Positive regulation by small RNAs and the role of Hfq

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Bacterial small noncoding RNAs carry out both positive and negative regulation of gene expression by pairing with mRNAs; in *Escherichia coli*, this regulation often requires the RNA chaperone Hfq. Three small regulatory RNAs (sRNAs), DsrA, RprA, and ArcZ, positively regulate translation of the sigma factor RpoS, each pairing with the 5' leader to open up an inhibitory hairpin. In vitro, *rpoS* interaction with sRNAs depends upon an (AAN)₄ Hfq-binding site upstream of the pairing region. Here we show that both Hfq and this Hfq binding site are required for RprA or ArcZ to act in vivo and to form a stable complex with *rpoS* mRNA in vitro; both were partially dispensable for DsrA at 37 °C. ArcZ sRNA is processed from 121 nt to a stable 56 nt species that contains the pairing region; only the 56 nt ArcZ makes a strong Hfq-dependent complex with *rpoS*. For each of these sRNAs, the stability of the sRNA • mRNA complexes, rather than their rate of formation, best predicted in vivo activity. These studies demonstrate that binding of Hfq to the *rpoS* mRNA is critical for sRNA regulation under normal conditions, but if the stability of the sRNA • mRNA complex is sufficiently high, the requirement for Hfq can be bypassed.

Sigma 38 | translational control | Sm-like protein | RNA–protein interactions

Small regulatory RNAs (sRNAs) are an important part of bacterial environmental response pathways (1–4). sRNAs are *trans*-acting posttranscriptional regulators that most often regulate gene translation by base-pairing to target mRNAs (3–5), in concert with the RNA chaperone Hfq (6, 7). Hfq, a hexameric ring protein with structural and sequence homology to Sm proteins (8), is known to stabilize sRNAs in vivo and facilitate sRNA pairing to targets in vitro (7). Hfq binds preferentially to single-stranded RNA, interacting with U-rich RNA on the proximal side of its central pore (9) and with A-rich RNA on its distal face (10, 11). Hfq binds both to sRNAs and to many of their target mRNAs (3, 12, 13), suggesting that Hfq binding might bring the RNAs together. However, the precise mechanism by which Hfq stimulates RNA pairing is not fully understood.

One of the most extensively studied targets of sRNA regulation is the *rpoS* mRNA leader, which encodes the σ^S subunit for RNA polymerase, an important transcription factor for stress response genes (1, 14). Hfq is necessary for expression of RpoS in vivo (15–17). Translation of the *rpoS* mRNA is self-repressed by a stem loop in its 5' leader which blocks ribosome access (18) (Fig. 1). Three different Hfq-binding sRNAs, DsrA, RprA, and ArcZ, positively regulate translation by base-pairing to the same region in the *rpoS* leader, releasing self-repression (Fig. S1) (19–21). Each of these sRNAs is expressed under a different stress condition, allowing synthesis of RpoS and therefore expression of the RpoS regulon in response to a variety of different stresses.

Previous biochemical experiments demonstrated that the ability of DsrA and RprA to anneal to *rpoS* mRNA is facilitated by Hfq (22). A long 5' leader was found to be essential for Hfq-dependent annealing of these sRNAs to *rpoS* mRNA (23, 24). Hfq binds the *rpoS* leader site specifically, increases the rate of *rpoS* mRNA • DsrA base pairing 30 to 50-fold, and stabilizes the final mRNA • sRNA complex in vitro (23). The ability of Hfq to strongly bind the *rpoS* leader and facilitate *rpoS* mRNA pairing with DsrA depended on two single-stranded A-rich elements—an

Fig. 1. sRNA activation of *rpoS* translation requires Hfq. The *rpoS* mRNA leader forms an inhibitory secondary structure that is relieved by Hfq-dependent DsrA, RprA, or ArcZ binding.

