

# Translational activation of *rpoS* mRNA by the non-coding RNA DsrA and Hfq does not require ribosome binding

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## ABSTRACT

At low temperature, translational activation of *rpoS* mRNA, encoding the stationary phase sigma-factor,  $\sigma^S$ , involves the small regulatory RNA (sRNA) DsrA and the RNA chaperone Hfq. The Hfq-mediated DsrA-*rpoS* interaction relieves an intramolecular secondary structure that impedes ribosome access to the *rpoS* ribosome binding site. In addition, DsrA/*rpoS* duplex formation creates an RNase III cleavage site within the duplex. Previous biochemical studies suggested that DsrA and Hfq associate with the 30S ribosomal subunit protein S1, which implied a role for the ribosome in sRNA-mediated post-transcriptional regulation. Here, we show by ribosome profiling that Hfq partitions with the cytoplasmic fraction rather than with 30S subunits. Besides, by employing immunological techniques, no evidence for a physical interaction between Hfq and S1 was obtained. Similarly, *in vitro* studies did not reveal a direct interaction between DsrA and S1. By employing a ribosome binding deficient *rpoS* mRNA, and by using the RNase III cleavage in the DsrA/*rpoS* duplex as a diagnostic marker, we provide *in vivo* evidence that the Hfq-mediated DsrA/*rpoS* interaction, and consequently the structural changes in *rpoS* mRNA precede ribosome binding. These data suggest a simple mechanistic model in which translational activation by DsrA provides a translationally competent *rpoS* mRNA to which 30S subunits can readily bind.

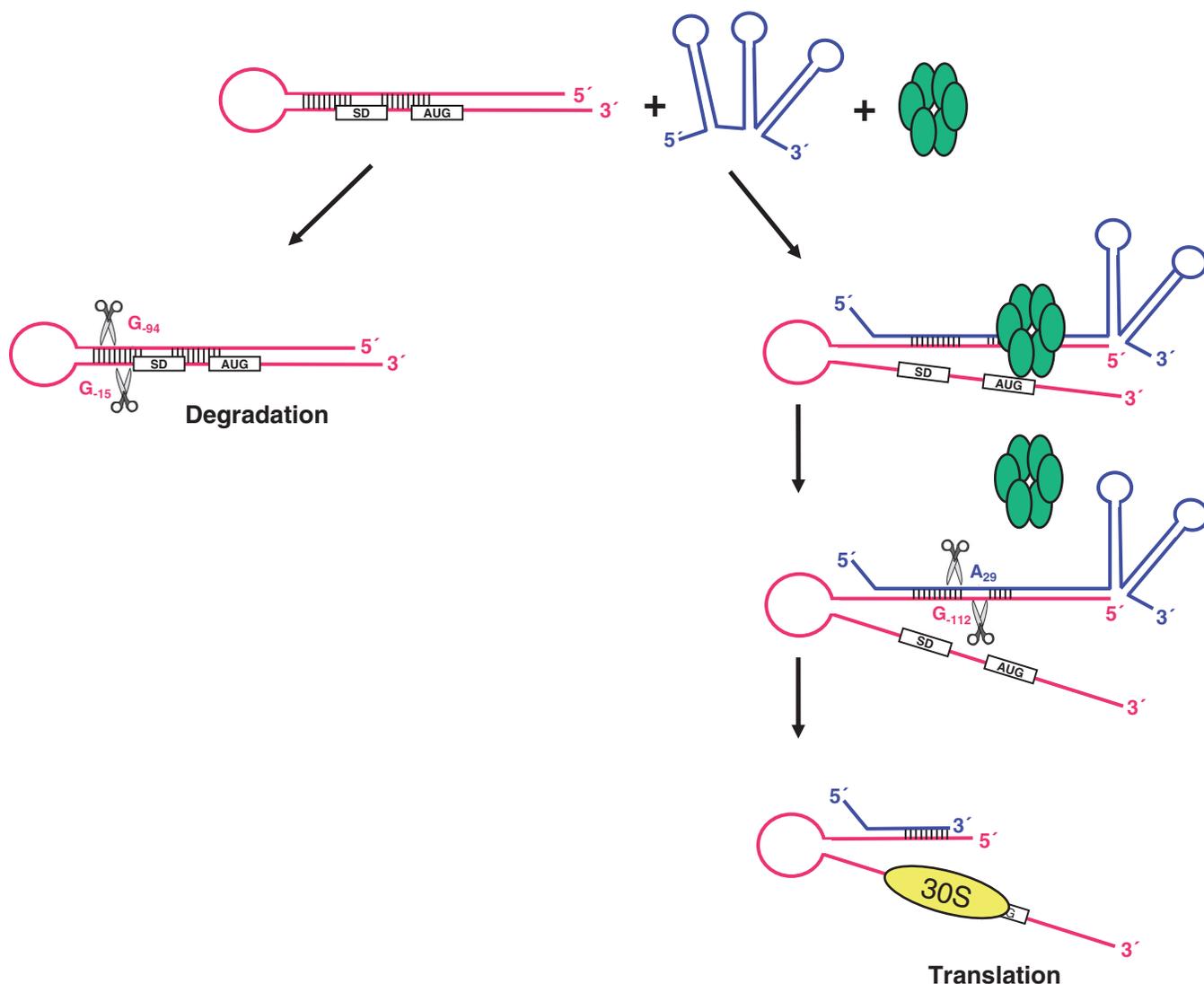
## INTRODUCTION

Bacterial *trans*-encoded small regulatory RNAs (sRNAs) are transcribed in response to various stresses. These

sRNAs, which are mainly encoded in intergenic regions, regulate gene expression primarily at the post-transcriptional level through base-pairing with the target mRNA. This can result in either translational activation or repression of the mRNA, whereby the latter mechanism of regulation appears to be predominant in bacteria (1). The majority of the sRNAs from enteric bacteria require the hexameric RNA chaperone protein Hfq for function. Hfq has been shown to protect sRNAs from degradation (2), and to facilitate annealing of sRNAs with the target mRNA (3–5), which may entail unfolding of both, the sRNA and the mRNA (6–10). Several lines of evidence suggest that the *Escherichia coli* Hfq hexamer (Hfq<sub>6</sub>) has distinct binding surfaces for sRNAs and mRNA, and that both ligands can bind simultaneously to Hfq<sub>6</sub>. While sRNAs appear to require the proximal site, and in particular the inner core of Hfq<sub>6</sub> for binding (11), the C-terminal extension of *E. coli* Hfq appears to be crucial for mRNA binding (10).

