through increases in both translation and protein stability (2, 65). Genetic evidence suggests that RpoS is degraded by the energy-dependent ClpXP protease (52) with the help of other factors (42, 48, 58).

The *E. coli hfq* gene product, host factor I (HF-I), was discovered through its role in the in vitro replication of Qβ, an RNA bacteriophage (19, 20, 30). The function of HF-I in uninfected cells has been unknown, but it is an RNA-binding protein associated with ribosomes (5, 31, 53), and *hfq* mutants are pleiotropic (60). In recent work, it has been shown that *S. typhimurium* and *E. coli* mutants defective in the *hfq* gene have substantially reduced expression of *rpoS* (3, 43). The pleiotropy of *hfq* mutants may be partly explained by the requirement of HF-I for efficient translation of *rpoS*.

Here we describe genetic experiments performed to define the role of HF-I in *rpoS* expression by the isolation of suppressors that improve expression of *rpoS-lac* in an *hfq* mutant background. A number of mutants were isolated; many of these substantially decrease the dependence of *rpoS* expression on *hfq* function. A model is presented to explain the effect of these mutations on *rpoS* expression through destabilization of an RNA secondary structure that is postulated to inhibit translation. A preliminary test of the importance of two RNA stems predicted by the model was carried out with compensatory mutations. The results of this test strongly support the model for a base pair of one proposed stem, while no suppression or compensation was seen for a base pair in the other stem. These results are discussed in terms of the possible involvement of sequence-specific interactions of *rpoS* RNA with regulatory factors, as well as other models.

MATERIALS AND METHODS

Bacterial strains and construction. The wild-type *S. typhimurium* strain LT-2 does not carry the *lac* operon. Strain TE6134 (*hfq-1*::Mud-Cam) was constructed by transduction from TE5314 (1, 3) into the recipient strain, LT-2. The high-frequency generalized transducing bacteriophage P22 mutant HT*105/1 int-201* (50) was used for all transductions in *S. typhimurium* by standard methods (9).

Protein fusions and operon fusions to the *lac* genes were constructed with a DNA fragment derived from the *E. coli rpoS* gene and were described in detail previously (3). The *rpoS-lac* protein fusion was constructed by digesting pMMkatF2 (obtained from P. C. Loewen [44]) with *Cla*I and *Eag*I, filling in the ends with the Klenow fragment of DNA polymerase I, and cloning the 1.6-kb fragment into pRS552 (56), which had been digested with *Eco*RI and *Bam*HI and filled in. The *rpoS-lac* construction regenerates the *Eco*RI and *Eag*I sites flanking the inserted segment. This fusion extends from the *Cla*I site, upstream of *nlpD*, to codon 73 of the *rpoS* gene. For the purpose of recloning the *Nco*I-*Eag*I segment, which was recovered from *rpoS* mutants and sequenced, into an unmutagenized fusion vector, the *rpoS-lac* fusion was cloned into a derivative of

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pRS552 from which the *Nco*I and *Eag*I sites had been removed by a standard fill-in procedure.

All *lac* fusions were transferred to the chromosome of an *E. coli recD* mutant by linear transformation as described previously (15). Phage P22 transducing lysates were grown in *E. coli* (14, 15) and were used to transduce the fusions into the *S. typhimurium* chromosome. Each resulting strain carries a *lac* fusion in single copy as an insertion of a Kan-promoter-*lac* fragment in the *put* operon. Fusions containing mutations were usually recovered from the *S. typhimurium* chromosome onto a plasmid in a P22-mediated transductional cross, exactly as described previously (15). In one case, the mutation was recovered by PCR. Chromosomal DNA was prepared as described previously (3, 22).

Media and growth conditions. Bacteria were grown at 37°C in Luria-Bertani (LB) medium, prepared as described in reference 54. Plates were prepared by using nutrient agar (Difco) with 5 g of NaCl per liter. Antibiotics were added to final concentrations as follows: sodium ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; and kanamycin sulfate, 50 μg/ml. MacConkey lactose agar was prepared as described previously (40).