A₆ element and an (AAN)₄ repeat element—that lie upstream of the self-inhibitory stem (Fig. S2). When *rpoS* leader RNAs were truncated to less than 200 nt or when both A-rich elements were mutated, Hfq bound the *rpoS* leader nonspecifically and had a modest (twofold) effect on pairing with DsrA (23) (summarized in Fig. 24). Interestingly, although mutation of both A-rich elements was required to eliminate specific Hfq binding to the *rpoS* leader, mutating the (AAN)₄ repeat was sufficient to render the leader insensitive to Hfq stimulation of pairing with DsrA (23) (Fig. 24), implying that Hfq must be recruited to specific sequences within the *rpoS* leader for positive control of *rpoS* expression by DsrA.

We have used the *rpoS* system and the detailed knowledge of its behavior to address major questions about Hfq function and to compare, in vivo and in vitro, different regulators of the same target. We show that Hfq-binding sites on the mRNA target play a direct and critical role in sRNA-mediated activation and that Hfq acts by stabilizing sRNA complexes with the *rpoS* leader.

Results

Essential Site for Hfq in the *rpoS* 5' Leader. The in vivo roles of the extended *rpoS* leader and the A-rich elements in the stimulation of *rpoS* translation were measured using a translational fusion of the entire *rpoS* leader and the first 30 nt of the *rpoS* coding sequence to *lacZ* (Fig. 2B, green bar). This fusion is stably integrated at the chromosomal *lacZ* site; expression of the leader is under the control of the pBAD promoter (Fig. 2B).

Expression of the fusion was measured either in the presence of an empty vector (expression dependent upon chromosomally encoded sRNAs) or after overexpression of one of the three sRNAs that stimulate RpoS translation (Fig. 2C). As expected, overexpression of DsrA, RprA, or ArcZ significantly activated the expression of the full-length fusion (Fig. 2C, left-most bars). Hfq is known to be critical for *rpoS* translation (16), and consistent with this, deleting *hfq* from the wild-type strain reduced basal level *rpoS* expression significantly (Fig. 2D, left-most white

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Fig. 2. *rpoS::lacZ* fusions activated by DsrA, RprA, and ArcZ. (A) A summary of in vitro results from (23) showing the importance of *rpoS* leader length and an (AAN)₄ element (red box) for the action of Hfq. The numbers indicate the nucleotide at the 5' end of the *rpoS* leader RNA, relative to the natural start; the in vitro RNAs used both previously and in this work extended 12 nt into the ORF. The "double-mutant" construct had the properties of the (AAN)₄ mutant. Structure of the 5' leader and sequence of the mutations in the A-rich elements are shown in Fig. S2. (B) The *rpoS* leader constructs carrying the truncations and mutations described in Fig. 2A were fused to *lacZ* to create translational fusions under the control of the arabinose-inducible P_{BAD} promoter; the (AAN)₄ and A₆ point mutations were introduced into the full-length fusion rather than the long fusion shown in Fig. 2A. The specific strains are described in Table S1. (C) sRNA activation of *rpoS* leader fusions. Strains containing the vector pBRplac (black bars) or plasmids overexpressing DsrA, RprA, or ArcZ, were grown in LB containing arabinose and IPTG at 37 °C to stationary phase before β-galactosidase activity was measured. (D) sRNA activation of *rpoS* leader fusions in an *hfq*⁻ background. Same as in C, with *hfq::cat* derivatives; white bars contain vector plasmid. Note that y axis values are significantly less in D than in C.

bar; note different scale for Fig. 2 C and D graphs). However, multicopy DsrA, but not RprA or ArcZ, was still capable of stimulating translation (Fig. 2D, blue bar), albeit the final expression level was significantly lower than in an *hfq*⁺ host (compare to Fig. 2C). The ability of DsrA to act to stimulate *rpoS* translation in the absence of Hfq has previously been shown (16) and is investigated further below.

Derivatives of the fusion were constructed containing the truncations of the *rpoS* leader shown in Fig. 2A. We focus first on *rpoS* leader derivatives that had a large effect on Hfq regulation in vitro. In vitro, the (AAN)₄ sequence was required for Hfq-dependent annealing of DsrA (23). Consistent with in vitro observations, mutating the (AAN)₄ sequence reduced basal expression of the fusion significantly (Fig. 2C, black bars), close to that seen in an *hfq* mutant (Fig. 2D). Therefore, in vivo, as in in vitro, this sequence is necessary for expression of *rpoS* by the sRNAs expressed from the chromosome.