Translational activation of *E. coli rpoS* mRNA, which encodes the stress sigma factor,  $\sigma^S$ , by the sRNA DsrA at low temperature (12) has served as a paradigm for studying the molecular mechanism(s) underlying this intricate regulation. Several studies (13–15) showed that DsrA activates *rpoS* translation by base-pairing with the 5' *rpoS* leader, which relieves an intra-molecular stem-loop structure (Figure 1) that sequesters the *rpoS* ribosome binding site (rbs). The RNA chaperone Hfq is necessary for DsrA-mediated regulation of *rpoS* mRNA (16). Recent studies (10,17) have dissected at least two functions of Hfq in this process. Hfq was shown (i) to bind upstream of the DsrA/*rpoS* annealing site, which in turn accelerated the rate of DsrA annealing to *rpoS* (17) and (ii) to induce conformational changes in DsrA (10), which could facilitate base-pairing between DsrA and *rpoS*. In addition to its function in *rpoS* translational activation, DsrA base-pairing with the *rpoS* leader stabilizes the *rpoS* transcript by re-directing RNase III cleavage in its 5' untranslated region (UTR). During logarithmic growth

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**Figure 1.** Model for translational activation of *rpoS* mRNA by DsrA and Hfq. Left, in the absence of DsrA and Hfq, the rbs of *rpoS* is sequestered by intra-molecular base-pairing. As a consequence of RNase III cleavage, the *rpoS* mRNA eventually becomes prone to RNase E dependent decay (18). Right, DsrA/*rpoS* duplex formation is facilitated by Hfq. Upon annealing of DsrA/*rpoS*, Hfq is released (6,15) and RNase III cleavage may occur at A<sub>29</sub>/G<sub>-112</sub> in the DsrA/*rpoS* duplex (18). The 30S subunit can then readily bind to the rbs of the 5' truncated *rpoS* mRNA. DsrA and *rpoS* RNAs are shown in blue and red, respectively. Hexameric Hfq is in green and RNase III cleavage sites are indicated by scissors.

and in the absence of DsrA the double-stranded portion of *rpoS* mRNA is cleaved at positions -15/-94 by RNase III (Figure 1), which is accompanied by rapid decay of the mRNA coding sequence (18). However, after DsrA/*rpoS* annealing, RNase III cleavage occurs within the DsrA/*rpoS* duplex (Figure 1), and as a result of translation, the mRNA seems to become stabilized (18).

The sRNA DsrA has been reported to interact with the small ribosomal subunit (19), and more recently with ribosomal protein S1 (20). Protein S1 has been shown to bind to poly-U rich stretches located upstream of the rbs of *E. coli* phage mRNAs, which suggested that S1 can serve as a general translational enhancer by increasing the local concentration of the translation initiation determinants on the 30S subunit (21). Besides, S1 is required for translation initiation of structured mRNAs (22,23), which may be attributed to its helix-destabilizing activity

(24). Based on co-sedimentation experiments (25) and immuno-diffusion studies (26), Hfq was reported to associate with 30S subunits, and an 'interactome' study revealed that several ribosomal proteins, including protein S1, co-purified with tagged Hfq protein (27). In addition, a co-sedimentation analysis suggested that RNA polymerase bound protein S1 interacts directly with Hfq (28). Based on their finding that DsrA binds to 30S subunits, Worhunsky *et al.* (19) suggested a model, wherein 30S-bound DsrA would serve to increase the local concentration of DsrA with ribosome associated Hfq and/or *rpoS* mRNA, and by inference that ribo-regulation of *rpoS* by DsrA could be a ribosome based mechanism.

In this report, we present several experiments that collectively argue against a model, wherein translation activation of *rpoS* mRNA by Hfq and DsrA occurs at the

ribosome. We show that (i) Hfq fractionates with the cytoplasmic ribosome-free fraction. By employing Far-western blotting we obtained (ii) no evidence for a physical interaction between Hfq and S1. Filter binding experiments with S1 protein purified to homogeneity and with S1-proficient 30S subunits did not (iii) corroborate a direct interaction between S1 and DsrA. Moreover, (iv) RNase III cleavage was shown to occur in the DsrA/*rpoS* duplex in the absence of ribosome binding signals on *rpoS* mRNA, suggesting that Hfq-DsrA mediated translational activation of *rpoS* precedes ribosome binding.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

The *E. coli* strain BW25113 (*lacI<sup>f</sup> rrnB<sub>T14</sub> ΔlacZ<sub>WJ16</sub> hsdR514 ΔaraBAD<sub>AH33</sub> ΔrhaBAD<sub>LD78</sub>* (29), and the isogenic *hfq* and *rpoS* deletion strains JW4130 and JW5437, respectively, were obtained from the Keio gene knockout collection (30). The strains were grown in Luria-Bertani (LB) medium, and ampicillin (100 μg/ml), kanamycin (25 μg/ml), tetracycline (30 μg/ml) or chloramphenicol (15 μg/ml) were added to the medium where appropriate.

### Construction of plasmids

Plasmid pARpoSwt, carrying the *rpoS* gene including its own promoter sequence was constructed as follows: the fragment containing the *rpoS* gene was obtained by means of PCR using the *rpoS* forward primer 5'-AAGA ATTCAAGCCTGCACAAAATTCACCGTTGCTGT TGC-3', and the reverse primer (5'-AATCTAGATGGG CATCGGACCTTTTATTGTGCACAGA-3'). The PCR product was inserted into the ClaI and NruI sites of pACYC184, yielding plasmid pARpoSwt. Plasmid pARpoSmut2, bearing *rpoS* with both *rpoS* SD sequences mutated was prepared by means of site-directed mutagenesis using a two-step PCR procedure. Both the *rpoS* SD sequence AGGAG (nt from -12 to -8, with regard to the A (+1) of the *rpoS* start codon) and the internal SD sequence AGGGAG (nt from +143 to +148) were changed to TAAA and AATATT, respectively, by means of PCR and by using the mutagenic primers 5'-CAGCGTATTCTGACTCATAAGGTGGTT TAAACCCGTGATCCC-TTGAC-3' and 5'-CCAACAC ACGCTGTGTGGAATATTGCGATAACAGTTCCCTC TTCGGCCAAA-3', respectively (altered nucleotides are underlined). The resulting PCR product was inserted into the ClaI and NruI sites of pACYC184, yielding plasmid pARpoSmut2. The mutations at the internal SD sequence did not change the reading frame of *rpoS* mRNA (Figure 5A). All modifications were confirmed by sequencing.