β-Galactosidase assays. Cells were centrifuged and resuspended in Z buffer (100 mM KPO_4 [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$) and then permeabilized by treatment with sodium dodecyl sulfate and chloroform (40). Assays were performed in Z buffer containing 50 mM β-mercaptoethanol by using a kinetic method and a plate reader (Molecular Dynamics). Activities (changes in optical density at 420 nm [ΔOD_{420}] per minute) were normalized to the cell density (OD_{650}) of the actual sample; activity was always compared to appropriate controls assayed at the same time. For experiments employing cultures grown to different densities, the number of cells harvested was adjusted to provide approximately equal cell density in the assay. In each case, the results are from a single experiment; each experiment was repeated several times, with similar results.

Construction of mutations. Localized mutagenesis was carried out as described previously (9, 29). Site-directed mutations in the *rpoS* ribosome binding site (RBS) region were constructed by PCR with segment overlap extension, with minor modifications (28). In this method one DNA fragment containing the desired mutation is synthesized in an initial PCR and then is used as a primer in a second PCR. This method allows substitution of mutations incorporated into the primer sequence without the need for a nearby restriction site. As a template, we used a single 441-bp DNA fragment cloned into pUC119 and pUC120 (61). The templates for the first and second reactions were thus oriented oppositely, so that the final product could be amplified with a single *lac* primer. All constructs were sequenced completely between the *Nco*I and *Eag*I sites.

DNA sequencing. Sequencing of double-stranded plasmid DNA was done as previously described (14) by using Sequenase version 2.0 (U.S. Biochemical Corp.) according to the manufacturer's instructions. Plasmid DNA for sequencing was isolated by column chromatography (Qiagen).

Computer analysis. RNA secondary structures were analyzed by MFOLD and several plotting methods from the University of Wisconsin Genetics Computer Group suite of programs (13).

RESULTS

Mutations that increase *rpoS-lac* expression. In previous studies, it was found that *hfq* mutants of *S. typhimurium* and *E. coli*, which are defective in HF-I, show substantially reduced synthesis of the RpoS sigma factor (3, 43). As determined with an *rpoS-lac* protein fusion, in the *hfq* mutant, expression is four- to sixfold lower under inducing conditions (stationary phase in LB medium), while expression of *rpoS-lac* operon fusions is barely affected (3, 4). Pulse-labeling shows that the rate of synthesis of RpoS is decreased in the *hfq* mutant; therefore, it is likely that translation of *rpoS* is specifically defective. The mechanism of this dependence is not yet known.

To investigate the role of HF-I in *rpoS* control, we sought mutations that alter the expression of *rpoS*. We looked for mutations that increase *rpoS-lac* expression in an *hfq* mutant background, anticipating that some such mutations might function as suppressors of the *hfq* mutant phenotype. We relied on the fact that an otherwise-wild-type *S. typhimurium* strain carrying a single-copy *rpoS-lac* fusion (to codon 73) displays a Lac^+ phenotype on MacConkey lactose plates, whereas an *hfq* mutant with the same fusion is Lac^-. A transducing phage P22 lysate was grown on strain TE6253 (Kan^r *rpoS-lac* [pr]), mutagenized with hydroxylamine in vitro (29), and used to transduce *hfq* mutant strain TE6134 to Kan^r on MacConkey lactose plates. Rare Lac^+ colonies were recovered and characterized, and a total of 11 hydroxylamine-induced mutations were analyzed. These mutations all mapped to the region between the *Nco*I and *Eag*I sites shown in Fig. 1 (see Materials and Methods for details).

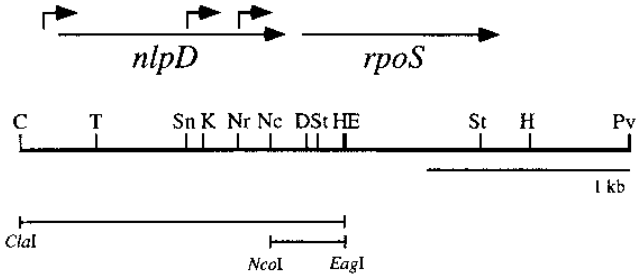

FIG. 1. Map of the *rpoS* region. The center line is a restriction map of the 3.0-kb *Cla*I-*Pvu*II DNA fragment from pMMkatF2 (44) that includes the *E. coli nlpD* and *rpoS* genes, which are shown above with three promoters (marked by arrows) that can express *rpoS* (33). Indicated at the bottom are two restriction fragments used extensively in this work: the *Cla*I-*Eag*I fragment used to make the *lac* fusions and the *Nco*I-*Eag*I fragment used to make substitutions of mutations. Restriction site abbreviations: C, *Cla*I; T, *Tth*111I; Sn, *Sna*BI; K, *Kpn*I; Nr, *Nru*I; Nc, *Nco*I; D, *Dra*I; St, *Stu*I; H, *Hpa*I; E, *Eag*I; Pv, *Pvu*II.