Activation of the (AAN)₄ mutant fusion was also tested in experiments in which DsrA, RprA, or ArcZ was overproduced (Fig. 2C). Again, consistent with in vitro experiments using DsrA (23), mutating the (AAN)₄ site reduced activation by overproduced RprA and ArcZ to around 25% of that seen for the wild-type fusion (Fig. 2C). Strikingly, however, DsrA was still able to stimulate translation, to 70% or more of that seen with the wild-type fusion.

Deletions of the leader ("short" and "minimal") that remove the A-rich sequences [both A₆ and (AAN)₄; Fig. 2A, red bars] behave similarly to the (AAN)₄ mutant; they are not stimulated by RprA or ArcZ overexpression, but they are stimulated by DsrA (Fig. 2C). By contrast, a longer fusion containing both A-rich sites was regulated similarly to the full-length fusion (Fig. 2C, "long fusion").

These data strongly support an essential role for the (AAN)₄ site to allow Hfq-dependent *rpoS* translation. For RprA and ArcZ, *rpoS* translation is fully Hfq dependent even when these sRNAs are overproduced, and this site is essential. DsrA, when

overproduced, can partially bypass Hfq (Fig. 2D); it can also bypass the need for the (AAN)₄ site.

Structure of the *rpoS* Leader. We next consider changes to the *rpoS* leader that did not affect sRNA binding in vitro, but nonetheless modulated *rpoS* translation in vivo. As noted above, mutations in the A₆ motif behaved similarly to the wild-type leader in vitro (23) (Fig. 2A). In vivo, mutation of the A₆ motif reduced both the basal and sRNA-activated expression compared to wild type, indicating that the A₆ mutation is not completely benign (Fig. 2C). Interestingly, the basal expression in cells carrying the A₆ mutant was the same in *dsrA*⁺ and *dsrA*⁻ cells (Fig. S3), indicating that perhaps the basal level of DsrA is not sufficient for effective regulation when this site is deleted or mutated. An A₆/(AAN)₄ double mutant fusion responded to the sRNAs in the same way as the (AAN)₄ mutant fusion (Fig. 2C and D).

As summarized in Fig. 2A, the "long" *rpoS* leader (missing the first 253 nt but retaining both A-rich sequence elements) also behaved like the full-length fusion in vitro. This fusion is fully stimulated by all three sRNAs (Fig. 2C), but, in contrast to the other *rpoS* fusions, is not activated by overproduced DsrA in an *hfq* mutant (Fig. 2D), and has reduced basal expression compared to the full-length fusion (Fig. 2B and Fig. S3). Among all the leader truncations, the fusion containing the full-length 576 nt *rpoS* leader had the highest level of basal expression and was the most strongly stimulated by sRNA overexpression (Fig. 2C).

These data suggest that although the long (323 nt) leader mimics the 576-nt full-length leader RNA in vitro (23) (Fig. 2A), missing upstream sequences reduce its ability to be activated by the basal level of DsrA present at 37 °C in vivo, or by the level of overexpressed DsrA found in an *hfq* mutant (Fig. 2D and Fig. S3, long). This lower basal activity of the long leader could be due to a loss of additional 5' regulatory elements or a more repressive RNA structure.

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Fig. 4. sRNAs binding the *rpoS* leader at 37 °C. (A) sRNA titrations of the long *rpoS* leader in the absence of Hfq. DsrA (blue circles), RprA (orange squares), ArcZ56 (brown diamonds), and ArcZ121 (red triangles) were mixed with the long *rpoS* leader RNA and subjected to native gel electrophoresis and the formation of a complex calculated as described in *Materials and Methods*. (B) As for A, with the addition of Hfq.

bind the *rpoS* leader tightly, consistent with all three sRNAs activating *rpoS* expression in *hfq*⁺ cells (Fig. 2C). In the absence of Hfq, the affinity of RprA and ArcZ56 for *rpoS* is greatly reduced, whereas the affinity of DsrA for *rpoS* is nearly unchanged, consistent with the in vivo result that DsrA, but not RprA or ArcZ, activates *rpoS* expression in an *hfq*[−] background (Fig. 2D).