Plasmid pS1 used for the purification of S1 protein was constructed as follows. The *rpsA* gene encoding protein S1 was amplified from genomic *E. coli* DNA by PCR using the forward primer (5'-AAAAGGCGCCATGACTGAAT CTTTGCTCAACTC-3') containing an EhoI (bold) restriction site and the reverse primer 5'-GGGAGCTCG

AGAATTACTCGCCTTTAGCTGC-3') containing an XhoI (bold) restriction site. The PCR product was cleaved with EhoI and XhoI, and inserted into the corresponding sites of the pProEX-HTb expression vector (Invitrogen). The resulting plasmid encodes an N-terminal His<sub>6</sub>-tagged S1 protein of which the tag can be removed by TEV protease cleavage.

### RNA preparation for *in vitro* studies

DsrA RNA was synthesized from a DNA template generated by PCR as described previously (18). The 5' segment of *rpoS* mRNA (corresponding to nt from -564 to +188), were amplified from pARpoSwt and pARpoSmut2 plasmids, respectively, using the forward primer 5'-GCTCTAGATAATACGACTCACTATAGG GTGAACAGAGT-GCTAACAAAATGTTGCCGAAC A-3' that contained a T7 promoter sequence (underlined), and the reverse primer 5'-CCAAGGTAAAGCTGAGTC GC-3'. The corresponding *rpoS* transcripts were synthesized with T7-RNA polymerase (18). The transcripts were dephosphorylated with calf intestine phosphatase (MBI Fermentas), 5'-end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP (Hanke Lab. Products, Vienna) and gel-purified on 6% polyacrylamide-8M urea gels following standard procedures. The RNA concentration was determined by measuring the A<sub>260</sub>.

### Primer extension analysis

*E. coli* strains JW5437 and JW4130 carrying plasmids pARpoSwt and pARpoSmut2, respectively, were grown at 25°C to ensure DsrA synthesis (12). At an OD<sub>600</sub> of 0.6, 20-ml aliquots were withdrawn for total RNA purification. In parallel, 2-ml samples were withdrawn to determine the RpoS levels by quantitative immunoblotting (see below). Total RNA was purified from culture aliquots using the hot phenol method (31). Primer extension analysis was performed using AMV reverse transcriptase (Promega) together with 15 μg of purified total RNA primed with the *rpoS*-specific [<sup>32</sup>P] 5'-end-labeled oligonucleotide (5'-TCCGTTCTCATCAAATTCGCA TC-3'). The extension products along with a sequencing ladder, which was prepared using the 5' segment (nt from -564 to +188) of *rpoS* mRNA as a template, were resolved on a 10% sequencing gel. As a loading control, the 5S rRNA levels were determined in the corresponding total RNA preparations by primer extension as described (18). The resulting signals were visualized by a PhosphoImager (Molecular Dynamics). The RNase III specific cleavage signals at G<sub>-112</sub> in *rpoS* mRNA and the 5S RNA signals were quantified by ImageQuant software. The experiment was carried out in triplicate. For determination of the relative RNase III cleavage efficiency at G<sub>-112</sub> the respective *rpoS*-specific cleavage signals were normalized to that of the 5S RNA signals (Figure 6B).

### Northern blot analysis

The steady-state levels of DsrA in strains JW5437 and JW4130 carrying plasmids pARpoSwt and pARpoSmut2, respectively, were determined by northern-blot analysis using 10 μg RNA of the same

RNA preparations used for the primer extension analyses (see above). The RNA samples were denatured for 5 min at 65°C in loading buffer containing 50% formamide, separated on 8% polyacrylamide/8 M urea gels, and then transferred to nylon membranes by electroblotting. The RNA was crosslinked to the membrane by exposure to UV light. The membrane was hybridized with DsrA-specific [<sup>32</sup>P] 5'-end-labeled oligonucleotide (5'-TC GTTACACCAGGAAATCTGATGT-3') and the hybridization signals were visualized using a PhosphorImager (Molecular Dynamics).

#### Western blot analysis

The RpoS and L9 (loading control) protein levels were determined in strains JW5437 and JW4130 harboring plasmids pACYC184 (control), pARpoSwt and pARpoSmut2, respectively. The strains were grown at 25°C in LB medium until they reached an OD<sub>600</sub> of 0.6, at which time 2-ml aliquots were withdrawn, pelleted and boiled in protein sample buffer. Equal amounts of total protein were separated on 12% SDS-polyacrylamide gels and blotted to a nitrocellulose membrane. The blots were blocked with 5% dry milk in TBS buffer, and then probed with anti-RpoS (NeoClone, Madison) and anti-L9 (provided by Dr I. Moll) antibodies to detect the RpoS and L9 proteins, respectively. The antibody-antigen complexes were visualized with alkaline-phosphatase conjugated secondary antibodies and BCIP/NBT solutions following standard procedures. The Hfq and S1 protein levels in purified 30S ribosomes and Hfq, S3 and L14 protein levels in ribosomal fractions were determined analogously using specific antibodies against Hfq (Pineda, Berlin) and both ribosomal proteins (provided by Dr I. Moll).

#### Far-western blotting

Far-western blotting was performed according to the protocol of Wu *et al.* (32) with some modifications. Briefly, Hfq and S1 (25 pmol each) were electrophoresed on a 12% SDS-polyacrylamide gel and then electroblotted onto a nitrocellulose membrane at 15 V for 30 min. After transfer, the proteins were denatured by washing the membranes with 6 M guanidine-HCl in AC buffer (10% glycerol, 100 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 0.1% Tween-20, 1 mM DTT) containing 2% milk powder for 30 min at room temperature. In order to re-nature the proteins, the membranes were treated with three successive washes in AC buffer (including 2% milk powder) containing 3, 1 and 0.1 M guanidine-HCl, respectively, for 30 min at room temperature (first wash) or at 4°C (last two washes). Finally, the membranes were incubated in AC buffer (including 2% milk powder) overnight at 4°C. Then, the membranes were washed twice with AC buffer and incubated with Hfq (150 pmol as a hexamer) or S2 (25 pmol) in 10 ml AC buffer for 3 h at room temperature. The membranes were washed four-times for 10 min with AC buffer. Then the membranes were incubated according to a standard western-blot protocol with the first antibodies (anti-Hfq

or anti-S2 antibodies) and afterwards with the corresponding second antibodies conjugated to alkaline phosphatase. The antibody-antigen complexes were visualized as described above.

