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The Sm-like Hfq Protein Increases OxyS RNA Interaction with Target mRNAs

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Summary

The *Escherichia coli* host factor I, Hfq, binds to many small regulatory RNAs and is required for OxyS RNA repression of *fhlA* and *rpoS* mRNA translation. Here we report that Hfq is a bacterial homolog of the Sm and Sm-like proteins integral to RNA processing and mRNA degradation complexes in eukaryotic cells. Hfq exhibits the hallmark features of Sm and Sm-like proteins: the Sm1 sequence motif, a multisubunit ring structure (in this case a homomeric hexamer), and preferential binding to polyU. We also show that Hfq increases the OxyS RNA interaction with its target messages and propose that the enhancement of RNA-RNA pairing may be a general function of Hfq, Sm, and Sm-like proteins.

Introduction

The OxyS RNA is a 109 nucleotide, untranslated RNA induced in response to oxidative stress in *E. coli*. This small RNA (sRNA) acts both as a global regulator that activates and represses the expression of multiple genes and as an antimutator that protects cells against DNA damage (Altuvia et al., 1997). Previous studies showed that OxyS RNA represses translation of two of its target genes: *fhlA*, encoding a transcriptional activator, and *rpoS*, encoding the alternate sigma factor σ^s (Altuvia et al., 1998; Zhang et al., 1998). OxyS RNA repression of *fhlA* is achieved through two basepairing interactions (Altuvia et al., 1998; Argaman and Altuvia, 2000). One site overlaps the ribosome binding site, and a second site resides within the coding sequence of the *fhlA* mRNA. The OxyS RNA-*fhlA* mRNA basepairing prevents ribosome binding and thus represses translation. The mechanism of OxyS RNA repression of *rpoS* translation is less clear, but has been shown to require the RNA binding protein Hfq (Zhang et al., 1998).

Hfq (HF-I) was identified as a bacterial host factor required for replication of the Qβ RNA bacteriophage (Franze de Fernandez et al., 1968; reviewed in Blumenthal and Carmichael, 1979). The 11.2 kDa *E. coli* Hfq protein is heat stable and has been reported to exist as a pentamer (Kajitani et al., 1994) or a hexamer (Franze de Fernandez et al., 1972; Kamen et al., 1972; Carmichael et al., 1975) in solution. The protein initially was calculated to be present at 2,500 molecules per cell in exponential growth (Carmichael et al., 1975), but more recent estimates are 30,000–60,000 molecules per cell (Kajitani et al., 1994; Talukder et al., 1999). The majority of Hfq is detected in the cytoplasm in association with ribosomes, while a minor fraction associates with the nucleoid (Kajitani et al., 1994; Talukder et al., 2000).

The inactivation of the *hfq* gene in *E. coli* causes a variety of phenotypes and alters expression of many proteins (Tsui et al., 1994; Muffler et al., 1997) indicating that Hfq acts as a pleiotropic regulator. Hfq destabilizes the *mutS*, *miaA*, and *hfq* mRNAs (Tsui et al., 1997) and stimulates *ompA* mRNA decay by interfering with ribosome binding, thus exposing the mRNA for endonuclease cleavage (Vytvytska et al., 1998, 2000). Hfq also activates *rpoS* mRNA translation (Muffler et al., 1996; Brown and Elliott, 1996, 1997; Cunning et al., 1998). This activation requires, at least in part, the function of two sRNAs, DsrA and RprA, which basepair with the upstream region of the *rpoS* leader (Majdalani et al., 1998, 2001). Hfq binds to the OxyS, DsrA, and RprA RNAs as well as to many other sRNAs present in *E. coli*, and we have proposed that all of these Hfq-bound sRNAs are involved in translational regulation (Wassarman et al., 2001). In addition to roles in modulating mRNA stability and translation, Hfq has been shown to stimulate elongation of poly(A) tails by poly(A) polymerase I (Hajnsdorf and Regnier, 2000).

Despite the abundance of Hfq and its apparent general role in cell regulation, the mechanism of Hfq action still is unclear. Here we report that Hfq shares homology and properties with the Sm family of proteins. We delineate the Hfq binding site on the OxyS RNA. We also show that Hfq is required for OxyS RNA repression of both *fhlA* and *rpoS* and enhances OxyS RNA binding to both of these target mRNAs.

Results

Hfq Requirement for OxyS RNA Repression of Both *fhlA* and *rpoS*

We previously observed that OxyS RNA repression of *rpoS* translation requires Hfq (Zhang et al., 1998). To determine whether the protein is necessary for other OxyS RNA functions, we examined OxyS RNA repression of *fhlA* translation in strains lacking Hfq. The effect of OxyS RNA constitutively expressed from a plasmid (poxyS) on β-galactosidase expression from *rpoS-lacZ* and *fhlA-lacZ* fusions was compared in wild-type and *hfq-1* mutant backgrounds (Tables 1 and 2). Unlike the Hfq requirement for high basal levels of *rpoS-lacZ* expression, the basal levels of *fhlA-lacZ* expression are not affected in the *hfq-1* mutant. As reported previously (Altuvia et al., 1997), both fusions were repressed in the wild-type strains carrying poxyS. However, the repres-

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Table 1. Effect of *hfq-1* Mutants on OxyS Repression of *fhlA-lacZ* and *rpoS-lacZ* Fusions

Strain	Vector[a]	poxyS[a]	Fold Repression
rpoS-lacZ	650 ± 120	120 ± 25	× 6
rpoS-lacZ hfq-1	170 ± 8.1	150 ± 17	× 1
fhlA-lacZ	41 ± 3.8	1.8 ± 0.5	× 23
fhlA-lacZ hfq-1	41 ± 5.6	36 ± 6.1	× 1

Cm^r derivatives of pKK177-3 and poxyS were used with the *rpoS-lacZ* fusion strains, and amp^r derivatives were used with the *fhlA-lacZ* fusion strains.
[a] Average (in Miller units, with standard deviation) of three independent β-galactosidase activity assays.

sion of both fusions was eliminated in the *hfq-1* mutant background. OxyS RNA constitutively expressed from the chromosome (*oxyR2* mutant strain) also did not repress the fusions if the strain carried the *hfq-1* allele (data not shown). In addition, OxyS RNA repression of the endogenous *fhlA* gene, as monitored by immunoblots, was eliminated in the *hfq-1* mutant background (data not shown). Thus, Hfq is required for OxyS RNA repression of both *fhlA* and *rpoS*.

Hfq Similarity to Sm Proteins

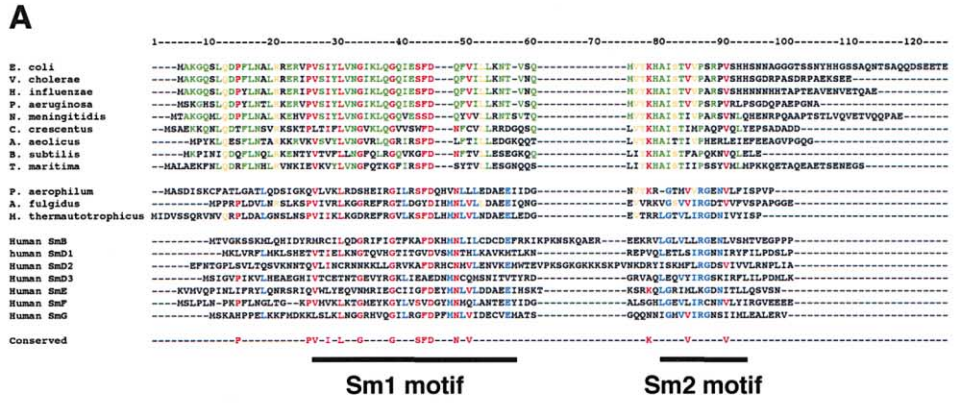
Searches for sequence homology using the BLAST algorithm showed that the Hfq protein is conserved in other bacteria (Figure 1A). These searches revealed that Hfq has significant similarity with the Sm1 sequence motif found in Sm and Sm-like (Lsm) proteins in archaea and eukaryotes. Sm and Lsm proteins are associated with spliceosomal snRNAs and are an integral part of the splicesome, and Lsm proteins also appear to have a role in mRNA degradation (reviewed in Kambach et al., 1999; He and Parker, 2000; Pannone and Wolin, 2000; Will and Lührmann, 2001). Intriguingly, the highly conserved glycine and aspartate residues near the site of RNA binding in Sm proteins (Törö et al., 2001; Urlaub et al., 2001) are absolutely conserved among the Hfq homologs. The Sm2 sequence motif defined for the Sm family members is not evident in the Hfq proteins; the highly conserved arginine and glycine residues defining this domain are not conserved. However, the Hfq homologs share a second region of particularly high conservation, which contains a number of conserved hydrophobic residues that can be aligned with hydrophobic residues found in the Sm2 sequence motif.