In contrast to the equilibrium binding results, our kinetic experiments revealed no significant differences in the rates by which DsrA, RprA, or ArcZ56 bind the long *rpoS* leader without Hfq present. The addition of Hfq conferred modest increases to the rate by which DsrA and RprA bound *rpoS* (3.5-fold and 2.5-fold, respectively), and a much larger 35-fold increase to the rate of ArcZ56 binding (Table 1 and Fig. S7). Substituting the (AAN)₄ mutant version of the long *rpoS* leader in our kinetic experiments eliminated the effect of Hfq on the RprA binding rate, and reduced the effect of Hfq on the DsrA binding rate to 2.5-fold, as expected (23). The (AAN)₄ mutation also substantially reduced the effect of Hfq on the binding rate of ArcZ56, although the rate remained significantly larger than for RprA or DsrA (Table 1 and Fig. S7).

Regulation by ArcZ sRNA. Whereas Hfq made stable complexes with the full-length but not the processed form of ArcZ, the *rpoS* leader bound the processed but not the full-length form of ArcZ, suggesting different roles for Hfq in the processing and *rpoS* binding of this sRNA. In vivo, the processed form of ArcZ is totally lost in an *hfq* mutant (Fig. S5); it was also totally absent when a plasmid directly expressing the short (processed form) was induced in an *hfq* mutant. This instability in an *hfq* mutant strongly suggests an interaction with Hfq, even though the complexes are heterogeneous in vitro. The short form of ArcZ was as active as the full-length ArcZ for regulation of *rpoS*, in the presence of Hfq, and, as for the full-length ArcZ, the short form was totally inactive in the absence of Hfq (Fig. S8). As noted below, the short form of ArcZ was unable to act on the (AAN)₄ mutant (Fig. S8), so that stabilization of the short sRNA is not the only role for Hfq for the action of ArcZ.

Discussion

A-rich Element is Necessary for Hfq Regulation of sRNA Activation.

RpoS translation is a useful and well-studied model system for studying the mechanisms of sRNA regulation and the role of the RNA chaperone protein Hfq. In *Escherichia coli*, the *rpoS* mRNA is the target of sRNAs that can either repress or activate its translation in response to different stimuli (20, 29, 30). Three activating sRNAs, DsrA, RprA, and ArcZ, are induced by different environmental cues and have different structures but activate translation of *rpoS* in the same manner: by disrupting a self-inhibitory stem in the 5' leader (Fig. S1).

Here we combine genetic and biophysical approaches to show that, in the case of DsrA, RprA, and ArcZ, Hfq regulates sRNA activity by modulating the strength of the sRNA • mRNA complex. A single-stranded (AAN)₄ repeat element, identified previously as being required for Hfq enhancement of DsrA binding *rpoS* mRNA in vitro, is also required for RprA and ArcZ activation of *rpoS* translation. This implies that Hfq must interact directly with the *rpoS* mRNA, as well as with the sRNAs. In addition, we present a biophysical explanation for why DsrA, but not RprA or ArcZ, acts independently of Hfq when overexpressed.

The good agreement between our in vitro and in vivo results is gratifying, because the in vitro binding studies used 323-nt fragments of the *rpoS* leader and an indirect assay, RNA • RNA binding, to test the ability of DsrA to activate *rpoS* in the presence of Hfq. Sun and Wartell found that RprA binding to *rpoS* mRNA also depends on an extended 5' leader in vitro (24). Our in vivo results now demonstrate that the same (AAN)₄ site within the extended 5' leader is critical for activation of *rpoS* translation by both RprA and ArcZ.