#### Filter binding assay

Binding of DsrA to 30S ribosomes purified from strain JW4130 as well as to S1 and Hfq proteins was assayed as follows. [<sup>32</sup>P] 5'-end-labeled DsrA RNA (10 nM) was mixed with different amounts (0–320 nM) of ribosomes or (0–640 nM) proteins in VD buffer [10 mM Tris (pH 7.4), 60 mM ammonium chloride, 6 mM 2-mercaptoethanol, 6 mM magnesium acetate]. The ribosomes were incubated in VD buffer for 15 min at 37°C before addition of DsrA. The complexes were incubated for 20 min at 25°C and then transferred to a Minifold II slot-blot filtration apparatus (Schleicher and Schuell) equipped with a 0.45-µm nitrocellulose membrane (Schleicher and Schuell). The membrane was washed twice with VD buffer, dried and exposed to a Molecular Dynamics PhosphorImager for 2 h for signal visualization.

Binding of the 5' segment of [<sup>32</sup>P] 5'-end-labeled *rpoS* wt and *rpoS*mut2 RNAs to 30S ribosomes purified from BW25113 strain was assayed in a similar way, with the exception that the mRNAs were pre-incubated with non-labeled DsrA in order to remove the secondary structure in *rpoS* leader that prevents ribosome binding. Labeled *rpoS* mRNA (10 nM) was mixed with equimolar amounts of DsrA, heated for 3 min at 85°C in VD buffer lacking magnesium and then slowly cooled to 25°C. Magnesium acetate was then added to a final concentration of 6 mM. Different concentrations (0–160 nM) of 30S ribosomal subunits purified from strain BW25113 were pre-activated at 37°C for 15 min in VD buffer, mixed with the DsrA/*rpoS* complexes and incubated for 20 min at 25°C. The filter binding assay and autoradiography were performed as described above. After visualization of the signals with a PhosphorImager, the individual slots were sliced and the radioactivity retained was measured by a scintillation counter. The obtained cpm values were expressed as percentage of the total input radioactivity (cpm value obtained with corresponding labeled 10 nM *rpoS* mRNA before the transfer onto the membrane) as shown in Figure 5C. The filter-binding experiments were carried out in duplicate and the results were averaged.

#### Preparation of ribosomal protein S1, Hfq, 30S ribosomes and depletion of S1 protein from purified 30S subunits

Protein S1 was purified from strain BL21(DE3)pLysS carrying the expression plasmid pS1 which encodes a N-terminally His-tagged S1 protein. Following growth to an OD<sub>600</sub> of 0.6, induction with 1 mM IPTG for 4 h at 28°C, and lysis of the cells in lysis buffer (500 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8, 200 µg/ml PMSF, 5 µg/ml lysozyme, 20 µg/ml DNaseI, 20 mM imidazole), the His-tagged S1 protein was purified by affinity chromatography on Ni-NTA agarose (QIAGEN) according to the manufacturer's instructions. Subsequently, the column

was washed with two volumes of washing buffer (500 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8) containing 20 mM imidazole. Finally, (His)<sub>6</sub>-S1 protein was eluted with a 60–500 mM gradient of imidazole in washing buffer and the His<sub>6</sub>-tag was removed from the purified protein by digestion with TEV protease (Invitrogen). Typically, 1 mg protein was incubated with 20 U TEV-protease for 3 h at room temperature. Then, the protein sample was incubated with 500 µl Ni-NTA agarose over-night at 4°C to bind the His<sub>6</sub>-tag and impurities. The mixture was then loaded on a Spin-X column (Costar) and the purified His<sub>6</sub>-tag free S1 protein was eluted by centrifugation. Pure S1 protein was dialysed against and stored in VD buffer.

30S ribosomal subunits devoid of initiation factors were prepared from *E. coli* strains BW25113 and the isogenic *hfq* deletion strain JW4130 as described (33). 30S ribosomes were depleted for S1 by affinity chromatography of 30 A<sub>260</sub> units of sucrose gradient purified ribosomes using poly(U)-Sepharose 4B (GE Healthcare) as described (23). The depletion of ribosomes for S1 was verified by western-blotting (Figure 4B).

The details of the procedure for Hfq purification on Ni-NTA agarose will be described elsewhere (our unpublished data). Both, Hfq and S1 (before TEV cleavage) were treated with micrococcal nuclease (Fermentas) in VD buffer containing 10 mM CaCl<sub>2</sub> for 60 min at 37°C. After digestion the proteins were re-purified on a Ni-NTA agarose column.

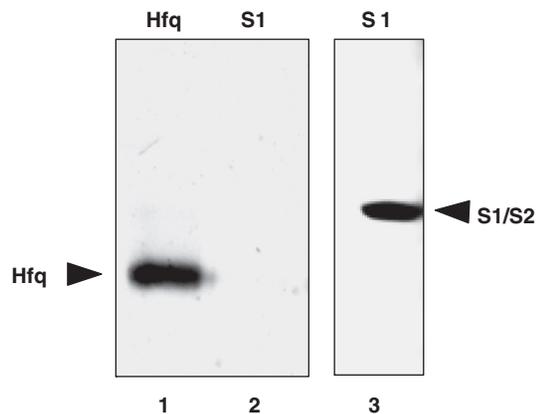
### Ribosome profiling and immuno-localization of Hfq

After growth in LB medium to an OD<sub>600</sub> of 0.8, the cells were collected by centrifugation at 5000g for 5 min. The pellet was resuspended in 250 µl of VD buffer, and lysozyme and DNase I were added to a final concentration of 100 µg/ml and 20 U/ml, respectively. Following incubation on ice for 10 min, the cells were centrifuged for 10 min at 14000g to remove cell debris. The lysate was layered onto a 10–30% (Figure 3A) or 5–20% (Figure 3B) sucrose gradient prepared in VD buffer. The gradients were centrifuged for 16 h at 120 000g in a Beckman SW 40 rotor, 0.7 ml fractions were collected and analyzed by measuring the A<sub>260</sub> using an ISCO UA-6 spectrophotometer. The proteins from 0.7-ml fractions were precipitated with 10% TCA and centrifuged for 10 min at 29 000g. The pellets were washed with acetone, boiled in protein sample buffer, and the proteins were separated on a 12% SDS-polyacrylamide gel. The fractions were probed for Hfq and ribosomal proteins S3 and L14 by quantitative immunoblotting, using the corresponding antibodies as described above.

