The C-terminal domain of *Escherichia coli* Hfq is required for regulation

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**ABSTRACT**

The *Escherichia coli* RNA chaperone Hfq is involved in riboregulation of target mRNAs by small trans-encoded non-coding (ncRNAs). Previous structural and genetic studies revealed a RNA-binding surface on either side of the Hfq-hexamer, which suggested that one hexamer can bring together two RNAs in a pairwise fashion. The Hfq proteins of different bacteria consist of an evolutionarily conserved core, whereas there is considerable variation at the C-terminus, with the γ- and β-proteobacteria possessing the longest C-terminal extension. Using different model systems, we show that a C-terminally truncated variant of Hfq (Hfq<sub>65</sub>), comprising the conserved hexameric core of Hfq, is defective in auto- and riboregulation. Although Hfq<sub>65</sub> retained the capacity to bind ncRNAs, and, as evidenced by fluorescence resonance energy transfer assays, to induce structural changes in the ncRNA DsrA, the truncated variant was unable to accommodate two non-complementary RNA oligonucleotides, and was defective in mRNA binding. These studies indicate that the C-terminal extension of *E. coli* Hfq constitutes a hitherto unrecognized RNA interaction surface with specificity for mRNAs.

**INTRODUCTION**

The *Escherichia coli* host factor I/Q (Hfq) was first described as an accessory factor of the phage Qβ replicase (1) and its importance in cellular physiology became evident when the broadly pleiotropic phenotypes of an *E. coli* hfq mutant were characterized (2). The observations that Hfq is involved in expression of the *rpoS* gene encoding the stationary sigma factor, σ<sup>S</sup> (3,4), in iron metabolism (5,6), in stability control of several mRNAs (7–9) and small non-coding regulatory RNAs (ncRNAs) (10–13), in riboregulation of target mRNAs by ncRNAs (5,10,14–17) and that it acts as a virulence factor in several bacterial pathogens (18–21) has recently sparked great interest in this highly conserved bacterial protein (22).

Most data on Hfq–RNA interactions stem from studies on ncRNAs. Hfq-binding sites on ncRNAs have been demonstrated to coincide with cleavage sites of the *E. coli* major ribonuclease RNase E, and Hfq was shown to protect ncRNAs from degradation (11,12). Hfq binds to OxyS, DsrA, RprA, RyhB, Spot42 and SgrS RNAs as well as to other ncRNAs identified in *E. coli* (10,13,14,16,17,23–27). These ncRNAs are involved in translational regulation of their cognate mRNAs. By forming RNA duplexes in the close vicinity or within the translational initiation region of target mRNAs, ncRNAs can either activate or silence translation, whereby the latter mode of action appears to be predominant (28). Hfq has been shown to stimulate in vitro annealing of Spot42 RNA with *galK* mRNA (16), of OxyS with *fhlA* mRNA (14), of RyhB with *sodB* mRNA (13,27) as well as that of SgrS with *ptsG* mRNA (17). It has been demonstrated in some cases that Hfq is dispensable once the interaction between the ncRNA and the target mRNA has taken place (13,14,29). It appears possible that the molecular mechanism by which Hfq brings about these interactions entails unfolding of ncRNAs. However, recent in vitro studies did not reveal significant changes in the secondary structure of the ncRNAs DsrA (29,30) and RyhB (27).

In contrast, the Hfq RNA chaperone activity induced structural changes in the 5′ untranslated regions of the RyhB target *sodB* (27) and of the MicA target *ompA* (31,32), which could facilitate the interaction of these ncRNAs with their target mRNAs. At least for *ompA* mRNA it was shown that the structural changes induced by Hfq prevailed upon proteolysis of the protein, another criterion, which classified Hfq as an RNA chaperone (31).

The 3D structures of the *Staphylococcus aureus* Hfq homologue (33), the N-terminal 72 amino acid (aa) of *E. coli* Hfq (34) and of *Pseudomonas aeruginosa* Hfq (35) revealed that it has a hexameric ring-shaped structure,
and corroborated the idea that it belongs to the large family of Sm- and Sm-like proteins. These proteins are involved in RNA processing in eukaryotes and bind to various RNAs, primarily recognizing short U-rich stretches, known as SM sites (36). An A/U-rich region preceded or followed by a stem-loop structure has likewise emerged as a common RNA-binding motif for Hfq (27,30,31,37).

A prevailing question concerns the interaction of a Hfq-hexamer with RNA substrates. The X-ray structure of *S. aureus* Hfq complexed with a single-stranded oligoribonucleotide showed that it binds to the protein in a circular conformation around a central basic cleft of the hexameric ring (33). Mutational studies suggested that one *E. coli* Hfq-hexamer provides two binding surfaces. These studies implicated the known binding cavity along the inner rim made up of six potential nucleotide-binding pockets (38) as well as the proximal amino acid residues R16 and F39 (39) in the interaction with the ncRNA DsrA. In addition, in the same studies the amino acid residues Y25, I30 and K31, located on the distal site of the hexamer, have been shown to be required for poly(A) binding, which culminated in the hypothesis that one Hfq-hexamer can bring together two RNAs in a pairwise fashion (38).