DNA sequence. For each mutation, the sequence of the *Nco*I-*Eag*I segment was determined; the positions and identities of the mutations are presented in Fig. 2. *S. typhimurium* fusion strains that carry the mutations indicated in Fig. 2 in single copy in the chromosome were reconstructed; results from β-galactosidase assays of stationary-phase LB cultures of these strains are also presented in Table 1. Each mutation increases *rpoS-lac* expression in the *hfq* mutant background; the range of increase observed is 2- to 11-fold. The level of expression in a wild-type background was also determined for each mutant, and dependence on *hfq* was determined by computing the ratio of the two activities (Table 1). All of the mutations decrease the dependence of *rpoS-lac* expression on *hfq* function; five of these exhibit a particularly large decrease in *hfq* dependence, and the G118A mutant is essentially independent of *hfq*. We also constructed chromosomal *rpoS-lac* operon fusions bearing each of the 11 mutations and found that expression was identical to that in the wild type for all mutants (4). From these results, we concluded that mutations in the region directly upstream of *rpoS* can dramatically increase its expression at the translational level, while at the same time decreasing its dependence on *hfq* function.

Models. The mutations listed in Table 1 were first grouped by location in the DNA sequence and then ranked by *hfq* dependence. There is a cluster of mutations with strong effects that lies about 100 bp upstream of the *rpoS* AUG codon, a second cluster in the RBS region directly upstream of the AUG codon, also with strong effects, and three more mutations within the *rpoS* coding sequence. Although the first cluster lies within the *nlpD* coding sequence, none introduces a stop codon. Other experiments have shown that translation of *nlpD* is not required for HF-I's effect on *rpoS* expression (4, 33). One mutation within *nlpD* (C126U) and two of three within the *rpoS* coding sequence are predicted to be silent with respect to translation.

Since these mutations all increase *rpoS* expression, they may act by decreasing the stability of a base-paired structure(s) within *rpoS* mRNA. Stable base pairing is capable of limiting the access of ribosomes to the RBS region of an mRNA and could play a regulatory role (see Discussion). In this way, the effects of mutations in the upstream cluster might be explained if nucleotides in this region pair with partners in the RBS region. These considerations led to the structural model shown

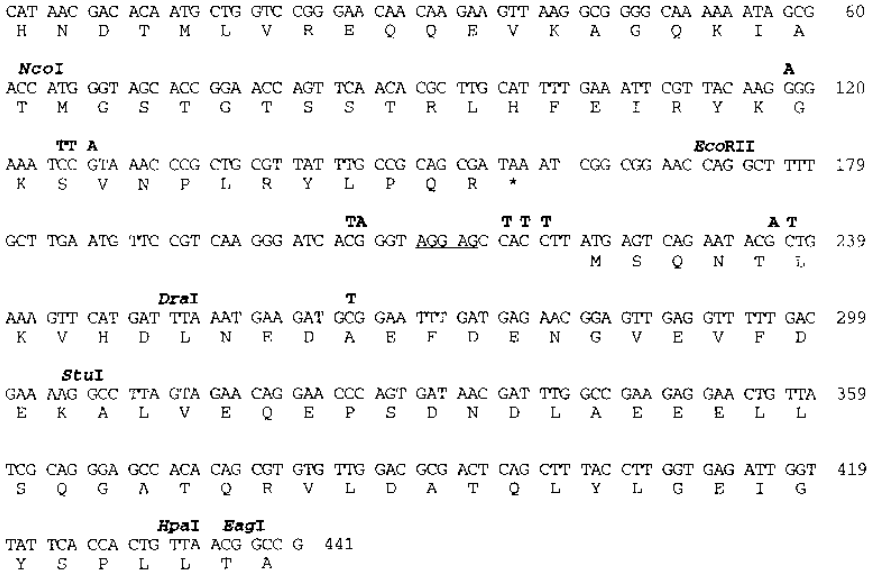

FIG. 2. Mutations affecting *rpoS* expression. The DNA sequence of the end of the *nlpD* gene and the beginning of the *rpoS* gene is shown. The predicted amino acid sequences of *nlpD* (stop codon at bp 157) and *rpoS* (start codon at bp 222) are also shown, and the *rpoS* Shine-Dalgarno sequence is underlined. Above the DNA sequence, individual boldface letters indicate the positions and identities of mutations that increase *rpoS* expression and confer *hfq* independence. These are all simple transitions except for the tandem C → T transitions at bp 216 and 218. Some restriction sites are also indicated in boldface. (Note: in the text and subsequent figures, mutations are identified by the sequence change in RNA rather than in DNA.)