Hfq was recently shown to strongly bind short RNAs with an AAN triplet repeat sequence (11) through a binding site that is physically distinct from the binding site for U-rich sequences (9, 10). An interesting possibility is that the A-rich sequences in the *rpoS* leader bind Hfq in a manner that prepares it to recruit sRNAs containing U-rich Hfq-binding sites. This could occur either through some type of bridging complex, or via the exchange of RNA ligands between Hfq hexamers; however, the precise mechanism by which Hfq brings these RNAs together is unknown.

Thermodynamic Threshold for *rpoS* Translation. We found that, in the absence of Hfq, DsrA binds the long *rpoS* leader ~19-fold more tightly than RprA and ~45-fold more tightly than ArcZ56, whereas ArcZ121 binding to *rpoS* was barely detectable (Fig. 4 and Table 1). This order of binding preference agrees with the free energies of sRNA-*rpoS* base pairs predicted by MFOLD (31). Hfq strongly stabilized the RprA • *rpoS* and ArcZ56 • *rpoS* complexes, by more than 50-fold, and by more than 1,000-fold, respectively.

The results nicely explain the difference between the *rpoS* fusion activation patterns of DsrA, RprA, and ArcZ, and suggest that there is a threshold of sRNA • *rpoS* complex stability required for *rpoS* translation to proceed (Fig. 5). In this model, one role of Hfq is to bring hybrid stability above this translation threshold (Fig. 5). Because the (AAN)₄ mutant *rpoS* leader is insensitive to Hfq, the complexes it makes with the sRNAs are not sufficiently stable, except in the case of DsrA, which binds the *rpoS* leader strongly enough at 37 °C that Hfq is not required (Fig. 5). One prediction from this conclusion is that strengthening the interaction between RprA or ArcZ and *rpoS*, for instance by replacing A–U base-pairs with G–C pairs, should render those sRNAs Hfq independent in vivo.

Although Hfq had only a modest effect on the stability of the DsrA • *rpoS* complex at 37 °C, previous results showed that Hfq stabilized this complex more than 20-fold at 25 °C (23). Therefore, it was initially surprising that DsrA overproduction was also

Fig. 5. Summary model. The population of open (activated) *rpoS* leader in the presence of sRNAs was simulated from the in vitro binding data [Table 1, (23)]: DsrA at 37 °C (dark blue), DsrA at 25 °C (light blue), and ArcZ (brown). Solid curves, no Hfq; dashed curves, with Hfq. Vertical dashed lines show the expected fraction of translatable *rpoS* leader when sRNAs are present at either endogenous or overexpressed levels. (Top) sRNA activation of the WT *rpoS* leader; (Bottom) sRNA activation of the (AAN)₄ mutant *rpoS* leader. It was assumed that the mutation does not affect sRNA binding, and that the presence of Hfq improves sRNA binding to the mutant by ~1.5-fold, similar to the kinetic behavior reported in ref. 23.

able to stimulate *rpoS* translation in an *hfq* mutant or in the (AAN)₄ mutant at 25 °C (Fig. S8). However, even in the absence of Hfq at 25 °C, DsrA binds the *rpoS* leader significantly more tightly than does either RprA or ArcZ. We therefore predict that at 25 °C, overproduced DsrA should still be able to bind the *rpoS* leader in the absence of Hfq. By contrast, endogenous levels of DsrA are expected to be insufficient for full *rpoS* activation in the absence of Hfq, in agreement with in vivo data (16) (Fig. 5). As at 37 °C, RprA, ArcZ, and the truncated version of ArcZ were not able to act at 25 °C without Hfq (Fig. S8).

That Hfq is still required for regulation of *rpoS* by endogenous DsrA suggests that the amount of sRNA in the cell is also important. High levels of sRNA, such as obtained by overexpression, make the system less sensitive to Hfq binding to the *rpoS* mRNA and to the stability of the mRNA • sRNA complex (compare vertical dashed lines, Fig. 5).