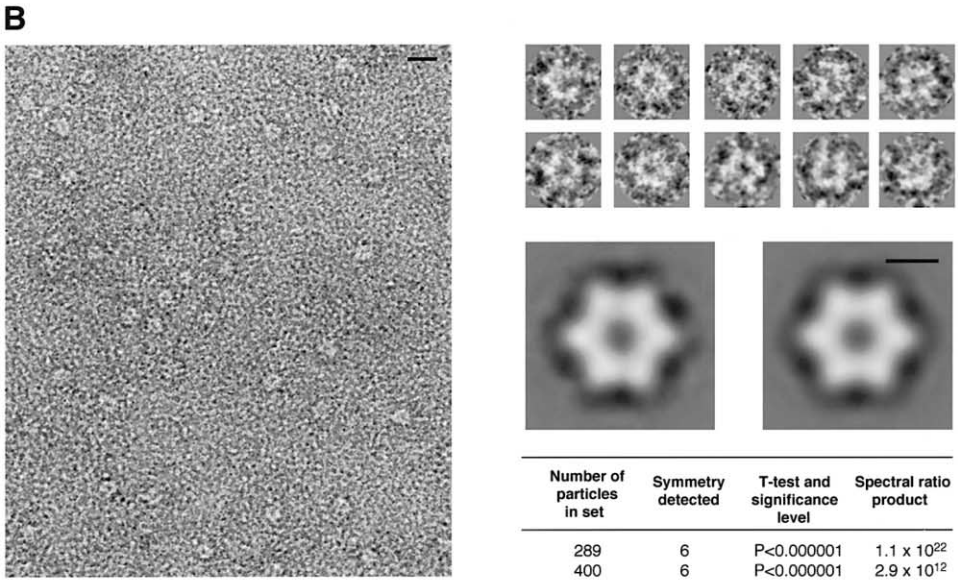
To learn more about the properties of Hfq, the native *E. coli* protein was overproduced and purified. Our purification scheme was based on previous strategies (Franze de Fernandez et al., 1972; Carmichael et al., 1975) and took advantage of both the heat stable nature of Hfq and its ability to bind poly(A). Consistent with previous measurements (Franze de Fernandez et al., 1972; Kamen et al., 1972; Carmichael et al., 1975), we found that, on gel filtration columns and in denaturing polyacrylamide gels, the purified Hfq protein migrates at a size consistent with a hexameric complex (data not shown).

We used electron microscopy to examine the shape and oligomeric state of Hfq protein. Negative staining showed ring-shaped particles ~70 Å in diameter (Figure 1B). Micrographs were digitized and analyzed using algorithms capable of detecting rotational symmetries

Table 2. Bacterial Strains and Plasmids

Strain	Relevant Genotype	Source or Reference
RO91	MC4100 [λRZ5 *rpoS742-lacZ* (hybr)] (amp^r)	Lange and Hengge-Aronis, 1994
GSO59	RO91 *hfq-1*::Ω (amp^r, kan^r)	Zhang et al., 1998
GSO38	MC4100 *φfhlA-lacZ* (kan^r)	Altuvia et al., 1997
GSO80	GSO38 *hfq-1*::Ω (kan^r, cm^r)	This study
MC4100	Δ(arg-lac)169	N. Trun
GSO81	MC4100 *hfq-1*::Ω (cm^r)	This study
GSO37	MC4100 Δ*oxyS2::cm*(cm^r)	This study
Plasmid		
pGSO4	poxyS (amp^r)	Altuvia et al., 1997
pGSO85	poxyS (cm^r)	Altuvia et al., 1997
pGSO146	pET21b-hfq (amp^r)	This study
pGSO100	pSP64-oxyS (amp^r)	Zhang et al., 1998
pGSO147	pSP64-T7/oxyS$_{G102C}$ (amp^r)	This study
pGSO136	pSP64-T7/oxyS$_{64\rightarrow109}$ (amp^r)	This study
pGSO137	pSP64-T7/spf (amp^r)	This study
pGSO138	pSP64-T7/fhlA$_{-107\rightarrow+96}$ (amp^r)	This study
pGSO148	pSP64-T7/fhlA$_{C-13G}$ (amp^r)	This study
pGSO103	pSP64-rpoS$_{-126\rightarrow+44}$ (amp^r)	Zhang et al., 1998
pGSO115	pCR2.1-T3/6S (amp^r, kan^r)	This study
pGSO113	pCR2.1-T3/5S (amp^r, kan^r)	This study
pGSO139	pCR2.1-T3/oxyS (amp^r, kan^r)	This study
pGSO140	pCR2.1-T3/oxyS$_{1\rightarrow90}$ (amp^r, kan^r)	This study
pGSO141	pCR2.1-T3/oxyS$_{1\rightarrow69}$ (amp^r, kan^r)	This study
pGSO142	pCR2.1-T3/oxyS$_{48\rightarrow109}$ (amp^r, kan^r)	This study
pGSO143	pCR2.1-T3/oxyS$_{48\rightarrow90}$ (amp^r, kan^r)	This study
pGSO144	pGEM2-T3/oxyS$_{48\rightarrow69}$ (amp^r)	This study
pGSO145	pGEM2-T3/oxyS$_{64\rightarrow90}$ (amp^r)	This study
pSA32	pRS552 fhlA32 (amp^r)	Argaman and Altuvia, 2000
pSA33	pRS552 fhlA32$_{C-13G}$ (amp^r)	Argaman and Altuvia, 2000





Number of particles in set	Symmetry detected	T-test and significance level	Spectral ratio product
289	6	P<0.000001	1.1×10^{22}
400	6	P<0.000001	2.9×10^{12}

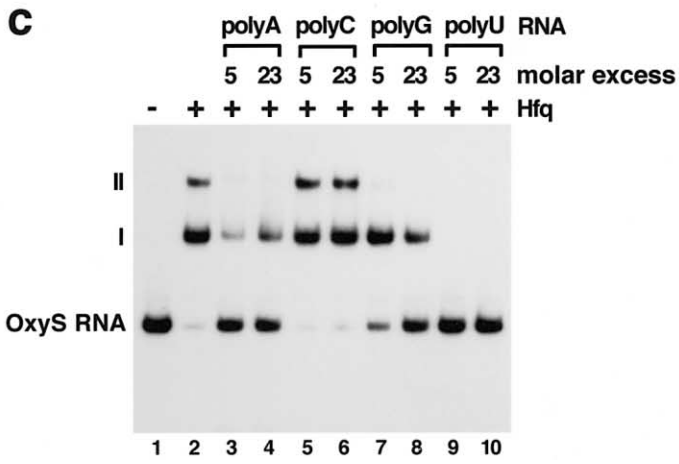


Figure 1. Hfq Similarity to Sm Proteins

(A) Alignment of a subset of Hfq homologs and archaeal and human Sm proteins created using the CLUSTAL W (1.74) program (http://molbio.info.nih.gov/molbio/gcglite/clustal17.html) with slight manual adjustment of residues in the linker region at position 60. Residues highlighted in red are conserved among the bacterial, archaeal, and human proteins; those in blue are conserved among the archaeal and human proteins; those in yellow are conserved among the bacterial and archaeal proteins; and those in green are conserved among the bacterial proteins. The Sm1 and Sm2 motifs defined by Hermann et al. (1995) are underlined.