in Fig. 3. Three stems, each containing at least three GC pairs, are connected by unpaired nucleotides. The *rpoS* Shine-Dalgarno sequence (53a) within the RBS region protrudes as a bulge between the second and third stems. All eight of the mutations that map to these two clusters (including one containing two substitutions) lie in stems of this structure. The three mutations mapping downstream, within the *rpoS* coding sequence, are not explained by the model. At present we do not understand their phenotypes, but we note that these are among the weaker mutations recovered.

Tests of RNA secondary structure. Even if secondary structure does limit *rpoS* expression, it may be questioned whether the specific structure shown in Fig. 3 is the relevant one. For example, computer analysis with MFOLD (13) identified a related RNA structure in which the top strand of stem I pairs with the bottom strand of stem II (not shown). Further analysis with MFOLD identified other potential structures, including one in which the RBS region would pair with a long stem lying immediately upstream. To assess the contributions of these predicted stems to a comparably folded RNA structure, the region from nucleotides 110 to 255 of Fig. 2 was analyzed by MFOLD. These boundaries were chosen because they lie in regions flanking the segment of interest that are predicted to be relatively unstructured. MFOLD calculated that all of the most stable structures, including these three possible pairing schemes for the RBS, had about the same free energy of formation (within 5%). These energies are not different enough to help us predict which structure is important in vivo.

We used a genetic strategy to test the contribution of speci-

TABLE 1. β-Galactosidase activities of *hfq*-independent *rpoS-lac* [pr] mutants

rpoS mutation	β-Galactosidase activity[a]		Wild-type/*hfq* mutant ratio
	Wild type	*hfq* mutant	
None (wild type)	158	39	4.1
G118A	162	146	1.1
C125U	699	428	1.6
C126U	569	323	1.8
G127A	425	176	2.4
C216U + C218U	352	184	1.9
G206A	816	384	2.1
C219U	255	90	2.8
C205U	365	127	2.9
C265U	194	80	2.4
G236A	341	112	3.0
C237U	291	103	2.8

[a] See Materials and Methods for explanation of the units.

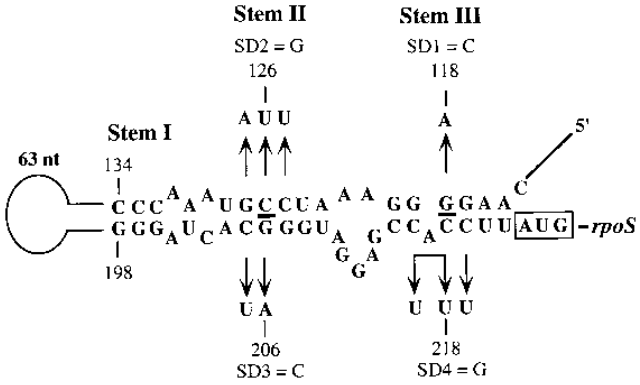

FIG. 3. Model of secondary structure including the RBS of *rpoS*. The folded structure shows the Shine-Dalgarno sequence looped out between two RNA stems (labeled II and III). Mutations that increase *rpoS* expression, obtained by localized mutagenesis, are indicated by arrows and letters. Positions numbered by base pair and designated SD1 through SD4 were altered by site-directed mutagenesis as discussed in the text. nt, nucleotides.