*Escherichia coli* Hfq homologues have been found in a number of Gram-negative and Gram-positive bacteria. The Hfq proteins of different organisms display an evolutionarily conserved common core consisting of amino acid residues 7–66, whereas there is considerable variation at the C-terminal end (14,16,34,40,41). The amino acid residues 7–66, whereas there is considerable evolutionarily conserved common core consisting of residues Y25, I30 and K31, located on the distal site of the hexamer, have been shown to be required for poly(A) binding, which culminated in the hypothesis that one Hfq-hexamer can bring together two RNAs in a pairwise fashion (38).

Studies on Hfq-mediated riboregulation have only been performed in *E. coli* and some close relatives, the Hfq protein of which contains an extended C-terminus. Here, we address the question whether the C-terminus of *E. coli* Hfq contributes to ncRNA-mediated riboregulation. Using different test systems, we show that a Hfq variant, comprising the first 65 N-terminal amino acid residues (Hfq<sub>65</sub>), is non-functional in *hfq*-auto-regulation, *RyB* mediated repression of *sodB* mRNA as well as in DsrA-mediated stimulation of *rpoS* mRNA translation. Hfq<sub>65</sub> displayed no gross defect in binding to the sRNAs RybB and DsrA, and as evident from real-time fluorescence energy transfer (FRET) assays, Hfq<sub>65</sub> retained the capacity to induce structural changes in DsrA. Using FRET, we further show that in contrast to full-length Hfq (Hfq<sub>wt</sub>), Hfq<sub>65</sub> is impaired in annealing of complementary RNA oligonucleotides. In addition, while Hfq<sub>wt</sub> was able to bind two non-complementary RNA oligonucleotides on the surface, Hfq<sub>65</sub> lacked this capacity. Moreover, Hfq<sub>65</sub> was defective in binding to all tested mRNAs. In summary, this study showed that amino acid residues following the conserved core of Hfq are involved in mRNA binding and thereby identifies a third interaction surface with specificity for mRNAs.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

The *E. coli* strains MC4100 (44), AM111 (MC4100 hflQ1::EcoRI) (4) and the corresponding F′ (*lac*) variants (41,44) have been described. They were grown in Luria–Bertani (LB) medium (45) or in M9 medium supplemented with 0.2% glucose, 2 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub> and 10 μg/ml thiamine. Where indicated, glucose was substituted by 0.2% succinate and the iron chelator 2,2′-dipyridyl (50 μM final concentration) was added to the medium. Ampicillin (100 μg/ml), kanamycin (25 μg/ml), tetracycline (30 μg/ml) or chloramphenicol (15 μg/ml) were added to the medium where appropriate to maintain plasmids.

**Construction of plasmids**

The plasmid pRB381 (46) derivatives pHfq131 (37) and pRsodB-lacZ (15), which bear inducible *hflQ-lacZ* and *sodB-lacZ* gene fusions, respectively, have been described. The plasmid pAHfq used for the synthesis of Hfq<sub>wt</sub> protein was constructed as follows. The *hflQ* gene along with the *lac* promoter was recovered on a PvuII fragment from plasmid pUH5 (6), and ligated into the EcoRV–NruI sites of pACYC184 (New England Biolabs Ltd., UK). Plasmid pAHfq<sub>65</sub>, encoding the Hfq<sub>65</sub> protein was constructed as follows: the fragment containing the *hflQ<sub>65</sub>* gene was obtained by means of PCR using the *hflQ* forward primer (5′-GCTCTAGAATATAATAGTTAATCTATTTAAGAAGGAGATATACATATGGCTAAGGGGCAAT-3′) (italics), and the reverse primer (5′-TTTTTGTGAATCTTACAAAGACGGAAAGACGGAACAGTAGAAGAATC-3′), containing a Xbal site (italics), and the reverse primer (5′-TTTTTGTGAATCTTACAAAGACGGAAAGACGGAACAGTAGAAGAATC-3′), which contains two stop codons (underlined) after the triplet encoding Ser65 (bold) as well as for phage Qβ replication (43).

The construction of the plasmids used for purification and in vivo synthesis of *S. aureus* Hfq (Hfq<sub>sa</sub>) and *Bacillus subtilis* Hfq (Hfq<sub>bs</sub>), respectively, is described in Supplementary Data. The plasmids pUHfq<sub>65</sub>, pHfq131 and pRB381 derivatives pHfq131 (37) and pRB381 (46) have been described. The plasmid pRB381 (46) derivatives pHfq131 (37) and pRB381 (46) have been described.

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The plasmids pUHfq<sub>wt</sub>, pUsod and pUryB, which served as templates for in vitro synthesis of hflQ<sub>126</sub>, sodB<sub>192</sub> and RyhB RNAs, respectively, have been described (6,13,37).