(B) Hexameric ring structure of Hfq. (left) Electron micrograph of negatively stained Hfq protein. Bar = 150 Å. (right) Examples of individual images used to generate the averaged image (n = 689) of Hfq (left), which shows a pronounced 6-fold symmetry and differs very little from the 6-fold symmetrized version (right). Bar = 25 Å.

(C) Hfq binding to OxyS RNA competed by polyU. Labeled OxyS transcript was incubated without or with purified Hfq protein. A 5- or 23-fold molar excess (calculated for monomers of OxyS and polyN) of polyA, polyC, polyG, and polyU RNA was added as competitor.

(Kocsis et al., 1995). We detected 6-fold symmetry in two independent data sets (Figure 1B). This symmetry is most strongly expressed at a radius of 30 Å, around the outer rim of the particle. No other order of symmetry was found to be statistically significant. Image averaging depicts Hfq as a hexagram with scalloped edges measuring ~55 Å between the midpoints of two opposite sides and ~73 Å between vertices. A heavily stained area, ~18 Å across, at the center of the particle may represent an axial pore or channel.

Sm proteins bind RNA with a high preference for polyU. To investigate whether Hfq exhibits this property, we carried out gel mobility shift assays. Labeled OxyS RNA was incubated with purified Hfq and analyzed on native polyacrylamide gels. Two shifted complexes (I and II; Figure 1C, lane 2) were observed with 3 pmol Hfq. Only complex I was observed with lower concentrations of Hfq (1 pmol), and complex II predominated in the presence of higher concentrations of Hfq (10 pmol) (data not shown). We then examined the ability of an excess of unlabeled polyA, polyC, polyG, or polyU to complete for OxyS RNA binding to Hfq. As shown in Figure 1C, polyU efficiently competed for all Hfq binding, polyA and polyG led to a reduction in the formation of complex II, and polyC had no effect on Hfq binding. Thus Hfq exhibits preferential binding to polyU, but also shows some binding to polyA and polyG.

Hfq Binding to the OxyS RNA Linker

In vivo methylation experiments are consistent with the OxyS RNA secondary structure shown in Figure 2A (Altuvia et al., 1997). To determine what regions of the OxyS RNA are required for Hfq binding, we performed a minimal binding analysis. The 5′ or 3′ end-labeled OxyS RNA was subjected to partial alkali hydrolysis. The resulting hydrolysis ladders were incubated with purified Hfq protein, and the bound and unbound RNAs were separated on a native polyacrylamide gel. The fragments present in the unbound, complex I, and complex II regions of the gel were excised, purified, and separated on a denaturing sequencing gel (Figure 2B). OxyS RNA fragments with stem-loop **a** alone or with stem-loop **c** and only a portion of the linker region were not bound by Hfq. The 5′ end-labeled fragments containing the regions 1–55 to 1–59 and 1–68 and longer as well as 3′ end-labeled fragments containing regions 71–109 and longer were found in complex I. These results indicate that binding in complex I requires sequences near position 70 of the OxyS RNA linker or sequences in loop **b** if stem-loop **b** is disrupted. Complex II was only formed with 5′ end-labeled fragments containing the regions 1–91 and longer and 3′ end-labeled fragments containing 44–109 and longer.

We also assayed the ability of oligonucleotides complementary to specific regions of OxyS to block Hfq binding (Figure 2C). The OxyS-Hfq interaction was strongly competed by oligonucleotides complementary to stem-loop **b** and the linker region (anti-$OxyS_{52\rightarrow71}$ and anti-$OxyS_{64\rightarrow83}$). No other oligonucleotides blocked binding. None of the oligonucleotides bound Hfq directly (data not shown), indicating that the observed competition by anti-$OxyS_{52\rightarrow71}$ and anti-$OxyS_{64\rightarrow83}$ was not due to titration of Hfq away from OxyS RNA. Gel mobility shift assays with labeled oligonucleotides also indicated that all of the oligonucleotides basepaired with the OxyS RNA (data not shown). However, anti-$OxyS_{76\rightarrow92}$ bound less efficiently than the other oligonucleotides, possibly due to the shorter length and lower T_m of this oligonucleotide. It is conceivable that this reduced basepairing is the reason why anti-$OxyS_{76\rightarrow92}$ did not affect OxyS-Hfq. Nevertheless, the results of the oligonucleotide blocking are consistent with Hfq binding near position 70 of the OxyS RNA.

To test whether the linker region is sufficient for Hfq binding, we assayed the ability of OxyS RNA derivatives with a series of 5′ and 3′ deletions to compete for Hfq binding to the labeled full-length $OxyS_{1\rightarrow109}$ RNA (Figure 2D). The OxyS-Hfq interaction was competed by the full-length transcript and by the $OxyS_{48\rightarrow109}$ and $OxyS_{64\rightarrow109}$ derivatives, showing that stem-loop **a** is not required for Hfq binding for derivatives containing stem-loop **c**. Binding also was competed by $OxyS_{1\rightarrow90}$, but not by $OxyS_{48\rightarrow90}$ and $OxyS_{64\rightarrow90}$, showing that Hfq is able to bind in the absence of stem-loop **c**, but only if stem-loop **a** is present. The $OxyS_{1\rightarrow69}$ and $OxyS_{48\rightarrow69}$ were unable to compete. Together, these results suggest that, in addition to the linker, Hfq binding requires the presence of a stem-loop on one side of the single-stranded region.

We previously examined the effects of OxyS RNA deletions in vivo and reported that a derivative carrying a deletion of 1–63 could repress both the *rpoS-lacZ* and *fhlA-lacZ* fusions, while a derivative with deletion of nucleotides 1–90 could only repress the *fhlA-lacZ* fusion (Altuvia et al., 1997). However, these derivatives carry an A/U-rich 47 nucleotide 5′ extension that could confer Hfq binding. To avoid this problem, we generated plasmids that would express OxyS RNA derivatives with the native 5′ end in vivo. We were unable to construct the full-length RNA, suggesting that overexpression of native OxyS RNA might be lethal. However, a derivative lacking stem-loops **a** and **b** (poxy$S_{64\rightarrow109}$) showed nearly wild-type repression of the *fhlA-lacZ* and *rpoS-lacZ* fusions, while a derivative lacking stem-loops **a** and **b** and the linker region (poxy$S_{91\rightarrow109}$) showed no repression. Thus the linker region is required for native OxyS RNA repression of both *fhlA* and *rpoS*.

OxyS RNA Stem-Loop Opening upon Hfq Binding

It has been proposed that Hfq mediates the access of Qβ replicase to phage plus strand RNA by melting out its 3′ end (Miranda et al., 1997; Schuppli et al., 1997) and activates *rpoS* mRNA translation by altering secondary structures encompassing the ribosome binding site (Muffler et al., 1997; Cunning et al., 1998). To determine whether Hfq binding causes changes in OxyS RNA structure, labeled OxyS transcript was incubated without or with Hfq and then subjected to partial RNase T2 digestion (Figure 3). RNase T2 cleaves 3′ of single-stranded nucleosides, with preference for unpaired A residues. Similar RNase T2 cleavages were observed for the 5′ and 3′ end-labeled transcripts, and no significant differences were detected between 3 or 10 pmol Hfq. Most of stem **a** and most of stem-loop **c** were resistant to RNase T2 cleavages, and these did not change in the presence or absence of Hfq. RNase T2 cleavage was detected for loop **a**, the entire stem-loop **b**, and most of the linker region. With the addition of Hfq, the region