Kinetics of Interaction is Less Important Than Stability. Whereas the in vitro equilibrium binding results closely correlate with the in vivo fusion expression data, the kinetic data do not follow this pattern. DsrA, RprA, and ArcZ bind the long *rpoS* leader with very similar rates in the absence of Hfq, and only ArcZ's binding to *rpoS* is accelerated more than 3.5-fold in the presence of Hfq. These results show that, at least in the conditions used in this study, the strength of the final sRNA • *rpoS* complex is a better predictor of whether translation will occur than the rate of complex formation. Many studies have examined the role of Hfq in facilitating RNA • RNA binding (32), and Hfq is clearly capable of increasing the rate of duplex formation. However, the results presented above suggest that Hfq's effect on the rate of RNA • RNA binding does not drive its in vivo effects on sRNA activity.

Three Different Patterns for *rpoS* Regulation. Three different sRNAs, with different structures, different upstream regulators, and different negative targets all target *rpoS* by binding in the same region (Fig. S1). Yet these three sRNAs have very divergent *rpoS* binding behavior, highlighting the variations on how sRNAs act and how Hfq helps them to regulate their targets.

DsrA binds Hfq tightly, pairs well with *rpoS* with or without Hfq, and that ability to pair without Hfq also bypasses the need for the (AAN)₄ motif when DsrA is overexpressed. RprA binds Hfq tightly as well, but is dependent upon both Hfq and the (AAN)₄ motif for pairing and regulation. Thus, the role of Hfq binding to the (AAN)₄ motif is an intrinsic part of regulation, and possibly is the major role for Hfq in this system.

ArcZ is unique in that, although it is totally dependent upon Hfq and the (AAN)₄ repeat in vivo, it is cleaved to an abundant shorter RNA form (26, 27). Expressing just the short form is sufficient to activate *rpoS* translation (Fig. S8). Remarkably, the full-length (121 nt) and processed (56 nt) versions of ArcZ have completely different binding activities. Although ArcZ121 binds Hfq well, it interacted very poorly with *rpoS*, with or without Hfq. In contrast, ArcZ56 was deficient in Hfq binding, forming mostly heterogeneous complexes (Fig. 3), but bound *rpoS* detectably in the absence of Hfq and very strongly in the presence of Hfq (Fig. 4). We do not yet know what about the full-length sRNA interferes with binding to *rpoS*, but apparently this sRNA has evolved to be active only after processing.

General Implications. These results outline the complexity of Hfq-dependent regulation, and the critical role that Hfq-binding sites on the mRNA play in this process. sRNAs with different architectures and different in vitro binding activities all manage to regulate the same target effectively. Tight Hfq binding to a target mRNA is clearly not sufficient, given the lack of proper regulation of fusions in which the (AAN)₄ motif is mutated, but the A₆ motif, sufficient for specific Hfq binding, is present (Fig. 2). The example of DsrA and its independence from Hfq and (AAN)₄ confirms results from other studies (33) that Hfq aids the interaction of two RNAs, but is not essential under conditions where the sRNA and mRNA can form a stable complex on their own. Finally, the results demonstrate that the stability of regulatory RNA complexes, rather than the kinetics of their formation, correlates best with in vivo activity. It will be of interest to see if these findings can be extrapolated to negatively regulated targets as well.

Materials and Methods

Bacterial Strain Construction and Handling. All *E. coli* strains used in this study are derivatives of the wild-type MG1655 and are listed in Table S1. Mutations in *dsrA* and *hfq* were introduced by P1 phage transduction, as described previously (34). Truncations to the *rpoS-lacZ* fusion were obtained by PCR amplifying the sequence contained in strain PM1409 with the appropriate oligonucleotides (see Table S1) and recombining the obtained PCR products in strain PM1205, as described previously (35). Mutations in the *rpoS-lacZ* fusion were obtained in a similar manner, except plasmids containing the desired mutant *rpoS* sequence were the PCR templates (see Tables S1 and S2). The pArcZ-56 plasmid was constructed by PCR amplifying the *arcZ* gene from strain MG1655 with oligonucleotides ArcZ-56-for and ArcZ-rev. The PCR product was then digested using the EcoRI and AatII endonucleases and introduced by ligation into the pBR-plac plasmid digested with the same enzymes (36). Transformation to introduce plasmids was as described in ref. 37.