## RESULTS

### Far western-blotting did not confirm a physical interaction between S1 protein and Hfq

Two lines of evidence suggested that Hfq might interact with ribosomal protein S1 of the 30S subunit (27,28). To test this putative interaction we employed Far-western-blotting, and used S1 and Hfq proteins purified to

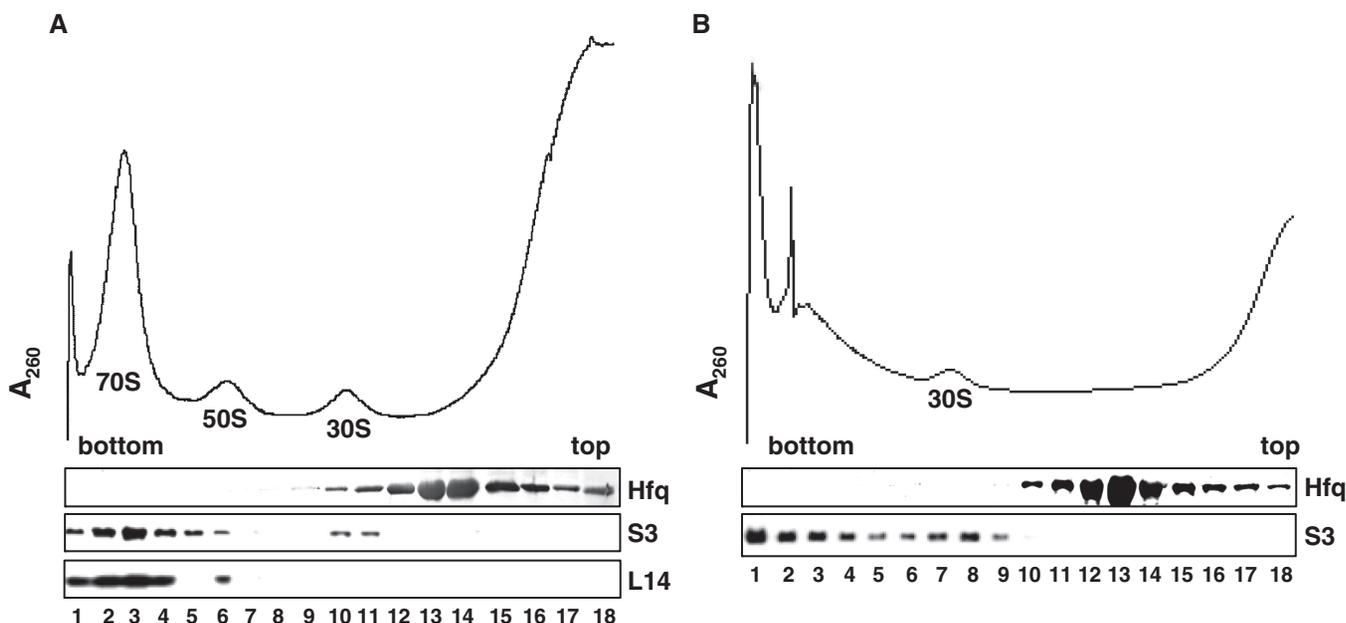


**Figure 2.** Far western-blotting does not reveal an interaction between S1 and Hfq. Hfq (25 pmol) (lane 1, control) and 25 pmol of S1 (lanes 2 and 3) were electro-blotted onto nitrocellulose membranes. After re-naturation of the proteins the membranes were incubated with 150 pmol Hfq hexamer (lane 2) and with 25 pmol S2 protein (lane 3). As described in ‘Materials and Methods’ section, the S1–S2 complex was visualized by immuno-detection using anti-S2 antibodies (lane 3), whereas no S1–Hfq complex could be detected with anti-Hfq antibodies (lane 2). Lane 1, Hfq protein was detected by anti-Hfq antibodies (control).

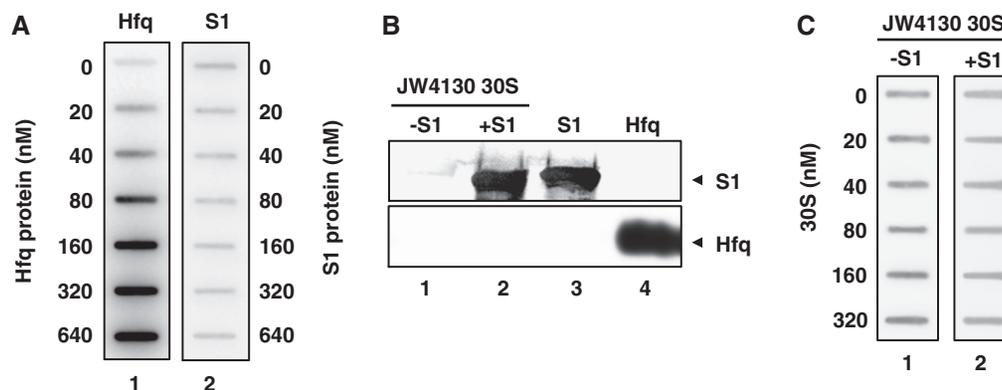
homogeneity. For Far-western-blotting, S1 protein was first subjected to SDS-polyacrylamide electrophoresis and then blotted onto nitrocellulose. The protein was then re-natured and the blot was incubated with Hfq protein (Figure 2, lane 2) or purified ribosomal protein S2 (Figure 2, lane 3), and binding was assessed by immuno-detection with anti-Hfq or anti-S2 antibodies. As anticipated from previous studies (34), S2 protein (positive control) interacted with protein S1 (Figure 2, lane 3), whereas Hfq did not (Figure 2, lane 2). This could not be attributed to a lack of Hfq-antibody binding, as they recognized Hfq protein that was co-electrophoresed next to S1 (Figure 2, lane 1). Moreover, *in vitro* co-immunoprecipitation studies with Hfq-antibodies performed with homogeneous Hfq and S1 preparations did likewise not reveal a physical interaction between both proteins (Supplementary Figure S1).

### Hfq localizes predominantly to the cytoplasm

Immunodiffusion and co-sedimentation experiments (25,26) suggested that Hfq associates with ribosomes. To assess the cellular distribution of Hfq, we performed ribosome profiling experiments in conjunction with quantitative immunoblotting. An extract of *E. coli* BW25113 grown to an OD<sub>600</sub> of 0.8 was separated by 10–30% sucrose gradient centrifugation, and the individual fractions were probed for Hfq with anti-Hfq antibodies as well as with antibodies against ribosomal proteins L14 and S3, which served as markers for 50S and 30S subunits, respectively. As shown in Figure 3A, the majority of Hfq (>95%) partitioned with the ribosome free fraction, whereas only <5% (fractions 10–11) of the total of Hfq fractionated with 30S subunits, and no Hfq was found in the fraction containing 50S subunits or 70S monosomes. As Hfq is a rather abundant protein (35), we next asked whether the



**Figure 3.** Cellular partitioning of Hfq. (A) Ten to thirty percent sucrose gradient. Top: Sedimentation profile showing the free 30S, 50S subunits and 70S monosomes derived from extracts of *E. coli* wt BW25113. Bottom: Equal volumes of each protein fraction were subjected to western-blotting using anti-Hfq, anti-S3 or anti-L14 antibodies. (B) Five to twenty percent sucrose gradient. Top: Sedimentation profile showing free 30S subunits derived from extracts of *E. coli* wt BW25113. Note that 50S and 70S particles migrated close to the bottom of the gradient. Bottom: Equal volumes of each protein fraction were subjected to western-blotting using anti-Hfq and anti-S3 antibodies.