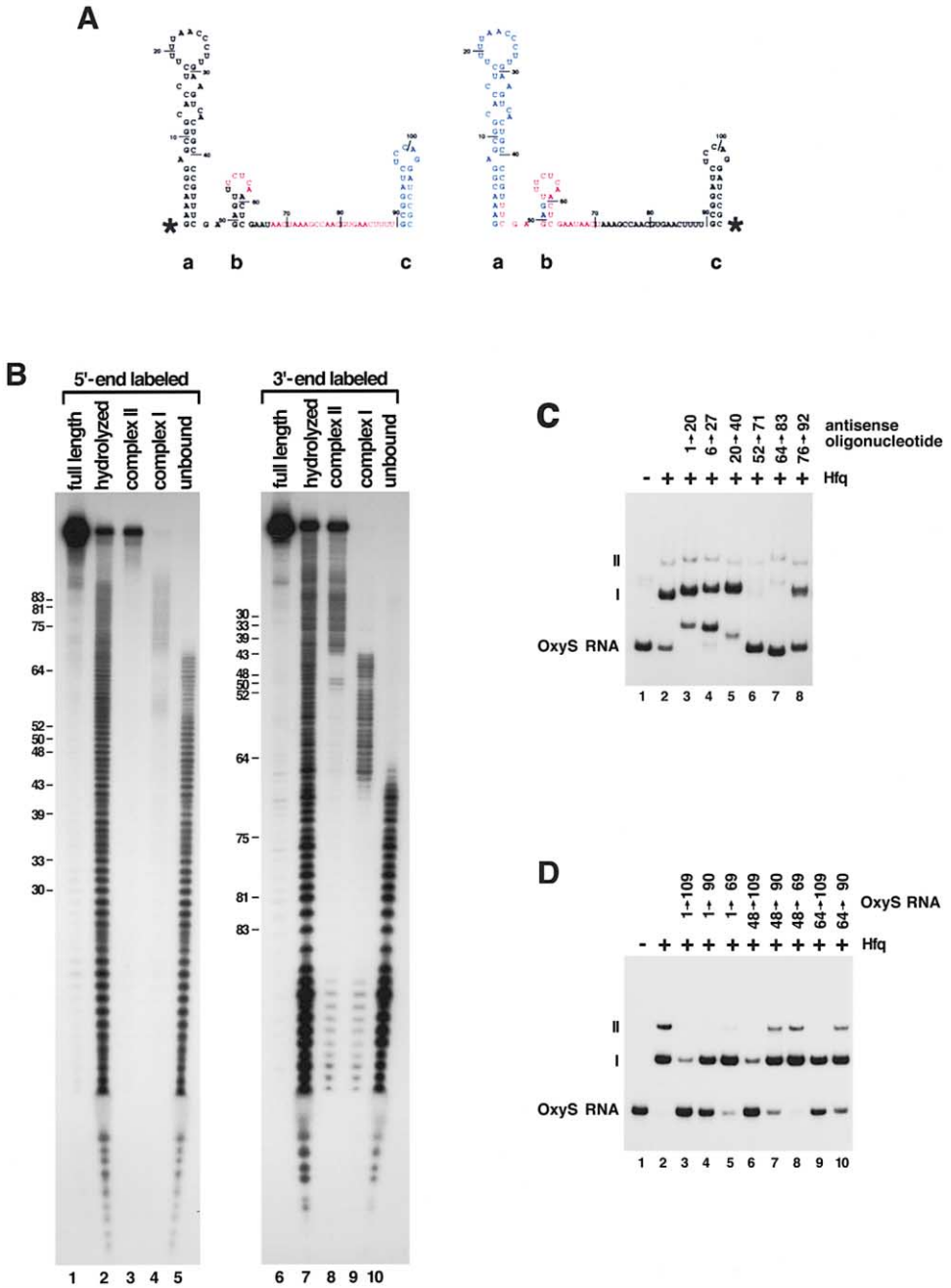


Figure 2. Hfq Binding to OxyS RNA Linker

(A) Predicted OxyS secondary structure based on the *mfold* program (http://www.ibc.wustl.edu/zuker/rna/form1.cgi). Regions of 5′ and 3′ end-labeled OxyS RNA (left and right panels, respectively) found in the unbound fraction (black) and in the OxyS RNA-Hfq complexes I (red) and II (blue).

(B) Minimal binding analysis of OxyS-Hfq interaction. 5′ or 3′ end-labeled OxyS transcript was subjected to limited alkaline hydrolysis. The resulting hydrolysis ladders were incubated with purified Hfq protein. Hfq-bound and unbound OxyS RNAs were separated on a native polyacrylamide gel and subsequently fractionated on a denaturing polyacylamide gel.

(C) Oligonucleotide blocking of OxyS binding to Hfq. Labeled OxyS transcript was incubated without or with a 5-fold molar excess of antisense oligonucleotide anti-$OxyS_{1\rightarrow20}$, anti-$OxyS_{6\rightarrow27}$, anti-$OxyS_{20\rightarrow40}$, anti-$OxyS_{52\rightarrow71}$, anti-$OxyS_{64\rightarrow83}$, or anti-$OxyS_{76\rightarrow92}$. The samples were subsequently incubated without or with purified Hfq protein. The altered mobility of non-Hfq-bound OxyS RNA, observed in lanes 3, 4, 5, 6, and 8, is due to basepairing with the competitor oligonucleotides.

(D) Competition with truncated OxyS RNAs. Labeled OxyS transcript was incubated without or with purified Hfq protein. A 5-fold molar excess of unlabeled $OxyS_{1\rightarrow109}$, $OxyS_{1\rightarrow90}$, $OxyS_{1\rightarrow69}$, $OxyS_{48\rightarrow109}$, $OxyS_{48\rightarrow90}$, $OxyS_{48\rightarrow69}$, $OxyS_{64\rightarrow109}$, or $OxyS_{64\rightarrow90}$ RNA was added as competitor.

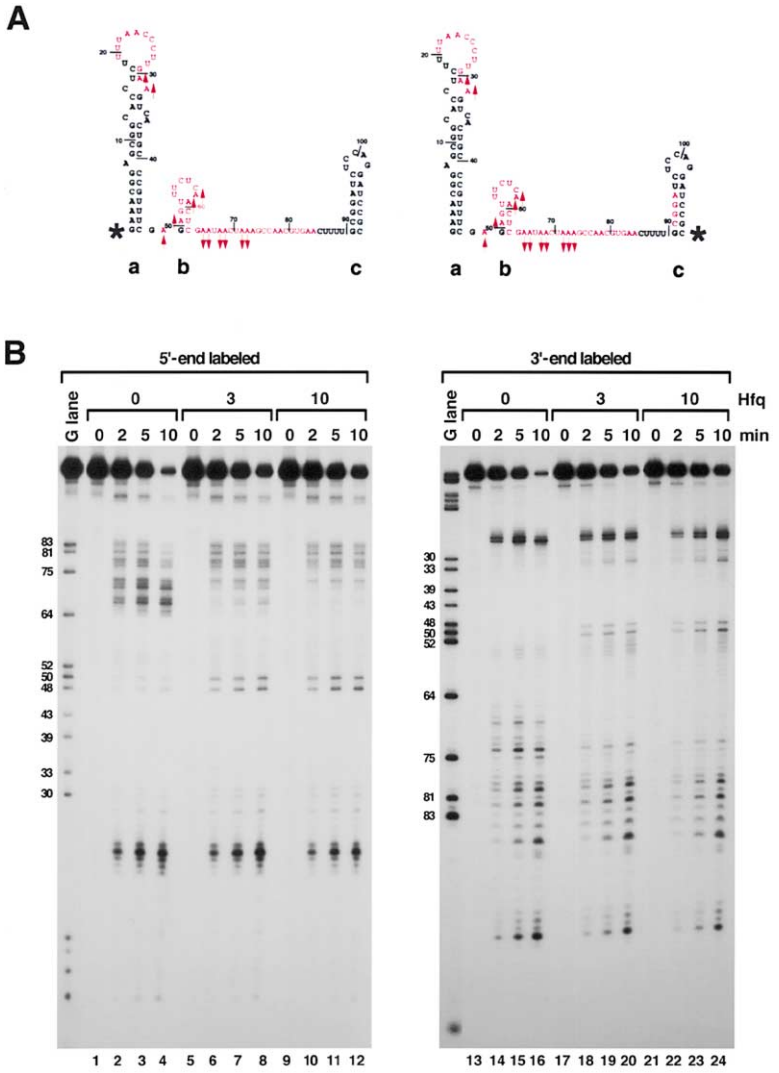


Figure 3. Changes in OxyS RNA Structure upon Hfq Binding

(A) Predicted OxyS secondary structure based on the *mfold* program. Red bases indicate RNase T2 cut sites. Upward arrows denote sites of increased cleavage in the presence of Hfq and downward arrows denote sites of decreased cleavage in the presence of Hfq.