β-Galactosidase Assays. Plasmid-containing bacteria were grown in microtiter plates containing LB with arabinose (0.2%), ampicillin (100 μg/mL), and IPTG (100 μM), for 6–7 h with agitation. OD₆₀₀ was determined, cells were lysed, and β-galactosidase activity was measured as described previously (20). The specific activities correspond to kinetic measurements of the V_{max}/OD600 and are calculated from averages of three or more independent experiments done in duplicate.

Transcription Template Construction. The RprA transcription template plasmid pUCT7RprA was constructed by amplifying by PCR the RprA sequences from a *plac-RprA* expression plasmid with primers containing a T7 promoter and Dral linearization site, and cloning into pUC18. Plasmids for transcribing ArcZ121 and ArcZ56 (pUCT7ArcZ121 and pUTt7ArcZ56) were cloned using the same approach. DsrA was transcribed from pUCT7DsrA (22). All sRNA transcripts begin with two or three nonnative G nucleotides added to facilitate transcription from a T7 promoter. Cloning primers are listed in Table S2.

Protein Purification. Hfq was overexpressed in *E. coli* as previously described (12) and the cells lysed by an Emulsiflex in lysis buffer (50 mM Hepes pH 7.5, 1 M NaCl, 1 M urea, 25 mM imidazole, 5% glycerol). The lysate was treated with DNase I and RNase A, incubated on ice for 1 h, and then cleared by centrifugation.

Consistent with previous observations (38), wild-type (untagged) Hfq was adsorbed onto a HiTrap Co²⁺ column. The column was washed first with lysis buffer and then extensively with wash buffer (50 mM Hepes pH 7.5, 1 M NaCl, 2 M urea, 25 mM imidazole, 5% glycerol), followed by elution with elution buffer (50 mM Hepes pH 7.5, 1 M NaCl, 250 mM imidazole, 5% glycerol). Desired fractions were pooled and dialyzed into Hfq storage buffer (50 mM Tris · HCl pH 7.5, 1 mM EDTA, 250 mM NH₄Cl, 10% glycerol by volume), and concentrated by ultracentrifugation before storage at –80 °C.

RNA Preparation. All four sRNAs were transcribed from the plasmids listed above after linearization with Dral. The rpoS323 RNA fragment was transcribed from a previously published PCR DNA template (23). All RNAs were transcribed with T7 RNA polymerase and purified by denaturing PAGE as previously described (39). RNA was ³²P labeled on the 5' end with γ -³²P-ATP or uniformly labeled by transcription in the presence of α -³²P-ATP as previously described (22, 23).

Native PAGE Assays. As previously described (22, 23), all RNA binding reactions were performed in annealing buffer (50 mM Tris · HCl pH 7.5, 250 mM NaCl, 250 mM KCl, used as 5X). For reactions without Hfq, its storage buffer was added to reactions instead. RNA was diluted in 10 mM Tris · HCl, 1 mM EDTA, pH 7.5, and renatured prior to use by heating at 75–80 °C for 1 min followed by 5 min at room temperature. All reactions were resolved on chilled non-denaturing 6% acrylamide gels in Tris-borate-EDTA, dried, and analyzed on a PhosphorImager.

Hfq titrations of the sRNAs, as well as kinetic and equilibrium sRNA • rpoS binding experiments, were carried out as previously described (22, 23), except the reaction temperature was 37 °C, cold sRNA concentration in kinetic experiments was 0.6 μ M, Hfq₆ when added was at 0.13 μ M, and carrier tRNA was omitted from the reactions.

Calculation of Binding Constants. Binding data were analyzed as previously described (22, 23), except that, for the equilibrium sRNA titrations of rpoS RNA, the fraction bound versus sRNA concentration was fit with the quadratic form of the single-site binding isotherm. For kinetic experiments, the fraction bound over time was fit to a single exponential rate equation. Equilibrium Hfq-binding experiments resolved at least two ribonucleoprotein complexes (S • H1 and S • H2) for each sRNA that were fit to a partition function for Hfq binding as previously described (22) and shown in Fig. S6.

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