Plasmid pUrpoS16 used for in vitro synthesis of rpoS<sub>652</sub> mRNA was constructed using primer C16 (5′-GGGGCTCAGAATACGCATCAGCATATAGTCGTTGAAACA-GAGTGCTAACAAATTTGGTC-3′), which contained a XbaI site (italics) and the T7 promoter sequence (underlined) followed by the 5′-terminal part of the 5′untranslated region of the *rpoS* gene (begins at the transcriptional start of *rpoS* mRNA.
when transcribed from the main P2 promoter) (47) and the reverse primer P21 (5’-AAAGAATTCTGGACGATGC
TTACCTACTCGCCGAAACG-3’), which contains an EcoRI site (italics) and is complementary to the sequence
following the stop codon of the rpoS gene. The PCR
product obtained with primers C16/P21 was cleaved with
XbaI and EcoRI, and ligated into the corresponding sites
of plasmid pUC18, resulting in plasmid pUrpos16.

Relative translational efficiency of hfq-lacZ
and sodB-lacZ fusions
Cultures of AM111F’ (pRHfq131) and AM111F’
(pRSodB-lacZ) co-transformed with the compatible plasmids
pACYC184 (control), pAHfq and pAHfq65, respectively,
were cultivated in LB or M9 medium (Figure 2)
at 37°C. At an OD_{600} of 0.5, the plasmid-encoded genes
were induced by addition of IPTG (1 mM). After 60 min,
triplicate aliquots were taken for the β-galactosidase
assays and for western blot analysis to verify Hfq wt or
Hfq65 production. In parallel, samples were withdrawn
for isolation of total RNA to determine the respective hfq-
lacZ and sodB-lacZ mRNA levels. The β-galactosidase
activity was determined from triplicate samples as
described (45) and the total RNA was purified by the
hot phenol method (48). The averaged β-galactosidase
activities obtained with the pRHfq131 and pRSodB-lacZ
constructs were normalized to the corresponding hfq-lacZ
and sodB-lacZ mRNA levels (= relative translational
efficiencies in Figure 2A and B). The corresponding lacZ
mRNA levels were determined by primer extension with
AMV reverse transcriptase (Promega GmbH, Germany)
using 5µg of total RNA primed with the lacZ-specific
5’-end labelled probe (5’-GGGAAGGCGATCGGT-3’)
and normalized to the 5S rRNA levels (internal control),
which were determined using primer R25 (5’-
GGTGGGACCCGCCGCTACGCGGCCCAGGC-3’).
The signals were visualized by a PhosphoImager
(Molecular Dynamics) and quantified by ImageQuant
software. Two independent sets of experiments were
performed.

Western blot analysis
The cellular levels of Hfq wt, Hfq65 and σ^{S} were determined
by quantitative immunoblotting. The σ^{S} levels were
determined in strain AM111F’ harbouring plasmids
pUC18 (control), pUH5 (Hfq wt) and pUHfq65 (Hfq65), respectively. The strains were grown at 28°C in LB
medium until they reached an OD_{600} of 0.3, at which time
IPTG was added to a final concentration of 1 mM. At an
OD_{600} of 1.0 equal amounts of cells were withdrawn
and boiled in protein sample buffer. The Hfq wt or Hfq65 levels
were determined at the times indicated in Figure 1A,
concomitantly with the determination of the relative
β-galactosidase values (Figure 2A and B), and the
quantification of the σ^{S} levels (Figure 2C), respectively.
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-Hfq (49) or anti-σ^{S}
(kindly provided by Dr F. Norel, Pasteur Institute, Paris)
antibodies. The antibody–antigen complexes were
visualized as described previously (49). The quantification
of the Hfq- or RpoS-specific bands was performed with
ImageQuant software.

Figure 1. Effect of Hfqwt on survival under nutrient limitation and on growth on succinate. (A) *Escherichia coli*
strains AM111(pAHgq) (closed square) and AM111(pAHgq5) (closed triangle) were grown in LB-medium, washed with M9-minimal medium,
and then resuspended to an OD_{600} of 0.05 in M9-minimal medium containing 0.2% succinate and 2,2’-dipyridyl (50µM final concentration). Growth
was followed by measuring the OD_{600} at the times indicated. The result of one representative experiment is shown.
The experiment was performed in duplicate. The error bars represent standard deviations. Bottom: determination of the levels of Hfq wt and Hfq 65 immunoblots (lower panels) showing the analysis was carried with equal amounts of total cellular protein as described in Materials and Methods section. Only the relevant sections of the strain AM111F represent data from duplicate experiments. The error bars represent standard deviations.

RNA preparation for in vitro studies

For hfq126 mRNA synthesis, the plasmid pUHfqwt digested with AflIII was used as a template for in vitro transcription with T7 RNA polymerase (Promega). To prepare sodB192 mRNA, ppoS652 mRNA and RyhB mRNA, the pUsod, pUrpoS16 and pUryhB plasmids digested with Asp718I, StuI and DraI, respectively, were used as templates. The run-off transcripts were purified on 6% polyacrylamide–8M urea gels following standard procedures. The mRNA concentration was determined by measuring the A260.