(B) RNase T2 footprinting. 5′ or 3′ end-labeled OxyS transcript was incubated without or with purified Hfq protein in the presence of 100 pmol of bovine serum albumin. After 10 min, the samples were treated with 0.5 units of RNase T2 for 0, 2, 5, or 10 min.

between nucleotides 65 and 73 showed less cleavage, indicating Hfq binding and protection of this region, consistent with the binding analysis described above. Interestingly, the addition of Hfq led to strongly enhanced cleavage at nucleotides 49 and 51 in stem-loop **b**. Hfq binding also was associated with partially enhanced cleavage at nucleotides 31 and 32 at the top of stem **a** and weakly enhanced cleavage at nucleotides 59 and 60 in stem-loop **b**. Thus Hfq promotes the formation of a structure with a more open conformation at the top of stem **a** and all of stem **b**. It is likely that stem **b**, comprised of only four basepairs, is relatively unstable. Thus, stem-loop **b** might be in equilibrium between unpaired and paired, with the unpaired conformation favored upon Hfq binding. In experiments carried out with RNase A, which cleaves 3′ of single-stranded C and U nucleotides, we observed decreased cleavage at nucleotides 67 and 71 and increased cleavage at nucleotides 35 and 58, consistent with the results of the RNase T2 digestion (data not shown).

Unchanged OxyS RNA Levels in *hfq-1* Mutant

Hfq has been shown to decrease the stabilities of the *mutS*, *miaA*, *hfq*, and *ompA* mRNAs (Tsui et al., 1997; Vytvytska et al., 1998, 2000) and to increase the stability of the DsrA RNA (Sledjeski et al., 2001). Therefore, we examined the OxyS RNA half-life in wild-type and *hfq-1* mutant cells (Figure 4). Expression of the chromosomally encoded RNA was induced by treatment with H_2O_2. After 5 min, rifampicin was added to stop further transcription, and the levels of OxyS RNA were examined over time. No significant differences in OxyS RNA half-life were detected in comparing the wild-type and *hfq-1* mutant strains. Interestingly, we found that the shorter OxyS RNA species, previously postulated to be cleavage products (Altuvia et al., 1997), were only detected in the wild-type strain. Possibly Hfq stabilizes the cleavage products or facilitates cleavage by increasing the accessibility for an endonuclease.

Increased OxyS RNA-Target mRNA Interaction upon Hfq Binding

Zhang et al. (2001) proposed that yeast Sm complex functions to aid the U1 snRNP-pre-mRNA interaction. In mobility shift assays using Hfq, labeled OxyS RNA, and unlabeled *rpoS* mRNA, we noticed supershifted bands (Figure 5A). These supershifted complexes could be due to Hfq facilitating an interaction between the

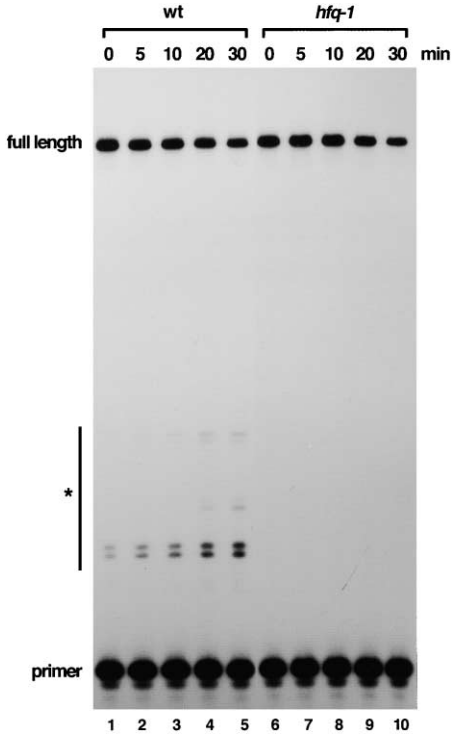


Figure 4. Unchanged OxyS RNA Half-Life in *hfq-1* Mutant

Exponentially growing cultures of wild-type (MC4100) and *hfq-1* mutant (GSO81) cells were treated with 0.2 mM H_2O_2 for 5 min. The samples then were treated with 100 μg/ml of rifampicin, and cells were collected after 0, 5, 10, 20, or 30 min. Total RNA was isolated and the OxyS RNA levels determined by primer extension assays. The asterisk indicates the regions of processing.

OxyS RNA and its target mRNAs. To test this hypothesis, Hfq and the OxyS RNA were incubated with the *fhlA* or *rpoS* transcripts (two OxyS targets) and 6S or 5S RNAs (two RNAs that are not OxyS targets). Supershifted bands were observed with Hfq and OxyS RNA incubated with the *fhlA* or *rpoS* mRNAs but not the 6S or 5S RNAs (Figure 5A, top panel). We next examined whether the *fhlA*, *rpoS*, 6S, and 5S transcripts could be supershifted by Spot42 RNA, another sRNA bound by Hfq (Wassarman et al., 2001). The *fhlA* and *rpoS* mRNAs are not known to be Spot42 RNA targets. Although the *fhlA* transcript was able to compete somewhat for Hfq binding to Spot42 RNA, none of the unlabeled RNAs was supershifted by Spot42 RNA (Figure 5A, bottom panel). Thus Hfq specifically increases the interaction between the OxyS RNA and its target mRNAs.

To assess whether continued presence of Hfq is required to detect the enhanced interaction between the OxyS RNA and its target mRNA, we removed Hfq by proteinase K digestion and phenol extraction (Figure 5B and data not shown). We still observed strong supershifted bands, although the bands were of somewhat faster mobility, consistent with the removal of Hfq. Faint bands of similar mobility were detected for samples containing only OxyS RNA and *fhlA* mRNA, indicating that some interaction between the RNAs can occur in the absence of Hfq, but this interaction is significantly enhanced in the presence of Hfq. We suggest that Hfq can be considered a chaperone; its action is required to facilitate the interaction between the OxyS RNA and its targets, but the protein is dispensable once the interaction has taken place.

A $fhlA_{C-13G}$ mutant with reduced basepairing to the OxyS RNA and a compensatory $OxyS_{G102C}$ mutant have been described in previous studies (Altuvia et al., 1998; Argaman and Altuvia, 2000). When the $fhlA_{C-13G}$ mutant was incubated with labeled wild-type OxyS RNA in the presence of Hfq, we observed reduced levels of the supershifted complexes (Figure 5C, left panel). No complexes were detected when the labeled $OxyS_{G102C}$ RNA was incubated with wild-type *fhlA* mRNA, but the supershifted bands were restored with $OxyS_{G102C}$ RNA, $fhlA_{C-13G}$, and Hfq (Figure 5C, right panel). These results show that Hfq only facilitates the interaction between RNAs able to basepair.

