RNA chaperone activity of the Sm-like Hfq protein

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The *Escherichia coli* Sm-like host factor I (Hfq) protein is thought to function in post-transcriptional regulation by modulating the function of small regulatory RNAs. Hfq also interferes with ribosome binding on *E. coli* *ompA* messenger RNA, indicating that Hfq also interacts with mRNAs. In this study, we have used stimulation of group I intron splicing *in vivo* and a modified *in vitro* toeprinting assay to determine whether Hfq acts as an RNA chaperone. Hfq was able to rescue an RNA 'folding trap' in a splicing defective T4 bacteriophage *td* gene *in vivo*. Enzymatic analysis showed that Hfq affects the accessibility of the *ompA* start codon, as well as other bases within the ribosome-binding site, explaining its negative effect on ribosome binding. We also show that the Hfq-induced structural changes in *ompA* mRNA are maintained after proteolytic digestion of the protein, which classifies Hfq as an RNA chaperone.

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INTRODUCTION

Escherichia coli host factor I (Hfq) was first described as a factor required for bacteriophage Q β replication (Fernandez *et al.*, 1968). The 11.2-kDa Hfq protein forms a hexameric ring-shaped structure, and belongs to the large family of Sm and Sm-like proteins that have RNA binding activity (Møller *et al.*, 2002; Schumacher *et al.*, 2002; Zhang *et al.*, 2002).

E. coli Hfq binds a number of small RNAs, including those transcribed from the genes *rprA* (Wassarman *et al.*, 2001), *dsrA* (Sledjeski *et al.*, 2001) and *oxyS* (Zhang *et al.*, 1998). All three of these RNAs modulate translation of the *rpoS* gene, which encodes the σ^S subunit of RNA polymerase, and Hfq is thought to be required for their function. In addition, we have shown that Hfq represses translation of the *E. coli* *ompA* gene by interfering with ribosome binding, which results in rapid degradation of *ompA* messenger RNA (Vytvytska *et al.*, 2000). Thus, Hfq appears to have both direct and indirect functions in the control of RNA translation, by its targeting of mRNAs and regulatory RNAs, respectively.

The nucleocapsid protein of human immunodeficiency virus and the hnRNP A1 protein enhance the activity of the hammerhead ribozyme (Herschlag *et al.*, 1994), and the *E. coli* proteins StpA

(Zhang *et al.*, 1995; Clodi *et al.*, 1999) and ribosomal protein S12 (Coetzee *et al.*, 1994) are known to facilitate splicing of the bacteriophage T4 *td* intron. Because these proteins are thought to assist in RNA folding by preventing misfolding or by resolving misfolded RNA structures, the term 'RNA chaperone' has been used to describe them (Herschlag, 1995). Another hallmark of RNA chaperones is their stimulation of intermolecular annealing events between complementary nucleic acids (Tsuchihashi & Brown, 1994). Hfq has been shown to stimulate the interactions between spot42 RNA and *galK* mRNA (Møller *et al.*, 2002), and between OxyS RNA and *fhfA* mRNA (Zhang *et al.*, 2002). In addition, an electron microscopy study indicated that binding of Hfq to the 3'-terminal region of phage Q β plus-strand RNA induces a conformational change that brings the 3'-end of the RNA close to the S-site/M-site domain. There, the RNA is presented to the bound replicase, enabling it to initiate replication (Barrera *et al.*, 1993). In this study, we have used an *in vivo* RNA-chaperone assay, using an RNA 'folding trap' in the T4 phage *td* intron (Clodi *et al.*, 1999), and an assay using *ompA* mRNA as a target *in vitro*, to test the RNA-chaperone activity of Hfq.