Electrophoretic mobility shift assays

Hfqwt and Hfq65 proteins used in gel mobility assays were purified from AM111(pUH5) and AM111(pUH65) cells, respectively, as described (43). Gel-purified mRNAs were 5'–end labelled with [\(\gamma\)-32P]-ATP (Amersham Pharmacia Biotech) and again purified on 6% polyacrylamide–8M urea gels. Labelled mRNAs (5 nM) were incubated without or with increasing amounts of purified Hfqwt, Hfq65, HfqSa, or Hfqdb proteins (as indicated in the legends to Figures 3C and 5 and in Supplementary Figure S3C) in a 10 µl reaction in binding buffer (10 mM Tris, pH 7.5, 60 mM NH4Cl, 5 mM β-mercaptoethanol, 2 mM MgOAc, 100 ng of yeast tRNA) for 5 min at 37°C and then for 10 min at 0°C. The samples were mixed with 40% glycerol to a final concentration of 10% and loaded on a native 5% polyacrylamide gel. Electrophoresis was performed in TAE buffer at 60 V for 12 h. Radioactive bands were visualized using a PhosphoImager.

FRET assays

This method is described in more detail in Ref. (50). Two complementary, fluorophore-tagged RNA 21-mers (Cy5-5'–AUGUGGAAAAUCUCUAGCAGU-3' (Cy5-21R+) and Cy3-5'–ACUGCUAGAGAUUUCCACAU-3' (Cy3-21R–)) were used in the annealing experiment shown in Figure 4A. For binding of non-complementary RNA oligonucleotides (Figure 4B), Cy5-21R+ and the RNA oligonucleotide Cy3-21R+ (Cy3-duplex) (5'-Cy3-CUUUCAUUGGUCGUGCUCUC3') were employed. The tagged RNA oligonucleotides were purchased from VBC-Biotech (Vienna, Austria). Using a Tecan GENios Pro microplate reader, the first oligoribonucleotide was injected into wells with or without Hfq protein (1 µM final Hfq–hexamer concentration), and the measurement was started with the injection of the second oligoribonucleotide. The reaction was performed in annealing buffer (50 mM Tris–HCl pH 7.5, 3 mM MgCl2 and 1 mM DTT) at 37°C. The final concentration of the RNAs was 5 nM in a volume of 40 µl. The reaction was allowed to proceed for 180 sec, and with Cy3 excited, donor and acceptor dye fluorescence emissions were measured once every second. The time-resolved ratio of the fluorescence emissions (FRET index FCy5/FCy3) was normalized to 1 at t180 and least-square fitted with Prism 4.03 (GraphPad Software Inc., San Diego, CA, USA) with the second-order reaction equation for equimolar initial reactant concentrations \(y = A \cdot [1 - 1/(k_{\text{ann}} t + 1)]; \ k_{\text{ann}} = \) observed annealing reaction constant, A = maximum reaction amplitude. The reaction curves were fitted with \(y = A \cdot [1 - 1/(k_{\text{db}} t + 1)];\)

Figure 2. Hfq65 is defective in auto- and riboregulation. (A) Relative translational efficiency of hfq131-lacZ mRNA in the absence of Hfq (white bar), in the presence of Hfqwt (black bar) and Hfq65 (grey bar) in strains AM111F (pRhfq131; pACYC184), AM111F (pRhfq131; pAHfq) and AM111F(pRhfq131; pAHfq65) grown in LB medium, respectively. (B) Relative translational efficiency of sodB-lacZ mRNA in the absence of Hfq (white bar), in the presence of Hfqwt (black bar) and Hfq65 (grey bar) in strains AM111F (pRnosB; pACYC184), AM111F (pRnosB; pAHfq) and AM111F (pRnosB; pAHfq65) grown in M9 medium, respectively. The averaged β-galactosidase values normalized to mRNA levels obtained in the absence of Hfq was set to 1 (white bar). The values obtained in the presence of Hfqwt (black bar) and Hfq65 were normalized to the control. The experiment was performed in duplicate. The error bars represent standard deviations. Bottom: determination of the levels of Hfq wt and Hfq 65 in the respective strains by quantitative immunoblotting (see Materials and Methods section). (C) Graphical representation of the σ5 levels in strain AM111F' harbouring plasmid pUC18 (lane 1; control), pUHfq65 (lane 2; Hfq 65) and pUH5 (lane 3; Hfq wt), respectively. The western blot analysis was carried with equal amounts of total cellular protein as described in Materials and Methods section. Only the relevant sections of the immunoblots (lower panels) showing the σ5 and Hfq-specific bands are depicted. Quantification of the western blot was done with ImageQuant software. Values were normalized to the σ5 signal obtained in the presence of Hfqwt in strain AM111F' (pUH5), which was set to 1. The results represent data from duplicate experiments. The error bars represent standard deviations.
$k_{db}$ = observed double binding reaction constant. The curves shown are representative; the observed reaction constants $k_{ann}$ and $k_{db}$ were calculated as the average of three individually fitted reactions.