Using coimmunoprecipitation, we also determined whether Hfq increases the interaction between the OxyS RNA and *fhlA* mRNA in vivo. Because *fhlA* mRNA levels are difficult to detect when expressed from the chromosome, we probed for *fhlA-lacZ* and $fhlA_{C-13G}$-*lacZ* fusion transcripts expressed from a plasmid (Argaman and Altuvia, 2000). Wild-type and Δ*oxyS2* mutant cells carrying the plasmids, and left untreated or treated with H_2O_2, were lysed, and cell extracts were subjected to immunoprecipitation with Hfq-specific antiserum. Selected and total RNA samples then were probed for the OxyS, *fhlA* or $fhlA_{C-13G}$, or Spot42 RNAs by primer extension (Figure 5D). OxyS RNA coimmunoprecipitated with Hfq in all samples where the sRNA was expressed. The wild-type *fhlA* mRNA most strongly coimmunoprecipitated with Hfq when the OxyS RNA was expressed; the mRNA was not selected from untreated cells and was selected at lower levels from treated Δ*oxyS2* mutant cells. In addition, almost no mutant $fhlA_{C-13G}$ mRNA was selected from H_2O_2-treated wild-type cells. Equal amounts of the Spot42 RNA coimmunoprecipitated with Hfq under all conditions, indicating that the differences in the levels of the *fhlA* and $fhlA_{C-13G}$ mRNAs are not due to differences in the sample precipitation. These findings suggest that the Hfq protein also enhances the basepairing interaction between the OxyS RNA and its target mRNAs in vivo, although Hfq also can bind some *fhlA* mRNA directly or indirectly through an as yet unidentified partner.

Discussion

Hfq Is a Bacterial Homolog of Sm Proteins

The *E. coli* Hfq protein has been shown to play a role in a variety of RNA transactions and is required for OxyS RNA repression of both *fhlA* and *rpoS* mRNA translation. Here we show that Hfq has hallmarks of the Sm and Lsm proteins that are integral parts of RNA processing and mRNA degradation complexes in eukaryotic cells: the presence of an Sm1 motif, formation of an oligomeric ring, and preferential binding to polyU.

Sm and Lsm proteins are defined by the conservation of two sequence motifs that encompass the loops shown to contact RNA (reviewed in Kambach et al., 1999; Will and Lührmann, 2001). An alignment between bacterial Hfq homologs and Sm proteins revealed that many of the residues conserved in the Sm1 motif are conserved in the bacterial Hfq proteins. Homology be-

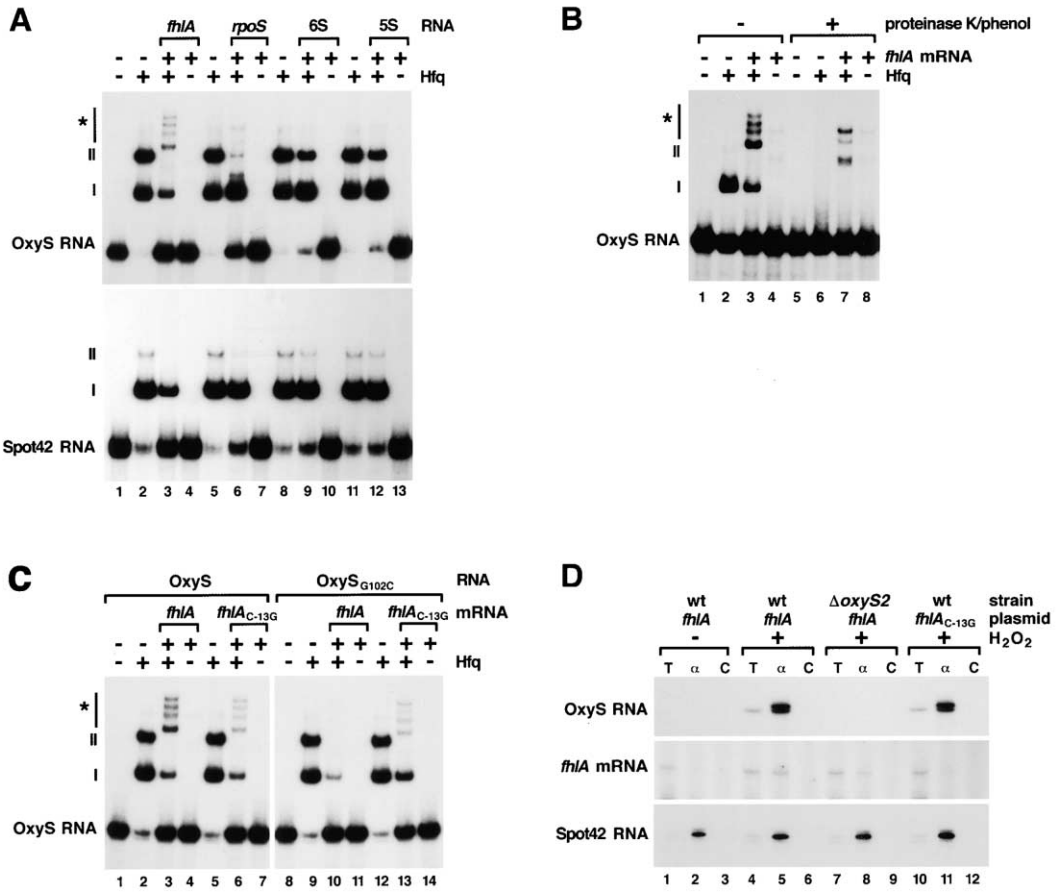


Figure 5. Increased OxyS RNA Interaction with *fhlA* and *rpoS* mRNAs in the Presence of Hfq

(A) OxyS RNA supershift with *fhlA* or *rpoS* mRNAs. Labeled OxyS or Spot42 transcript was incubated without or with purified Hfq protein. A 5-fold molar excess of unlabeled $fhlA_{-107\rightarrow+96}$, $rpoS_{-126\rightarrow+44}$, 6S, or 5S transcripts was added to the indicated lanes.

(B) Persistence of OxyS RNA supershift after Hfq removal. Samples treated as in lanes 1–4 of (A) were either left untreated or digested with 10 μg proteinase K and extracted with phenol:chloroform.

(C) Effect of mutations on OxyS RNA-*fhlA* mRNA supershift. Labeled wild-type OxyS or mutant $OxyS_{G102C}$ transcript was incubated without or with purified Hfq protein. A 5-fold molar excess of unlabeled wild-type *fhlA* or mutant $fhlA_{C-13G}$ transcripts was added as indicated. The asterisk marks region of supershifted bands in (A), (B), and (C). The multiple supershifted bands observed are probably due to OxyS RNA basepairing with multiple regions of the *fhlA* and *rpoS* mRNAs.

(D) Coimmunoprecipitation of Hfq, OxyS RNA, and *fhlA* mRNA. Extracts were prepared from wild-type (MC4100) and Δ*oxyS2::cm* mutant (GSO37) strains carrying the wild-type *fhlA-lacZ* fusion (pSA32) or the $fhlA_{C-13G}$ mutant derivative (pSA33) that were left untreated or exposed to 0.2 mM H_2O_2 for 5 min. Immunoprecipitations were carried out with control preimmune sera [C] or α-Hfq sera [α] and compared to the total RNA [T] present in 1/10 extract equivalent used in the immunoprecipitations. The extracted RNA was analyzed by primer extension with primers specific to the OxyS, *fhlA*, or Spot42 RNAs.

tween the Sm2 motif and the Hfq homologs is less obvious. However, Hfq proteins share a second region of high conservation containing a number of conserved hydrophobic residues that can be aligned with hydrophobic residues found in the Sm2 sequence motif. We propose that this second region of Hfq homology functionally substitutes for the Sm2 motif. Given the heat-stable nature of the *E. coli* Hfq protein, we also predict that the Hfq structure is very similar to the structures of Sm proteins which contain heat-stable β sheets.

The formation of oligomeric rings is another hallmark of Sm proteins. Eukaryotic Sm proteins form rings composed of seven different subunits (Achsel et al., 1999), while archaeal Sm proteins form rings with seven (Achsel et al., 2001; Collins et al., 2001; Mura et al., 2001; Törö et al., 2001) or six identical subunits (I. Törö, J. Basquin, H. Teo-Dreher, and D. Suck, personal communication). Our electron microscopy studies showed that Hfq also forms rings, whose diameter of ~70 Å is comparable to the 80 Å of eukaryotic and archaeal Sm and Lsm proteins (Achsel et al., 1999, 2001). Symmetry analysis revealed that Hfq is a hexamer, consistent with data obtained using other biochemical approaches. There are precedents for variations in the order of symmetry among different members of the same protein family. For example, the 20S proteasome is composed of heteromeric heptameric rings in the case of the yeast enzyme and of homomeric heptamers in the case of the archaeal enzyme, while the homologous bacterial peptidase ClpQ (HslV) forms homomeric hexamers (reviewed in Zwickl et al., 2000). It remains to be established whether the precise order of symmetry has implications for Hfq, Sm, or Lsm protein function.