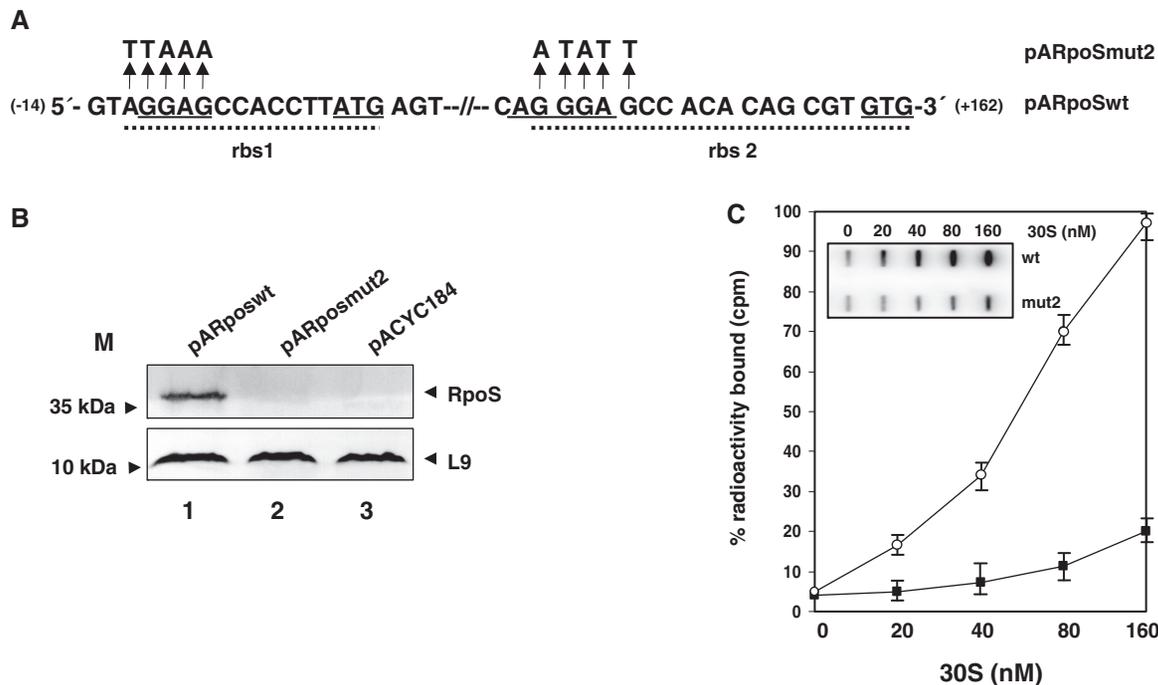


**Figure 4.** DsrA interactions with S1, Hfq and 30S ribosomes. (A) DsrA binding to S1 and Hfq proteins. [<sup>32</sup>P] 5'-end-labeled DsrA (10 nM) was incubated in the absence and in the presence of 20, 40, 80, 160, 320 and 640 nM of purified Hfq (panel 1; Hfq- concentrations are given for the monomer) and S1 (panel 2) proteins. The binding reactions were filtered through a nitrocellulose membrane and the RNA retained on the membrane was visualized by autoradiography. The K<sub>d</sub> for Hfq-hexamers binding to DsrA was calculated with ~20 nM, which is in agreement with a previous study (11). (B) Analysis of the S1/Hfq content of ribosomes purified from the *hfq* deletion strain JW4130 and of Hfq and S1 preparations by western-blot analysis using anti-S1 (upper panel) and anti-Hfq (lower panel) antibodies. Lane 1 contained 60 pmol of 30S subunits purified from JW4130 cells that were depleted for S1 protein. Lane 2 contained 60 pmol 30S ribosomes purified from JW4130 cells. Lanes 3 and 4 contained 60 pmol of S1 and Hfq protein, respectively, that were purified to homogeneity. Only the relevant sections of the immunoblots showing the S1- and Hfq-specific signals are shown. (C) DsrA binding to S1-proficient and S1-deficient 30S ribosomes. [<sup>32</sup>P] 5'-end-labeled DsrA (10 nM) was incubated in the absence and in the presence of 20, 40, 80, 160 and 320 nM of purified 30S from JW4230 cells that were depleted for S1 (lane 1) and in the absence or presence of the same amounts of S1-proficient ribosomes purified from JW4130 cells (lane 2). The binding reactions were filtered through a nitrocellulose membrane and the RNA retained on the membrane was visualized by autoradiography.

observed association of minute amounts of Hfq with 30S could result from an insufficient separation of the ribosomal particles from cytoplasmic contents. After separation of the same extracts by a 5–20% sucrose gradient followed by immuno-detection of Hfq and S3 protein, a co-sedimentation of Hfq with the 30S fraction was no longer observed (Figure 3B).

#### DsrA does neither bind to purified protein S1 nor to S1-proficient 30S subunits

Next, we re-examined whether the sRNA DsrA binds to purified S1 protein or to S1 proficient ribosomes. First, filter binding experiments were performed with gel-purified [<sup>32</sup>P] 5'-end-labeled DsrA and increasing concentrations of purified S1 and Hfq proteins. Hfq was used