DsrA RNA labelled at the 5'- and 3'-ends with the fluorophores Cy3 and Cy5, respectively, was obtained from Dharmacon (USA). Five nanometre of this RNA was incubated with indicated concentrations of Hfq or Hfq65 (Figure 3B) in a buffer containing 50 mM Tris–HCl pH 7.5, 3 mM MgCl₂, 1 mM DTT. After incubation at 25°C for 30 min, donor (Cy3) and acceptor (Cy5) emissions were measured, and the FRET index was calculated as $F_{Cy5}/F_{Cy3}$. The data were fitted in KaleidaGraph 3.51 (Synergy Software, Reading, PA, USA) with the equilibrium binding equation $y = y_0 + A/(1 + (K_{1/2}/c)^n_H)$; $A = \text{maximum amplitude}$, $K_{1/2} = \text{dissociation constant}$, $n_H = \text{Hill coefficient}$.

RESULTS

Hfq65 confers a reduced viability under nutrient limitation and does not support RyhB-mediated repression of growth on succinate

Although E. coli hfq– mutants are viable under laboratory conditions they show pronounced pleiotropic phenotypes (2). In an initial survival study, the hfq– strain AM111F’ was co-cultivated with the isogenic hfq+ strain MC4100F’ in minimal medium, and growth of both strains was followed over 4 days by scoring the total colony forming units (CFU). In contrast to MC4100F’ the CFU of AM111F’ declined rapidly from day one to day four, when the strain was close to extinction (data not shown). These data clearly indicated that Hfq is pivotal for survival of cells under nutrient limitation that can be reconciled with its requirement for riboregulation under adverse conditions (51). To test whether the C-terminus of Hfq is required for function, we therefore asked whether a truncated variant of Hfq, comprising the first N-terminal 65 aa, can sustain viability under nutrient limiting conditions. The growth and survival rates of the hfq– strain AM111, bearing the plasmid borne hfq65 allele [AM111(pAHfq65)] were compared with that of AM111 bearing the plasmid borne hfqwt gene [AM111(pAHfq)]. Both strains were grown in LB-medium, washed several times with minimal medium, and then resuspended to $1 \times 10^9$ cells/ml in M9 minimal medium supplemented with 0.2% glucose. As shown in Figure 1A, the CFU of strain AM111(pAHfq65) started to decrease $~$24 h after inoculation. While the CFU of AM111(pAHfq65) was reduced to $< 1\%$ over the 4 day observation period that of AM111(pAHfq) remained constant. As the CFU was determined in the presence of the selective antibiotic, it is unlikely that the reduced viability of AM111(pAHfq65) can be attributed to plasmid loss. In addition, quantitative
immunoblotting experiments after 48, 72 and 96 h did not reveal a significant difference in the level of Hfq wt and Hfq65 in the respective strains (Figure 1A).

We next sought for more direct in vivo evidence for the apparent dysfunction of Hfq 65. Masse´ and Gottesman (5) have shown that both Hfq and the ncRNA RyhB are required for translational repression of the sdhCDAB operon, resulting in the inability to grow on succinate or fumarate as the sole carbon source. To test whether RyhB-mediated negative regulation of the sdhCDAB genes is impaired in the presence of Hfq65, the strains AM111(pAHfq65) and AM111(pAHfq) were cultured in succinate-minimal medium in the presence of the iron chelator 2,2'-dipyridyl, which is known to induce RyhB synthesis (5). As expected, the strain AM111(pAHfq) did hardly grow in the presence of succinate, whereas similar growth rates were observed for AM111(pAHfq65) and the hfq– strain AM111(pACYC184) (Figure 1B), indicating that Hfq65 is non-functional in supporting RyhB-mediated negative regulation of the sdhCDAB operon.

Hfq65 is non-functional in hfq-autoregulation, in RyhB-mediated repression of sodB mRNA and in DsrA-mediated stimulation of rpoS mRNA

Three model systems were used to further corroborate the idea that the C-terminus of Hfq is required for post-transcriptional regulation. First, we tested whether Hfq 65 is affected in autogenous regulation (37). When compared to strain AM111 hfq–/C0 (pACYC184; pRhfq131), co-expression of the hfq gene from plasmid pAHfq and the hfq131-lacZ reporter gene from the compatible plasmid pRhfq131 in AM111 hfq–/C24 resulted in a 50% decrease of the relative translational efficiency of the hfq131-lacZ mRNA. In contrast, Hfq65 was defective in translational autocontrol, despite comparable intracellular levels of Hfqwt and Hfq65 (Figure 2A).