A third hallmark of Sm proteins is preferential binding

to polyU. Hfq binding to the OxyS RNA was clearly competed by polyU. However, we also observed some competition with polyA and polyG. These results are consistent with the findings of Carmichael, who developed an Hfq purification scheme based on Hfq binding to polyA-cellulose (Carmichael et al., 1975), but also found that Hfq bound to polyU-cellulose (Carmichael, 1975). Hfq protects the sequence AAUAACUAA from RNase T2 digestion, indicating the protein binds to this region of the OxyS RNA linker. This sequence does not match the consensus sequence determined for Sm binding ($PuAU_{4-6}GPu$) (reviewed in Will and Lührmann, 2001), but is comprised predominantly of A and U residues. Senear and Steitz (1976) reported that Hfq binds to the sequences ACCAAUACUAAAAAG and AAUAAAUUAUCA CAAUUACUCUUACG on the Qβ RNA. Although the OxyS and Qβ RNA sequences and the sequences of other sRNAs bound by Hfq are all A/U rich, no single consensus motif can be identified, suggesting that Hfq does not bind with high sequence specificity. This conclusion is in agreement with our finding that an A/U-rich extension derived from the vector could partially substitute for the OxyS RNA linker. As a result of the limited specificity, Hfq also may bind to more than one position on the OxyS RNA or may move along the RNA. It also is possible that one Hfq hexamer is bound in complex I while two hexamers are bound in complex II. Our deletion mapping indicates that Hfq binding does require the presence of at least one stem-loop in conjunction with the A/U rich linker. A similar requirement for a stem-loop has been suggested for some Sm proteins (reviewed in Will and Lührmann, 2001). Additional studies are needed to determine all of the Hfq contacts with OxyS RNA and the nature of the complexes found at higher Hfq concentrations.

Hfq Role in Increasing RNA-RNA Pairing

An important unresolved question is the role carried out by Hfq and also Sm and Lsm proteins. Sm and Lsm complexes have been proposed to modulate RNA-RNA and protein-RNA interactions (Tharun et al., 2000; Zhang et al., 2001), but it is not clear how the ring particles carry out these processes. We considered three possible roles, not mutually exclusive, for Hfq binding to the OxyS RNA: Hfq might act to change OxyS RNA structure, Hfq might affect the processing or stability of the OxyS transcript, and Hfq might increase the interaction between the OxyS RNA and its target mRNAs. The results of RNase T2 digestion experiments carried out in the absence and presence of Hfq indicate that Hfq binding leads to an opening of stem **b** and the top of stem **a**. Although Hfq binding does not lead to gross changes in the OxyS RNA secondary structure, the opening of the top of stem **a** might allow for better basepairing between loop **a** of the OxyS RNA and the *fhlA* mRNA. We did not detect a difference in the OxyS RNA half-life in comparing wild-type and *hfq-1* mutant cells. More products arising from OxyS RNA processing were observed for the wild-type strain, but we do not believe these processing products have a significant effect on OxyS RNA activity, given that they represent only a small fraction of the total OxyS RNA population. Most strikingly, mobility shift and coimmunoprecipitation assays showed that Hfq binding leads to enhanced interaction between OxyS RNA and its target mRNAs in vitro and in vivo. Thus, we suggest that the primary role of Hfq is to act as a chaperone to facilitate pairing between OxyS RNA and its targets. Other recent studies indicate that Hfq also increases the interactions between the Spot42 RNA and *galK* mRNA (Møller et al., 2002 [this issue of *Molecular Cell*]) and the DsrA RNA and *rpoS* mRNA (C.C. Brescia, R. Buchanan and D. Sledjeski, personal communication) indicating that enhancing RNA-RNA pairing is a general function of Hfq and probably the Sm and Lsm proteins.

Hfq could facilitate the RNA-RNA interactions by a number of different mechanisms. Hfq binding could lead to the exposure of RNA regions that are critical for pairing. Alternatively, or in addition, Hfq could enhance RNA-RNA interactions by one Hfq particle binding two RNAs or by two Hfq particles, each binding different RNAs, coming together. Given that Hfq is tightly associated with the ribosome, it also is conceivable that Hfq could increase the interaction between a sRNA and its target mRNA by bringing the sRNA into the proximity of the ribosome. Our experiments suggest that Hfq only mediates interactions between RNAs for which there is basepairing. There are two regions of complementarity, 7 and 9 bp in length, between OxyS RNA and *fhlA* mRNA (Altuvia et al., 1998; Argaman and Altuvia, 2000). Argaman and Altuvia (2000) reported that the kissing complex formed by basepairing at these two sites is very stable in vitro in the absence of Hfq. However, formation of the OxyS RNA-*fhlA* mRNA complex in vivo may be less favorable in the presence of high concentrations of competing RNAs, raising the need for Hfq to enhance or stabilize the interaction. No basepairing interaction has been shown between OxyS RNA and *rpoS* mRNA, but the linker region of OxyS RNA can be aligned with sequences near the ribosome binding site of *rpoS* mRNA. Pairing between the OxyS RNA linker and *rpoS* mRNA may explain why the linker in the OxyS derivative containing the 5′ extension was required to detect repression of the *rpoS-lacZ* but not the *fhlA-lacZ* fusion. Future experiments aimed at further delineating the interactions between the Hfq protein, sRNAs, and their targets should be imminently doable with the *E. coli* system. Although some Sm proteins contain additional domains and may carry out more complex roles than Hfq, it is likely that studies of Hfq will provide insight into the mechanism of action of the heteromeric Sm and Lsm protein complexes.

Experimental Procedures

Strain Construction and Bacterial Growth Conditions

The bacterial strains used in the study are listed in Table 2. The *hfq-1*::Ω (cm^r) allele in TX3912 (Tsui et al., 1997) was moved into RO91, GSO38, and MC4100, and the Δ*oxyS2::cm* allele in GSO35 (Altuvia et al., 1997) was moved into MC4100 by P1 transduction (Silhavy et al., 1984). Cultures were grown under aeration at 37°C in Luria-Bertani (LB)-rich medium, and growth was monitored at OD_{600}. Ampicillin (50 μg/ml), chloramphenicol (25 μg/ml), or kanamycin (25 μg/ml) was added where appropriate.

Plasmid Construction

The plasmids used in the study are listed in Table 2. All of the inserts were generated by PCR or direct annealing of oligonucleotides (see http://dir2.nichd.nih.gov/nichd/cbmb/segr/segrPublications.html for a complete list of all oligonucleotides used in this study). To

construct pGSO146, the PCR fragment was first cloned into pCR2.1 (Invitrogen, Rockville, MD). The fragment then was excised with NdeI and EcoRI and cloned into the corresponding sites of pET21b (Novagen, Madison, WI). To construct pGSO136-pGSO138, the PCR fragments were digested with EcoRI and HindIII and cloned into the corresponding sites of pSP64 (Promega, Madison, WI). To construct the pGSO113, pGSO115, and pGSO139-143, the PCR fragments were cloned directly into pCR2.1. To construct pGSO144 and pGSO145, the oligonucleotides were annealed and cloned into the EcoRI and HindIII sites of pGEM2 (Invitrogen). The mutations in pGSO147 and pGSO148 were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All DNA manipulations were carried out using standard procedures, and the sequences of all inserts were confirmed by DNA sequencing.