**Figure 5.** The *rpoSmut2* mRNA is not translated and shows a strongly diminished affinity for 30S ribosomes. (A) Alterations of the Shine Dalgarno (SD) sequences in the *rpoSmut2* RNA variant. Both ribosome binding sites (rbs) of the *rpoS* gene are indicated. The SD sequences and initiation codons are underlined. The nucleotide exchanges, which did not affect the *rpoS* reading frame, are marked by arrows. (B) RpoS protein synthesis in the *rpoS*- strain JW5437 carrying plasmids pARpoSwt (lane 1), pARpoSmut2 (lane 2) and pACYC184 (control, lane 3), respectively. The western-blot analysis was carried out with anti-RpoS and anti-L9 antibodies (loading control) using equal amounts of total cellular protein as described in 'Materials and Methods' section. Only the relevant sections of the western-blot showing the RpoS- and L9-specific signals are shown. (C) Affinity of the 5' segments (nt from -564 to +188) of *rpoS* wt and *rpoSmut2* mRNAs for 30S ribosomes. [<sup>32</sup>P] 5'-end-labeled *rpoS* wt (white circles) and *rpoSmut2* (black squares) RNA fragments (10 nM) were incubated in the absence and in the presence of 20, 40, 80 and 160 nM 30S subunits purified from *E. coli* strain BW25113. The binding reactions were filtered through a nitrocellulose membrane and mRNA retained on the membrane was determined by scintillation counting. The mRNA bound to ribosomes is expressed as percentage of the input radioactivity (~20 000 ± 500 c.p.m.), which was set to 100%. Inset: an autoradiograph of a representative slot blot showing binding of *rpoS* wt (wt) and *rpoSmut2* (mut2) RNA fragments to ribosomes. The graphical representation shown is an average of two independent experiments. The error bars represent standard deviations.

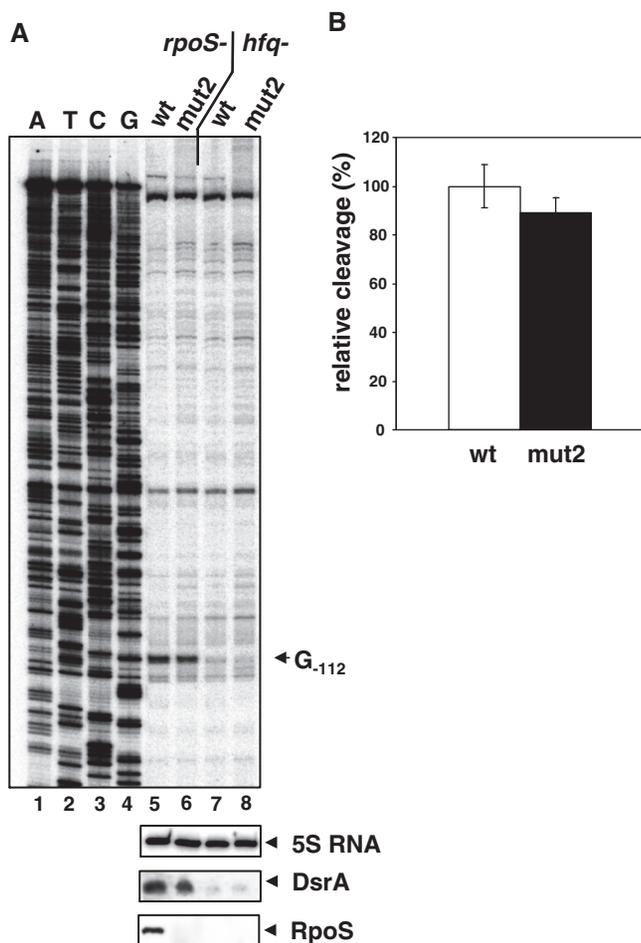
as a control as it binds DsrA with a high affinity (15). As shown in Figure 4A, the addition of increasing amounts of S1 protein did not result in an increased retention of DsrA on the filter, i.e. purified S1 protein (lane 2) did not show a significant affinity for DsrA, which was at the anticipated variance with Hfq protein (lane 1).

In addition, we tested whether DsrA binds to 30S ribosomes devoid of Hfq. S1-proficient and S1-depleted 30S particles were purified from the *hfq* deletion strain JW4130 as described in Materials and Methods section. Strain JW4130 was used to avoid impurities of Hfq in isolated 30S particles (Figure 3A), which could have obscured the results. The presence or absence of S1 in the corresponding ribosome preparations was verified by western-blotting. As shown in Figure 4B, lane 1, S1 was removed from the S1-containing 30S ribosomes purified from JW4130 (lane 2) after passage over a poly-U column. As anticipated both 30S preparations (± S1) were devoid of Hfq (Figure 4B, lanes 1 and 2). The binding of DsrA to both 30S preparations was assessed using filter binding experiments. As shown in Figure 4C, DsrA did not bind to 30S particles regardless of whether they contained S1 (lane 2) or whether S1 was absent (lane 1).

### Translational activation of *rpoS* mRNA by DsrA and Hfq is not a ribosome based mechanism

Although the *in vitro* experiments presented above did not provide evidence for an interaction of 30S ribosomes (or S1 protein) with DsrA or with Hfq protein, we sought for another means to scrutinize whether translational activation of *rpoS* mRNA by DsrA and Hfq occurs in the absence of ribosomes. To diminish ribosome binding of *rpoS* mRNA, both Shine and Dalgarno sequences present in *rpoS* mRNA (36) were modified (Figure 5A). The modified *rpoS* gene and the wild-type *rpoS* gene were then cloned into plasmid pACYC184, giving rise to plasmids pARpoSwt and pARpoSmut2, respectively.

As shown in Figure 5B, lane 2, the RpoS protein was not synthesized in the *E. coli rpoS*- strain JW5437 harboring plasmid pARpoSmut2. For verification, a filter binding experiment was performed to assess binding of the 5' portion of *rpoS* wt and *rpoSmut2* RNA to 30S ribosomes (Figure 5C). When compared to *rpoS* wt RNA binding of *rpoSmut2* was strongly diminished, which was in agreement with the apparent lack of translation of the latter mRNA (Figure 5B, lane 2).



**Figure 6.** DsrA/*rpoS* duplex formation does not require ribosome binding. (A) Primer extension analysis of total RNA isolated from the *E. coli rpoS* deletion strain JW5437 (lanes 5 and 6) and the *hfq* deletion strain JW4130 (lanes 7 and 8) harboring plasmids pARpoSwt and pARpoSmut2, respectively. The experiment performed with the *hfq*-strain JW4130 served as a control, as Hfq is pivotal for DsrA/*rpoS* annealing, and thus indirectly for cleavage by RNase III at nt G<sub>-112</sub> (18) within the *rpoS* leader (arrowhead). Lanes 1–4, *rpoS* RNA sequencing ladder. The 5S RNA levels (loading control) and the DsrA RNA levels were determined by primer extension- and northern-blot analysis, respectively, of the total RNA isolated from corresponding strain. Note that the stability of DsrA was reported to be greatly reduced in the absence of Hfq (2,16). The RpoS protein levels were determined by western-blotting from aliquots of the different strains concomitantly with total RNA preparation. The experiment was performed in triplicate. One representative autoradiograph is shown. (B) RNase III cleavage efficiency in *rpoS* wt and *rpoSmut2* RNA in strain JW5437. The RNase III-specific cleavage signals obtained after cleavage at G<sub>-112</sub> within *rpoS* wt (white bar) and *rpoSmut2* (black bar) were quantified and normalized to the corresponding 5S RNA levels. The relative cleavage efficiency obtained in RNA derived from strain JW5437(pARpoSwt) was set to 100%. The graphical representation shown is an average of three independent experiments. The error bars represent standard deviations.