Second, we employed the well-studied RyhB/sodB model system, wherein the ncRNA RyhB has been shown to repress translation initiation of sodB mRNA (encodes an iron containing superoxide-dismutase) in a Hfq-dependent manner (5,6,27,52). As shown

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**Figure 4.** Deletion of the Hfq C-terminus results in a decreased RNA annealing activity and abolishes the ability to bind two RNAs simultaneously. (A) Annealing of two fluorophore-labelled RNA 21-mers can be accelerated by RNA chaperones (top; 50) that can be monitored by FRET. Bottom, 5 nM of each RNA oligonucleotide Cy5-21R and Cy3-21R were annealed at 37°C either in the absence or in the presence of 1 µM Hfqwt or Hfq65 hexamer. FRET was calculated as ratio of acceptor/donor emission (FCy5/FCy3), normalized to 1 at t100s, and the data was fitted with the second-order reaction equation for equimolar initial reactant concentrations $y = A [1 - 1/(k_{ann} t + 1)]$. Hfqwt and Hfq65 increased the observed annealing reaction constant $k_{ann}$ 7- and 2-fold, respectively. (B) In a set-up with the non-complementary Cy5-21R and Cy3-21R (Cy3-duplex) RNA oligonucleotides, simultaneous binding of the two RNAs to a protein can be measured (top). Bottom, the time-resolved FRET index curves were fitted with $y = A [1 - 1/(k_{db} + 1)]$ to yield the dual RNA oligonucleotide binding rate constant $k_{db}$. The RNA oligonucleotides by themselves do not anneal, whereas Hfqwt can bind both RNAs simultaneously. Incubation with Hfq65 resulted in a $k_{db}$ 10-fold lower than with Hfqwt, indicating that the C-terminal truncation coincides with a severely reduced capacity for simultaneous binding of the two non-complementary RNA oligonucleotides. The FRET index in this graph was not normalized to indicate the reaction amplitudes.
in Figure 2B, Hfq65 was likewise defective in RyhB-mediated translational repression of a sodB-lacZ reporter gene despite comparable intracellular levels of Hfqwt and Hfq65.

Third, we asked whether Hfq65 can support DsrA-mediated stimulation of rpoS mRNA, encoding the stationary sigma factor, σS. The ncRNA DsrA has been shown to bind to the 5′-untranslated region of rpoS mRNA in a Hfq-dependent manner, which leads to an exposure of the rpoS translation initiation determinants, and thereby facilitates translation (53–55). σS synthesis was assessed in the presence of either Hfq or Hfq65 at 28°C, when the level of DsrA is known to be elevated (55). In contrast to Hfqwt, Hfq65 did not stimulate σS synthesis, again suggesting that the C-terminus of Hfq is involved in regulation (Figure 2C).

**Hfq65 binds ncRNAs**

We have previously shown that Hfq65 binds with almost unaltered affinity to the ncRNA DsrA (43). Recent studies (30,38) suggested that DsrA binds to the inner core of the Hfq-hexamer that would explain why binding to DsrA is retained by Hfq65. It appeared possible that the molecular mechanism by which Hfq brings about ncRNA–mRNA interactions entails unfolding of ncRNAs. However, recent in vitro studies did not reveal significant changes in the secondary structure of DsrA (29,30) and RyhB (27).

Nevertheless, using enzymatic probing and biophysical methods, Brescia et al. (30) suggested that Hfq might affect the tertiary structure of DsrA. Structural mapping of DsrA placed its 5′ and 3′ end at a distance of 24 consecutive nucleotides (30), which prompted us to employ a FRET assay to verify, (i) whether Hfq alters the structure of DsrA, and if so, (ii) whether this function is retained by Hfq65. Five nanomol full-length DsrA labelled with Cy3 and Cy5 at the 5′ and 3′ end (Figure 3A), respectively, was incubated at 37°C with increasing amounts of Hfq. The decrease in FRET (Figure 3B) upon binding of Hfq indicated a change in the DsrA tertiary (and possibly secondary) structure with the two 5′ and 3′-end positioned fluorophores (Figure 3A) moving apart. A similar picture was observed with Hfq65, indicating that the conserved core region of Hfq is not only capable of DsrA binding (43) but also sufficient to induce conformational changes in the ncRNA.

Hfq65 was likewise demonstrated to bind to the ncRNA RyhB. As calculated from the shift-assays (Figure 3C), Hfq65 bound to RyhB with only a ~2-fold lower affinity (16 nM and 35 nM for Hfqwt and Hfq65-hexamers, respectively) when compared with Hfqwt. Thus, the C-terminus of Hfq does not contribute significantly to binding of the studied ncRNAs.

**The C-terminus of Hfq provides a RNA-binding surface**

We next asked whether the defects observed in Hfq65-mediated regulation (Figure 2) could be attributed to the absence of a RNA-binding surface. To test this possibility, we first used the two complementary RNA oligonucleotides, Cy5-21R⁺ and Cy3-21R⁻, which were labelled at their 5′-ends with Cy5 and Cy3, respectively. Hfq stimulated annealing of these RNA oligonucleotides with a rate constant kₐₙₙ of 0.034 s⁻¹, which was ~7-fold higher than observed for self-annealing of the two RNA oligonucleotides (Figure 4A). In contrast, the observed annealing constant for Hfq65 was ~3-fold lower than that determined for Hfqwt, and with 0.011 the kₐₙₙ for Hfq65 was only approximately twice of that seen for self-annealing of the two RNA oligonucleotides (Figure 4A). Next, we used two non-complementary RNA oligonucleotides Cy5-21R⁺ and Cy3-21R⁻ (Cy3-duplex) in the FRET assay (Figure 4B). Under these conditions no significant increase in the FRET signal was observed in the presence of Hfq65 (Figure 4B), whereas an increase in FRET was observed for Hfqwt. Given the spatial considerations required for FRET under these conditions this result corroborated other data in that a Hfq-hexamer can bind two RNAs simultaneously (38,39,57). In contrast to Hfqwt, the dual rate binding constant kdb for Hfq65 was ~10-fold lower, suggesting that Hfq65 is severely impaired in simultaneous binding of the two non-complementary oligonucleotides.