Hfq Purification

Cultures (1 l) of BL21(DE3)/pLysS (Novagen) carrrying pET21b-hfq were grown to $OD_{600} = 0.6$ at 37°C. Hfq expression was induced by the addition of IPTG (5 mM final concentration). After an additional 3 hr at 37°C, cells were collected by centrifugation and resuspended in 25 ml of buffer C (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 50 mM NH_4Cl, 5% glycerol). Cells were lysed by sonication, treated with 375 U of DNase I at 4°C for 20 min, and cleared by centrifugation. The supernatant was heated at 80°C for 10 min, and insoluble material was removed by centrifugation. The supernatant was loaded onto a poly(A)-sepharose column at a flow rate of 1 ml per min. The flowthrough was reloaded twice. The column was rinsed with 35 ml buffer C, followed by 35 ml of buffer C containing 1 M NH_4Cl. The Hfq protein was eluted with 35 ml buffer C containing 1 M NH_4Cl and 8 M urea. Fractions containing Hfq protein were pooled and dialyzed, with two buffer changes, against 1 liter buffer C containing 0.25 M NH_4Cl. Glycerol was added to a final concentration of 10%, and the protein was stored at −80°C.

β-Galactosidase Assays

β-galactosidase activity was assayed using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate (Miller, 1972).

Electron Microscopy and Image Analysis

Hfq has a high affinity for carbon support films and had to be diluted to 0.5 μg/ml to obtain a satisfactory distribution of molecules. Specimens negatively stained with 1% uranyl acetate were observed with a CM120 electron microscope (FEI, Mahwah, NJ). Micrographs were recorded at 60,000× magnification and digitized at 2.3 Å/pixel using a SCAI scanner (Z/I Imaging, Huntsville, AL). Image processing was carried out using the PIC-III program (Trus et al., 1996). Particles were extracted from digitized fields using X3D (Conway et al., 1993). Symmetry analysis and correlation averaging were performed according to Kocsis et al. (1995). Particles were translationally aligned relative to a precentered, circularly symmetric, reference image obtained by averaging the data set after translational alignment. The data were then analyzed for rotational symmetry by two algorithms, respectively based on the "spectral ratio product" and the Student's t test. The latter test estimates the probability that the tabulated symmetries (Figure 1B) might have occurred at random.

For image averaging, prealigned Hfq particles were brought into azimuthal register by cross-correlation methods. Typically, a randomly selected particle was used as reference in a first cycle. Then, a second reference was generated by averaging these images after screening them with the OMO algorithm (Unser et al., 1986), which typically rejected ~30% of the data. Two more such cycles followed. To confirm the robustness of the results, the same procedure was followed starting with three different particles as the initial reference; consistent results were obtained. The resolution of averaged images was 18 Å according to the SSNR criterion (Unser et al., 1987).

Gel Mobility Shift Assays

Plasmids were linearized with HindIII, SmaI, DraI, or SspI digestion. OxyS, $OxyS_{G102C}$, $OxyS_{64\rightarrow109}$, Spot42, $fhlA_{-107\rightarrow+96}$, $fhlA_{C-13G}$, and $rpoS_{-126\rightarrow+44}$ RNAs were generated with T7 RNA polymerase (Invitrogen), while 6S, 5S, OxyS, $OxyS_{1\rightarrow90}$, $OxyS_{1\rightarrow69}$, $OxyS_{48\rightarrow109}$, $OxyS_{48\rightarrow90}$, $OxyS_{48\rightarrow69}$, and $OxyS_{64\rightarrow90}$ RNAs were generated with T3 RNA polymerase (Invitrogen). The transcripts were radioactively labeled either at 5′ end with [γ-^{32}P]ATP and T4 kinase (Invitrogen) or at the 3′ end with [α-^{32}P]pCp and T4 RNA ligase (Roche Molecular Biochemicals, Indianapolis, IN). The labeled RNAs were purified on a 8% polyacrylamide/7 M urea gel and eluted in buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 M NaOAc, 10 mM EDTA, and 1% SDS at 65°C for 1 hr, followed by ethanol precipitation. The RNA concentration was determined by measuring the OD_{260} of unlabeled RNAs exposed to the same treatment. For all of the binding reactions, 0.2 pmol of the labeled transcript, 100 ng of yeast RNA, 3 pmol of purified Hfq, and the indicated amounts of unlabeled RNAs or antisense oligonucleotides were mixed in 10 μl of 1× binding buffer (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$). The reactions were incubated at 37°C for 10 min, whereupon 1 μl of 10× loading buffer (1× TBE, 50% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol) was added. The samples were analyzed on a 6% native polyacrylamide gel run in 0.5× TBE at 4°C.

Minimal Binding Analysis

5′ or 3′ end-labeled full-length OxyS transcript (1 pmol) in 5 μl was treated with 0.5 μl of alkaline buffer (0.5 M NaOH, 10 mM EDTA) at 95°C for 50 s and immediately neutralized by the addition of 0.5 μl of acid buffer (0.5 M HOAc). After ethanol precipitation, the hydrolyzed RNA species were incubated without or with 3 or 10 pmol of Hfq protein and separated on a 6% native polyacrylamide gel as described above. Bound and unbound transcripts were visualized by autoradiography, excised from the gel, and eluted from the gel with 600 μl of elution buffer (20 mM Tris-HCl [pH7.5], 0.5 M NaOAc, 10 mM EDTA, 1% SDS) at 65°C for 1 hr. The RNAs were extracted with phenol:chloroform, ethanol precipitated, resuspended in 5 μl of FDM loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), and analyzed on a 10% polyacrylamide/7 M urea gel run in 1× TBE. RNase T1 and alkali digestion ladders of the end-labeled OxyS transcripts were used as molecular size markers.

RNase T2 Digestion

5′ or 3′ end-labeled full-length OxyS transcripts (0.2 pmol) were incubated without or with Hfq protein in 1× binding buffer containing 100 pmol BSA at 37°C for 10 min. Subsequently, 0.5 units of RNase T2 at 37°C was added to the indicated samples, and the incubation was continued for the indicated times. The samples were extracted with phenol:chloroform, ethanol precipitated, resuspended in 5 μl of FDM loading buffer, and analyzed on an 8% polyacrylamide/7 M urea gel run in 1× TBE.

Half-Life Determination

Cultures of MC4100 and GSO81 were grown to $OD_{600} = 0.4$. OxyS RNA expression was induced by adding 0.2 mM H_2O_2. After 5 min, the cells were treated with rifampicin (100 μg/ml final concentration). At the indicated times, cells were collected by centrifugation, and total RNA was extracted with Trizol reagent (Invitrogen). Primer extension assay was carried out using primer #188 as described previously (Zhang et al., 1998).

Immunoprecipitation

α-Hfq antiserum was generated by immunizing rabbits with purified Hfq protein (Covance, Vienna, VA). Cultures of MC4100 or GSO37 carrying pSA32 or pSA33 were grown to $OD_{600} = 1.7$. OxyS RNA expression was induced by adding 0.2 mM H_2O_2 for 5 min. Cells (equivalent to 10 OD_{600}) were collected by centrifugation, resuspended in 200 μl of lysis buffer (20 mM Tris-HCl [pH 8.0], 150 mM KCl, 1 mM $MgCl_2$, and 1 mM DTT) containing 10 U RNase inhibitor (Invitrogen), lysed with glass beads, and mixed with an additional 400 μl of lysis buffer. Serum (20 μl) and protein A Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ; 24 mg in 200 μl of Net2 buffer [50 mM Tris-HCl {pH 7.4}, 150 mM NaCl, and 0.05% Triton X-100]) were incubated at 4°C overnight and then washed 3 times with Net2 buffer. The serum-protein A Sepharose mix was then incubated with the cleared lysates (200 μl with an additional 10 U RNase inhibitor) at 4°C for 2 hr. The immunoprecipitated complexes were washed five times with Net2 buffer. RNA isolated by phenol:chloroform extractions and ethanol precipitation was analyzed by primer extension assays using primers #188 (Zhang et al., 1998),

#153 (TGT CCG AGA TCA CTC ATC GGT GTA TAT GAC), and #823 (CAG CCA AAT CCG ATT ACG).

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