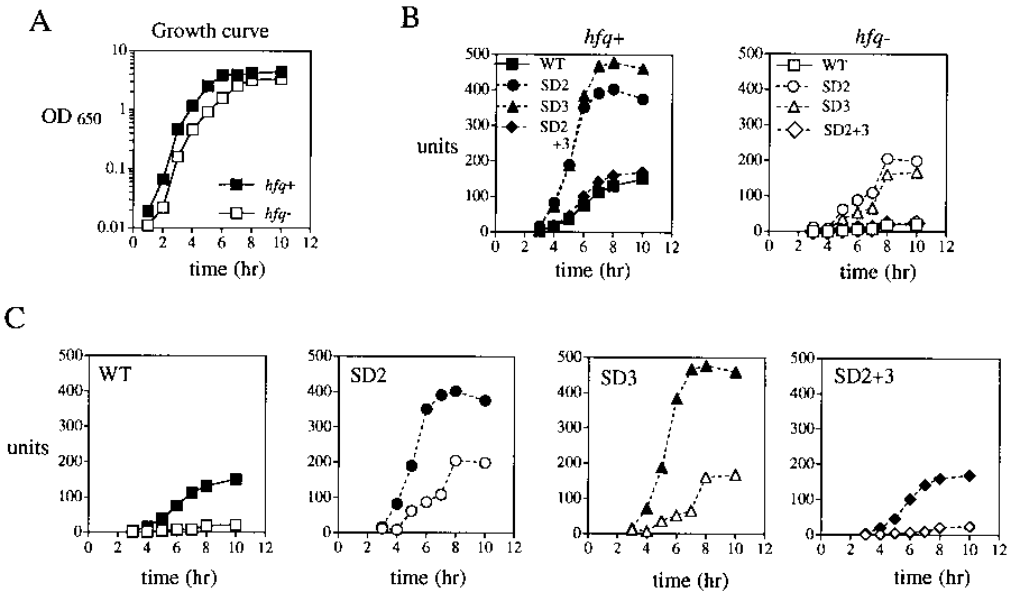

FIG. 4. Test of compensatory effects for mutants in stem II: SD2 and SD3. Mutations were placed in an *rpoS-lac* construct in single copy in the *S. typhimurium* chromosome in either a wild type (filled symbols) or an *hfq* mutant (open symbols) background. Cultures were grown to stationary phase in LB medium; at various times aliquots were taken and assayed for β-galactosidase activity. Shown are growth curves (A), summary plots comparing β-galactosidase activity versus time for different fusions in either an *hfq* mutant or wild-type background (B), and plots showing the effect of *hfq* status on individual fusions (C). WT, wild type.

fic base pairs to the proposed structure (18, 25). In this method, the partners of a putative GC pair are individually changed (G → C and C → G), and a double mutant (GC → CG) is also constructed. If only pairing ability and no other interaction is important for function, the single changes will confer a mutant phenotype but the double mutant will have compensatory changes that allow pairing and should restore the wild-type phenotype. We chose central base pairs in stems II and III for this test. The mutations are identified by the notations SD1 (site-directed mutation 1) to SD4, and the changes affect the positions identified by number in Fig. 3 (SD1 = G118C, SD2 = C126G, SD3 = G206C, and SD4 = C218G). The double mutations are designated SD1+4 and SD2+3. Each mutant segment was constructed by the site-directed method and placed in the fusion used for the previous mutant isolation and analysis, in single copy in the *S. typhimurium* chromosome. For each fusion, otherwise-isogenic paired *hfq*$^+$ and *hfq* mutant strains were constructed.

Cultures were grown in LB medium, samples were removed at various times along the growth curve, and the β-galactosidase activity was determined (Fig. 4 and 5). Figures 4A and 5A show growth curves (OD_{650} of the culture versus time) for one pair of *hfq*$^+$ and *hfq* mutant strains. (All strains of the same *hfq* genotype had superimposable growth curves.) Figures 4B and