We next asked whether DsrA/*rpoSmut2* and the DsrA/*rpoS* wt duplex formation occur with comparable efficiency despite the diminished binding of *rpoSmut2* to 30S particles (Figure 5C). To address this question, we utilized the RNase III cleavage upon DsrA/*rpoS* annealing as a diagnostic marker. Both plasmid borne copies of

the *rpoS* wt and *rpoSmut2* were expressed in the *rpoS*-strain JW5437. JW5437(pARpoSwt) and JW5437(pARpoSmut2) cells were cultivated at 25°C to an OD<sub>600</sub> of 0.6 when samples for RNA isolation and for western-blot analysis were withdrawn. Total RNA from either strain was purified and RNase III-dependent cleavage of *rpoS* wt and *rpoSmut2* mRNA was assessed by primer extension using a [<sup>32</sup>P] 5' end-labeled *rpoS*-specific primer. Both *rpoS* mRNAs were cleaved by RNase III at position G<sub>-112</sub> (18) with approximately the same efficiency (Figure 6A and B). The RNase III cleavage of *rpoSmut2* RNA did not result in synthesis of RpoS protein (Figure 5B) and the RNase III cleavage of *rpoSmut2* mRNA was clearly much greater than anticipated from its affinity for ribosomes (Figure 5C and 6B). Therefore, these experiments indicated that DsrA/*rpoS* duplex formation, and thus RNase III cleavage precedes ribosome binding.

## DISCUSSION

The aim of this study was to test the possibility whether Hfq-mediated translational activation of *rpoS* mRNA by DsrA is a ribosome-based mechanism. Far-western-blotting (Figure 2) and co-immunoprecipitation studies (Supplementary Figure S1) did not provide evidence for a physical interaction between Hfq and ribosomal protein S1, both of which were purified to homogeneity and then treated with micrococcal nuclease. As Far-western blotting verified the previously observed interaction between ribosomal proteins S1 and S2 (34), it seems unlikely that the observed lack of interaction between Hfq and S1 can be attributed to a lack of proper re-naturation of S1 protein on the filter. It seems rather possible that the reported co-purification of stoichiometric amounts of Hfq and S1 during RNA polymerase purification (28) resulted from a lack of proper RNase treatment. Thus, it is conceivable that the observed association between Hfq and S1 occurred through RNA as recently reported by Worrall *et al.* (37) for the previously implied interaction between RNase E and Hfq (38). In addition, using ribosomal profiling combined with quantitative immunoblotting, we have re-examined whether Hfq associates with 30S particles. When the sucrose gradients were optimized towards a better separation of 30S particles from cytoplasmic contents the Hfq protein clearly partitioned with the ribosome-free fraction. Therefore, the observed reactivity of 30S ribosomal fractions with Hfq antibodies in immuno-diffusion experiments (26) can probably be attributed to the cellular abundance of Hfq (35) and the resulting Hfq impurities present in the used 30S fractions. We would also like to note that treatment of the cell lysates with micrococcal nuclease before centrifugation resulted in a shift of the immunostained Hfq-specific band towards the top of the gradient (B. Večerek, unpublished results). This indicated that Hfq is in RNA complexes, and can explain why Hfq migrates close to 30S subunits in the gradient (Figure 3B).

In our hands neither purified protein S1 nor S1-proficient 30S subunits devoid of Hfq showed a

significant affinity for DsrA. This finding is at variance with the work of Koleva *et al.* (20), who found that S1 protein interacts with DsrA. One reason for their finding seems to be inherent to the S1 purification procedure. Recently, we have noticed that Hfq co-purifies with His-tagged proteins during affinity chromatography on Ni-NTA agarose. Thus, Hfq potentially co-purifies with any His-tagged protein. In fact, Hfq was also present in our (His)<sub>6</sub>-S1 preparation (Supplementary Figure S2A, lane 1). However, for this study, Hfq was removed by detaching the His-tag from S1 protein with TEV protease, followed by re-purification on Ni-NTA agarose (Supplementary Figure S2A, lane 2). As shown in Supplementary Figure S2B, the residual amounts of Hfq present in the (His)<sub>6</sub>-S1 preparation resulted in binding of DsrA, whereas no DsrA binding was observed to S1 protein purified to homogeneity (Figure 4A; Supplementary Figure S2B). In the work of Koleva *et al.* (20), the binding experiments were performed with S1-(His)<sub>6</sub> tagged protein. Therefore, it seems likely that the reported affinity of S1-(His)<sub>6</sub> for DsrA can be attributed to co-purified Hfq protein. However, Koleva *et al.* (20) have also identified an interaction between DsrA and S1 in the 30S subunit after photochemical crosslinking and subsequent mass spectrometry, which contrasts with our observation that DsrA does not bind to S1-proficient 30S subunits that were purified from the *hfq* deletion strain JW4130 (Figure 4C). In these experiments (20), the *in vitro* synthesized and body labeled DsrA RNA was not gel purified prior to photochemical crosslinking. Thus, it cannot be excluded that DsrA fragments rather than full length DsrA crosslinked to S1 protein (Schlax, personal communication).

Since our data did not reveal an interaction of Hfq with 30S subunits nor a direct one with S1 protein, and they did not support an interaction of DsrA with ribosomal protein S1, we considered it more likely that translational activation of *rpoS* mRNA by DsrA/Hfq does not require ribosome binding. To provide *in vivo* evidence for this idea the ribosome binding deficient *rpoSmut2* mRNA was constructed. In line with our hypothesis *in vivo* RNase III cleavage within the DsrA/*rpoSmut2* and the DsrA/*rpoS* wt occurred with a comparable efficiency despite the reduced affinity of the *rpoSmut2* mRNA for ribosomes. In our hands, these experiments can not be reconciled with a ribosome based translational activation of *rpoS* mRNA by DsrA and Hfq. They rather suggest that DsrA/*rpoS* duplex formation occurs in the absence of ribosomes, which could even occur during transcription, before the inhibitory secondary-structure forms. In any case, the 30S subunit would encounter a translationally competent mRNA to which it can readily bind (Figure 1).

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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