Finally, we tested whether Hfq65 is defective in binding to hq126 mRNA, sodB192 mRNA and rpoS652 mRNA to either of which Hfqwt was shown to bind (Figure 5). As shown in Figure 5A–C, Hfq65 did not bind to either mRNA fragment, which readily explained why Hfq65 was defective in auto- as well as riboregulation.

**DISCUSSION**

Although an A/U-rich region adjacent to a stem-loop structure has emerged as a RNA-binding motif for Hfq
(12,27,30), it remains poorly understood which sites of the Hfq-hexamer are involved in binding different RNA substrates. So far, mutational studies (38,39) revealed two independent RNA-binding surfaces, one of which is located on the proximal site of the hexamer and includes RNA interactions with the central cavity. As a poly(U) oligonucleotide competed with binding of the ncRNA DsrA (30), this binding site is apparently used by poly(U) (33) as well as by ncRNAs (30,38). Hence, this work corroborates mutational analyses (30,38), and verifies through studies on RyhB that the C-terminus is not instrumental for binding of ncRNAs. Moreover, as shown in Figure 3B, the C-terminus of Hfq is dispensable for Hfq to induce structural changes in DsrA. Taken together, these findings suggest that the evolutionary conserved common core of Hfq (34), i.e. the proximal surface of the Hfq-hexamer, specifies the ‘business surface’ for ncRNAs.

A second binding surface with a specificity for poly(A) has been located on the distal site of the hexamer with amino acid residue Y25, I30 and K31 being instrumental for binding to poly(A) stretches. In this case, poly(A) binding did not compete with DsrA binding, showing that both RNAs use indeed different binding surfaces (38). Binding of poly(A) to the distal site can also be reconciled with the unaltered binding affinity of a C-terminal Hfq deletion mutant, lacking the last 19 aa, and of Hfq65 for a polyadenylated mRNA (42) and for poly(A27) (Večerek, B., unpublished data), respectively. In addition, it has been shown that Hfq65 is proficient in phased Qβ replication and that mutations in K31 severely affect this function (43). Thus, it is possible that the distal site of Hfq is also pivotal for its function in Qβ replication. Moreover, the distal site could be involved in binding of Hfq to DNA A-tracts (58) that could explain the presence of Hfq in the nucleoid (59).

Equilibrium unfolding studies (42) indicated that the C-terminus contributes to the stability of the Hfq-hexamer. However, as Hfq65 forms stable hexamers in solution (43) and binds to ncRNAs (Figure 3) it seems less likely that the failure of Hfq65 to bind to hfq, sodB and rpoS mRNA (Figure 5) results from an altered stability. Therefore, we suggest that the C-terminus of Hfq provides a third RNA interaction surface with specificity for mRNAs, which can readily explain why negative translational autoregulation of hfq mRNA as well as Hfq-mediated riboregulation by the ncRNAs RyhB and DsrA was abolished in the presence of Hfq65 (Figure 2). Zambrano et al. (60) demonstrated that rpoS null strains died rapidly when mixed with rpoS+ strains. Therefore, the loss of viability of AM111(pHq65) upon entry into stationary phase (Figure 1A) can at least partially be attributed to the inability of Hfq65 to mediate translational activation of rpoS by DsrA. Similarly, as Hfq65 failed to bind to sodB mRNA (Figure 5), and thus to mediate translational repression of this mRNA by RyhB (Figure 2), it is reasonable to assume that the observed growth on succinate in the presence of Hfq65 and RyhB (Figure 1B) results likewise from the failure of the truncated protein to interact with the sodBCDAB mRNA.