RESULTS AND DISCUSSION

Assaying Hfq chaperone function *in vivo*

Semrad & Schroeder (1998) showed that the presence of a stop codon in the upstream exon of the phage T4 *td* gene inhibits RNA splicing. The splicing defect in the absence of translation was shown to be due to an exon–intron interaction that traps the pre-mRNA in a splicing-incompetent conformation. Making use of this 'folding trap' in the *td* intron, Clodi *et al.* (1999) have devised an *in vivo* RNA-chaperone assay that relies on suppression of the splicing defect. To test whether Hfq, like StpA (Clodi *et al.*, 1999), can rescue this folding trap, the *E. coli* *hfq*[−] strain AM111F' was transformed with plasmids carrying the wild-type *td* gene (pTZ18U–td Δ P6-2), or a mutant form of the gene that has a stop codon 82 nucleotides upstream of the 5' splice site (pTZ18U–tdKS82). The bacteria were also transformed with either plasmid pAZ205 (carrying the *stpA* gene), plasmid pDLE102 (carrying the *hfq* gene) or plasmid pACYC184 (an empty-plasmid control). As shown previously (Clodi *et al.*, 1999), overexpression of *stpA* (Fig. 1D, lane 2) stimulated splicing of the wild-type *td* gene, resulting in an approximately two-fold increase in the amount of spliced RNA (Fig. 1A, compare lanes 2 and 3; Fig. 1B). In contrast, expression of the *hfq* gene from the low copy-number vector pDLE102 had no measurable effect on the splicing of the wild-type *td* mRNA in the *hfq*[−] background (Fig. 1A, compare lanes 1 and 3; Fig. 1B). As determined by quantitative western

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Fig. 1 | *Escherichia coli* host factor I (Hfq) rescues the splicing defect of the exonic *tdKS82* mutant. (A) Splicing of the *td* intron and of the exonic *tdKS82* mutant, in the absence or presence of Hfq or StpA, was assayed by primer extension using the NBS2 primer, which is complementary to exon 2 of *td*, as described in Clodi *et al.* (1999). As shown on the right, primer extension in the presence of dideoxymethylenetriphosphate gave a 16 nucleotide (nt) product, whereas reverse transcription of cryptic and pre-messenger RNA resulted in primer +8 and primer +5 products, respectively. 'A' indicates the site of chain termination in the presence of dideoxymethylenetriphosphate. Other primer extension products have not been specified by Clodi *et al.* (1999). A representative autoradiograph from primer extension assays is shown. All assays were done using total cellular RNA isolated from the *E. coli* *hfq* strain AM111F'. Lanes 1–3 show the results of assays carried out using bacteria carrying a plasmid containing a wild-type *td* gene. Lanes 4–6 show results from assays using RNA isolated from bacteria carrying the pTZ18U-*tdKS82* (containing the mutant form of *td*). These bacteria were also transformed with one of the following plasmids: pDEL102 (encoding *hfq*; lanes 1 and 4), pAZ205 (encoding *stpA*; lanes 2 and 5) or pACYC184 (plasmid-only control; lanes 3 and 6). (B) Relative levels of splicing of the *td* intron and the *tdKS82* mutant in the presence and absence of Hfq and StpA. Average values from four independent experiments are shown. The numbering of the bars corresponds to the reactions shown in (A). Levels of splicing were normalized to total pre-mRNA levels. Splicing of the wild-type *td* intron in the absence of Hfq or StpA, corresponding to lane 3 in (A) was set to 1. (C,D) Determination of Hfq (C) and StpA (D) levels in the *E. coli* strains used for the *in vivo* RNA chaperone assay. Only the relevant section of the immunoblot is shown. Lanes 1–6, immunodetection of Hfq and StpA in the strains analysed in (A), lanes 1–6.

blotting, this may have been due to the low levels of Hfq expressed in strain AM111F' (pDLE102; pTZ18U-*td*ΔP6-2) (Fig. 1C, lane 1) as compared with the amount of StpA expressed in strain AM111F' (pAZ205; pTZ18U-*td*ΔP6-2) (Fig. 1D, lane 2). However, like StpA (Fig. 1A, lane 5; Fig. 1B), the expression of Hfq was sufficient to rescue the splicing defect of the exonic stop-codon mutant (Fig. 1A, lane 4; Fig. 1B). On a qualitative basis, and according to the definition of Clodi *et al.* (1999), the *in vivo* splicing assay suggests that Hfq functions as an RNA chaperone. Although this *in vivo* experiment does not permit distinction between direct and indirect effects of Hfq, it should be noted that the expression of Hfq from plasmid pDLE102 did not affect the StpA levels in *E. coli* strain AM111F' (Fig. 1D, compare lanes 1 and 3, and lanes 4 and 6, respectively).