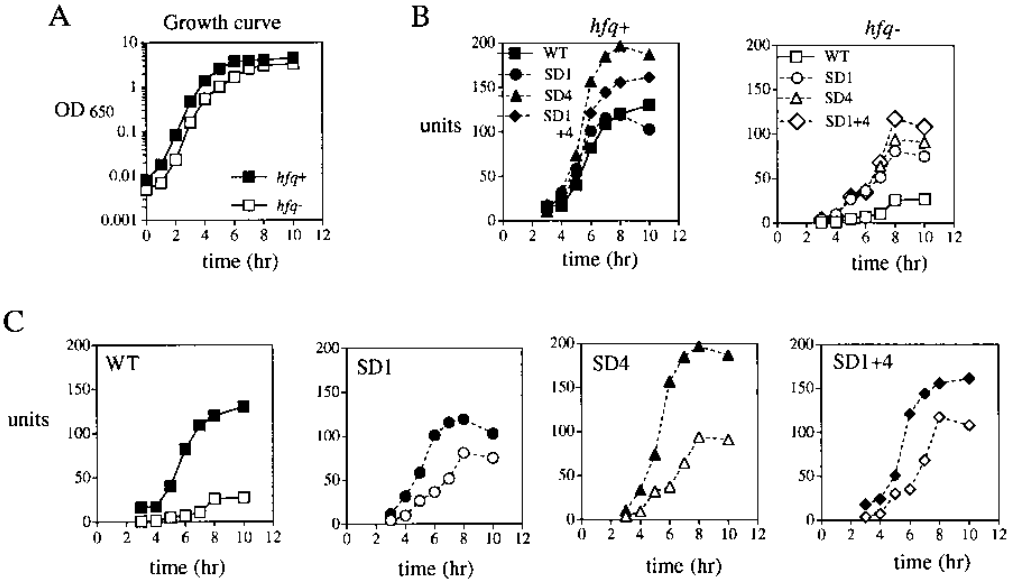

FIG. 5. Test of compensatory effects for mutants in stem III: SD1 and SD4. Mutations affecting stem III of an *rpoS-lac* construct, tested in single copy in the *S. typhimurium* chromosome. For other details, see the legend to Fig. 4.

5B show summary plots of activity versus time, allowing comparison of the mutant and wild-type *rpoS-lac* fusions in the same strain background, either *hfq* mutant or wild type. The effect of *hfq* status on individual fusions can be seen more clearly in Fig. 4C and 5C. Because the growth curves of the *hfq* mutants lag slightly and reach a lower terminal OD_{650}, each set of data was also analyzed to normalize for this difference, with no change in the results (4). We also constructed operon fusion versions of all of these mutants. In each case, expression was unchanged from that seen with the wild-type *rpoS-lac* operon fusion.

For stem II (Fig. 4), the individual mutations, SD2 and SD3, yielded strong mutant phenotypes, with an increase in expression in the *hfq* mutant background and a decreased dependence on *hfq* function (ratios of 1.9 and 2.8, respectively). The double mutant is indistinguishable from the wild type: it decreases expression in the *hfq* background and restores *hfq* dependence. These results are clearly consistent with a role for stem II in regulation of *rpoS* expression.

In the case of stem III (Fig. 5), the individual mutations, SD1 and SD4, yielded strong mutant phenotypes, with an increase in expression in the *hfq* mutant background and a lessened dependence on *hfq* (ratios of 1.1 and 2.0, respectively). In contrast to the results with stem II, however, for the stem III SD1+4 double mutant, the behavior is not like that of the wild type but instead is similar to that of the two single mutants. In this case the prediction of the simple pairing hypothesis is not fulfilled. This result does not necessarily mean that the model is incorrect but only that if it is correct there must also be additional sequence-specific interactions (see Discussion).

A related method of approaching the question of RNA secondary structure is a phylogenetic sequence comparison, which analyzes "experiments of nature" by looking at related organisms for the conservation of base-paired structures despite changes in the primary sequence (18). BLAST analysis revealed that the DNA sequence of this small region of *nlpD-rpoS* is 100% identical in *E. coli*, *S. typhimurium*, and *Salmonella typhi* and contains only one change in *Shigella flexneri*, at the beginning of the first stem. However, an important difference was found in *Yersinia entercolitica*, a species which also contains HF-I (45). The nucleotides in the bottom half of stem III, between the RBS and the ATG codon of *rpoS*, are not conserved, and this structure is predicted not to form. From this, we predict either that control of *rpoS* in *Y. enterocolitica* is not like that in other enteric bacteria or that the structure of stem III is not important for regulation.

DISCUSSION

Previous work has shown that the *hfq* gene, which encodes a small ribosome-associated RNA-binding protein, is required for efficient translation of *rpoS* (3, 43). In an *hfq* mutant strain of *S. typhimurium*, there is a four- to fivefold decrease in *rpoS* expression as determined with an *rpoS-lac* protein fusion, and there is a similar decrease in the synthesis of native RpoS protein. The effect of *hfq* status on *rpoS-lac* expression can be seen on MacConkey lactose plates: an *hfq* mutant carrying such a fusion forms Lac^- colonies, while a wild-type strain is Lac^+. Here, this screen was used with localized mutagenesis to isolate mutations that increase expression of an *rpoS-lac* protein fusion in an *S. typhimurium hfq* mutant host. A number of mutations linked to the fusion were found; the increase in *rpoS-lac* expression seen in these mutants ranged from 2- to 11-fold.