Both, Hfqwt and Hfq65, bound with comparable affinities to all three RNA oligonucleotides (K1/2 of ~50–230 nM; Supplementary Figure S1), whereby the K1/2 of Hfqwt for the RNA oligonucleotides was somewhat reduced when compared with Hfq65. Assuming two independent binding sites on Hfqwt for the RNA oligonucleotides, the K1/2 for Hfqwt would represent a mean value, which could explain the reduced affinity of Hfqwt for these substrates when compared with Hfq65. In any case, these results (Supplementary Figure S1) showed that neither Hfqwt nor Hfq65 discriminate against any of the RNA oligonucleotides used, and can therefore be readily reconciled with the idea that Hfq65 fails to accommodate two RNAs. Hfq65 stimulated annealing of the two complementary RNA oligonucleotides 2-fold (Figure 4A). We speculate that this moderate acceleration of self-annealing results from a reduction of Brownian molecular motion through immobilization of one RNA oligonucleotide. Obviously, the same effect cannot stimulate FRET with non-complementary RNA oligonucleotides, as they need to be fixed in close proximity. Recent studies (38), showed that amino acid substitutions at the proximal face including alterations within the Sm2 motif, i.e. at the ‘business surface’ for ncRNAs, had a minor effect on rpoS binding, and that poly(A) did not compete with rpoS mRNA binding. Taken together with our observations that the C-terminus of Hfq is required for rpoS mRNA binding (Figure 5), and that Hfq65 stimulated FRET with non-complementary RNA oligonucleotides (Figure 4B), the available data collectively suggest that the C-terminus of Hfq forms a major and perhaps independent interaction surface for (m)RNA. A truncated version of E. coli Hfq, consisting of the first 75 aa was functional in stimulating rpoS translation (41). Therefore, the sequence between amino acid 65 and 75 of Hfq will be of prime interest to tackle the C-terminal sub-domain required for binding of mRNAs. As the binding motif for Hfq appears to be similar in ncRNAs and mRNAs (12,27,30), the molecular features underlying the preference of the C-terminus for mRNA remains to be elucidated. As yet, structural data on the C-terminus of Hfq are lacking. The PONDR (predictor of natural disordered regions) algorithm (www.pondr.com) predicts that the C-terminus of Hfq is structurally disordered (Supplementary Figure S2) that is a hallmark of RNA chaperones (61). Such flexible regions are believed to provide conformational fluctuations that facilitate intermolecular interactions. The flexibility of the C-terminus could therefore provide the molecular basis for the interaction of Hfq with many mRNA substrates or proteins. Indeed, several proteins including RNA polymerase, ribosomal protein S1 (62), RNase E (63), polyA-polymerase (PAP I) and poly nucleotid phosphohydrolase (PNPase) (64) have been found in complex with Hfq. However, it remains to be elucidated in either case whether Hfq is in direct physical contact with these candidate proteins or whether these findings result from the spatial association of the transcriptional, translational and RNA decay machineries in bacteria.

As obvious from Figure 4B, Hfq65 can bind two non-complementary RNAs, whereas Hfq65 lacked this capacity. This seems somewhat at variance with band shift assays showing that Hfq only facilitates the interaction
between RNAs able to base pair (14). However, the RNA oligonucleotides used here may differ in their binding requirements from the natural substrates used by Zhang et al. (14), and monitoring binding in real time in solution most likely puts less constrains on the stability of RNA–protein complexes than gel electrophoresis. Nevertheless, as Hfq65 was defective in binding two RNA oligonucleotides one binding site for these substrates appears to be provided by the C-terminus.

An extended C-terminus is only found in Hfq proteins of γ- and β-proteobacteria (42). Interestingly, Hfq-mediated riboregulation of mRNAs by ncRNAs has unequivocally been demonstrated only in E. coli (51) and Salmonella enterica (65), and was implicated in Shigella (66) and Vibrio (67) species, all of which belong to the γ-proteobacteria. In contrast, with 73 aa and 77 aa, respectively, the firmicutes B. subtilis and S. aureus possess Hfq proteins with short C-terminal extensions (40). Ectopic expression of the S. aureus or the B. subtilis hfq gene in E. coli AM11111′hfq+ did not (i) inhibit growth on sucinate in the presence of RyhB (Supplementary Figure S3A), was unable (ii) to support RyhB-mediated repression of sodB translation and did not (iii) result in translational auto-repression of E. coli hfq mRNA (Supplementary Figure S3B). Consistent with these observations, neither the S. aureus nor the B. subtilis Hfq protein bound to hfq126 or sodB192 mRNA (Supplementary Figure S3C). Thus, phenotypically both proteins behaved like Hfq65. Despite of the heterologous E. coli system used, these experiments could imply that there are no other inherent features in these shorter Hfq variants that would compensate for the C-terminal extension present in the Hfq proteins of γ-proteobacteria. In addition, they lend further support to the notion that an extended C-terminus is required for riboregulation. In fact, no Hfq requirement has as yet been reported for ncRNA–mRNA interactions in B. subtilis (68,69) and in S. aureus (70,71). Moreover, in contrast to a reduced resistance towards several stress conditions and an attenuated virulence phenotype reported for hfq deletion mutants of several γ-proteobacteria including Vibrio cholerae (21), P. aeruginosa (43) or Salmonella typhimurium (72), a S. aureus hfq− mutant showed no detectable defects (73). This contrasts the somewhat reduced stress tolerance and virulence reported for a hfq− mutant of Listeria monocytogenes (20) that contains likewise a Hfq protein with a short C-terminal extension. In the latter bacterium, Hfq has been shown to confer stability to at least one ncRNA (74). Taken these reports, it remains to be seen whether Hfq-mediated riboregulation, i.e. translational silencing or activation of mRNAs by ncRNAs, is confined to γ- and perhaps β-proteobacteria. On the other hand, the elucidation of the molecular function of Hfq homologues with short C-terminal tails in firmicutes remains a further challenge.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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