DNA sequence analysis and reconstruction experiments showed that all the mutations map to a 380-bp *Nco*I-*Eag*I DNA fragment containing the *rpoS* RBS region and ATG codon. Eight of the 11 mutations and all those most independent of *hfq* lie in two clusters: one in the RBS region of *rpoS* and another within the last 13 codons of the upstream *nlpD* gene. All are G → A or C → U transitions (in RNA), as predicted for mutations induced with hydroxylamine. None of the mutations affects expression of an *rpoS-lac* operon fusion; therefore, all are presumed to affect translation of *rpoS*.

Each of the mutants also shows a decreased dependence on *hfq* function for *rpoS-lac* expression, although the strength of this effect varies. The mutations that give higher expression in the absence of HF-I tend to respond less to the HF-I that is present in the wild type. One exceptional mutant (with the G118A mutation) is essentially independent of HF-I yet retains relatively low *rpoS-lac* expression, with only a fourfold increase over the basal level.

One model for the *rpoS* mRNA structure in the region near the RBS is shown in Fig. 3. In this model all of the mutations that lie in these two clusters change positions that are base paired. Three adjacent stems, each with at least three GC base pairs, are linked by a few unpaired bases and separated by a long intervening loop of 63 nucleotides (which could also form a tight secondary structure). The *rpoS* Shine-Dalgarno sequence lies between stem II and stem III but is not base paired. This model was based mainly on the hypothesis that the mutations in the two clusters might alter a common base-paired structure sequestering the RBS. It also explains the similar phenotypes caused by mutations affecting bases paired in the structure: C126G and G206A, as well as G127A and C205U (Table 1).

Computer analysis reveals many possible alternative secondary structures for the *rpoS* mRNA; some of these differ in the RBS region (4). One model published previously invokes pairing with sequences downstream from the AUG codon (34). A preliminary test of the model shown in Fig. 3 was performed with compensatory site-directed mutations. The results of this test indicate that two mutations in stem II (SD2 and SD3) affect expression of *rpoS* as predicted: the combination of these two strong mutations gives mutual suppression and a completely wild-type phenotype. The simplest explanation is that a secondary structure including stem II forms and plays a role in setting the level of *rpoS* expression. We would be surprised if this result could be explained in any other way.

In contrast, the test of the predicted stem III whose results are depicted in Fig. 5 shows that an *rpoS-lac* protein fusion containing both mutations (SD1+4) has a phenotype similar to that of each single mutant. This result may mean that these positions are involved in base pairing but not with each other and that stem III is not present. Other potential structures are currently under investigation. Further tests by both genetic means and physical techniques will be required to establish the correct structure or structures.

An alternative explanation for these results is that one of the stem III mutations has an additional effect that also gives the mutant phenotype. In that case, the lack of suppression in the double mutant would be explained. For example, since the SD4 mutation (C218G) lies between the Shine-Dalgarno sequence and the AUG codon, it may increase the strength of the RBS. One might predict that if SD4 improves the strength of the RBS this would be additive with effects of opening the secondary structure, but this prediction conflicts with the observation that the phenotype of the SD1 mutant is similar to that of the SD4 mutant. However, it is not unprecedented for a mutation in the RBS region to have effects on expression only in the context of an inhibiting structure (12). For example, it may be that the *rpoS* RBS is already so strong that in the open con-

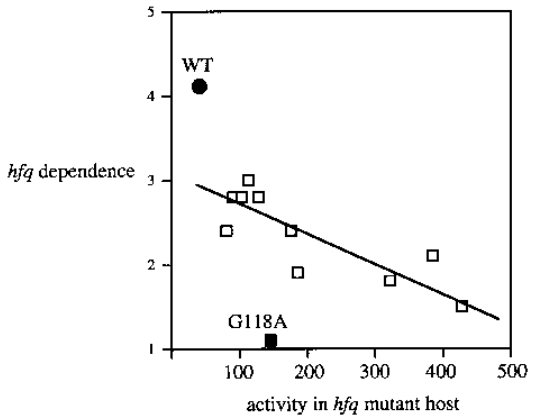

FIG. 6. Negative correlation of *hfq* dependence with level of *rpoS-lac* expression in the *hfq* mutant background. Data are replotted from Table 1. The line is a least-squares fit to the data, not including the wild-type fusion (filled circle) and G118A (filled square).

formation it is not binding limited and that in that context C218G has no effect. We note that in two other examples reported previously a G at −4 with respect to the AUG decreases translation (rather than increasing it, as seen here), but in both cases the wild-type nucleotide was A (51, 64). We have not yet tested the effect of A at this position for *rpoS*.

The mutations isolated in this study are suppressors of one aspect of the pleiotropic *hfq* mutant phenotype: they allow a high level of *rpoS* expression that is at least partially independent of *hfq* function. With the exception of the G118A and G118C (SD1) mutants, there is a correlation between the strength of expression in an *hfq* mutant background and the degree of independence of *hfq* (Fig. 6). The exceptional mutants (with mutations at G118) are essentially independent of *hfq* and yet have relatively low expression of *rpoS-lac*. This observation suggests the possibility that the noncompensatory mutant phenotype of the SD1+4 double mutant is due to a property of the SD1 mutation. For example, it could decrease an interaction with a hypothetical, negatively acting RNA-binding protein. Again, it is necessary to explain why the lack of this interaction does not have an additive effect; in this case we could imagine that the hypothetical protein binds only to a base-paired structure.

It seemed possible that the action of these suppressors, once understood, would help illuminate the mechanism of *rpoS* regulation by *hfq* (as in the Mu *com* system [63]). However, based on the mutants recovered so far, an equally valid model is that all these mutations (except possibly at G118) are simply bypass suppressors which accomplish the same end as HF-I but by a different mechanism. Many of the mutants allow levels of expression (with or without HF-I) that exceed those of the wild-type *rpoS-lac* fusion in cells containing HF-I. This can be explained by assuming that HF-I is much less than 100% effective at melting out or bypassing the effect of this structure in vivo.

There are many examples where translation is thought to be limited by a secondary structure which involves either the entire RBS or just the Shine-Dalgarno sequence or AUG codon, dating from the first work on natural mRNAs isolated from RNA phages (17, 21, 35, 36, 57). Translation-limiting secondary structures and mutations which destabilize them have also been characterized for a number of bacterial mRNAs (for examples, see references 6, 25, 47, 63, and 66; reviewed in references 10 and 24). Antisense RNAs can also work through formation of inhibitory structures (55). Conversely, at least some mRNAs may easily acquire inhibitory structures through mutation (51, 62). This is probably a consequence of the fact that the change of stability, and thus of expression, conferred by simple substitution within a secondary structure may be quite large (10, 11, 24).

We were initially surprised by the strong effects of C → U substitutions on *rpoS* expression, which we suggest are mediated by secondary structure. Unlike in DNA, GU pairs are allowed in RNA (8a). By this reasoning, the C125U, C126U, and C205U mutations would not be predicted to have a dramatic effect. However, GU is not actually equivalent to GC since the GU pair makes only two hydrogen bonds rather than three. Thus, the predicted stability of a stem with a C → U change is decreased by approximately 1 to 2 kcal/mol. The geometry of the bond is also different. Furthermore, there is a considerable amount of evidence that substitution of U for C in an RNA secondary structure can strongly affect a phenotype in vivo. In two cases involving RNase III cleavage sites (41, 49), GU pairs gave a strong mutant phenotype and so were thought to be disruptive of a structure which was stabilized by a GC pair at the same position. Since the sequence specificity of RNase III is relaxed (8, 32), it is argued that these effects are due only to weakened pairing.

There are also strong effects of a C → U substitution in structured RBS elements (11, 62). A change from C → U in the stem of a transcription terminator can negatively affect its function as well (38). However, since substitution of a CG for a GC base pair can decrease terminator efficiency in a way not predicted by simple base pairing models (7), it is difficult to be sure that there are no sequence-specific effects. In addition to protein-RNA interactions, another potential source of complications was found in a study of Tn*10* transposase translation (39), where C → U and U → C substitutions caused dramatic changes in regulation in vivo. In that study kinetic intermediates on a pathway to the final structure were identified in vitro. Either kinetic intermediates or other structures in equilibrium with a relatively unstable structure could affect activity if, for example, the alternative structure includes a stem with A that discriminates between C and U as a partner. Competing structures are yet another way to explain the results with the stem III mutants discussed above. Testing the existence of alternative structures in *rpoS* mRNA will be a challenge for future experiments.

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