Hfq binding affects the structure of the *ompA* RBS

Our previous studies have shown that Hfq blocks 30S-ribosome binding to *E. coli* *ompA* mRNA, and we observed that, unlike canonical translational repressors, Hfq does not protect a contiguous segment of the *ompA* ribosome binding site (RBS) from endoribonucleases (RNases) (Vytvytska *et al.*, 2000). 30S-ribosome binding protects the 5' untranslated region (UTR) of *ompA* from RNase E at cleavage site C, which is situated between the stem-loop structures hp1 and hp2 (Fig. 2C). Considering that the ribosome binds to the *ompA* RBS without destabilization of hp2, this result was in agreement with previous footprinting studies, which identified the region from -35 (±2) to +20 as the 30S boundaries (Hüttenhofer & Noller, 1994).

Changes observed in the cleavage pattern of RNA by endoribonucleases can be used to detect RNA chaperone activity. Taking

Fig. 2 Differential sensitivity to ribonucleases T1 and CV1 of *ompA*180 messenger RNA in the presence or absence of Hfq. Autoradiographs for positions -70 to +20 in the mRNA are shown. (A) Enzymatic footprinting by means of primer extension. Lanes 1-4, sequencing reactions. Lanes 5 and 6, primer extensions in the absence (-) and presence (+) of Hfq. Lanes 7 and 8, RNase CV1 cleavage in the absence and presence of Hfq. Lanes 9 and 10, RNase T1 cleavage in the absence and presence of Hfq. Lane 11, *ompA*180 mRNA was first incubated with Hfq, which was then removed by protease K digestion. RNase T1 was then added, and the cleavage sites were mapped by primer extension. Filled arrows mark positions where cleavage by CV1 was either inhibited (at positions -61, -27 and -14) or significantly increased (position -11) in the presence of Hfq. The unfilled arrow indicates the G of the AUG codon where cleavage by T1 was found to be inhibited in the presence of Hfq. The 5' and 3' ends of the *ompA* mRNA segment and the positions of the Shine-Dalgarno (SD) sequence and the AUG start codon are indicated. (B) RNase T1 cleavage of *ompA*180 mRNA labeled with ³²P at the 5'-end in the absence of Hfq (lane 1) or in the presence of a fifty-fold (+; lane 2) or ninety-fold (++; lane 3) molar excess of Hfq. Arrowheads indicate pronounced protection from T1 cleavage by Hfq. (C) Primary structure of the 5' untranslated region of *ompA*180 mRNA, showing the 5' terminal stem-loop structures (hp1 and hp2) and the 5' initial coding region (structure III in Rosenbaum *et al.*, 1993). The mRNA contains the first 180 nucleotides of the *ompA* gene. The A/U motif relevant for Hfq binding is indicated by a shaded bar. The SD sequence and the start codon (Met) are indicated, and the positions of the RNase E cleavage sites, C and D, are indicated by large arrows. The differences in the cleavage pattern by the RNases in the presence of Hfq are marked corresponding to (A) and (B).

into account the linear binding of the 30S ribosomal subunit, we examined the region from positions –70 to +20 of *ompA180* mRNA (comprising the first 180 nucleotides of the *ompA* mRNA and the 5′ untranslated region; see Fig. 2C) for Hfq-induced changes in RNase T1 and RNase CV1 cleavage patterns. This enzymatic footprinting analysis showed that the presence of Hfq results in changes in sensitivity (both increases and decreases) to these nucleases at different positions in the –70 to +20 region (Fig. 2A). We found that the presence of Hfq results in an increase in prematurely terminated primer-extension products within this mRNA segment (Fig. 2A, lanes 5 and 6). Therefore, we considered only protection from RNase cleavage and consistently occurring pronounced increases in RNase cleavage as relevant. As shown in Fig. 2A and C, these types of change in the pattern of cleavage by RNase CV1 were found at positions –61, –27 and –14 (protection), and at position –11 (pronounced increase compared to other signals). The alterations in cleavage at the first two positions (–61 and –27) affect nucleotide positions surrounding hp2, whereas the Hfq-induced increased sensitivity to RNase CV1 at position –11 affected the single-stranded conformation of the Shine–Dalgarno (SD) sequence. Moreover, the presence of Hfq rendered the G residue of the AUG start codon of *ompA* mRNA resistant to T1 cleavage, indicating that the start codon is not available for pairing with the anticodon of initiator transfer RNA in the presence of Hfq (Fig. 2A, lane 10). To verify these results, RNase T1 digestions were carried out in the absence or presence of Hfq, using *ompA180* mRNA labelled at the 5′ end with ³²P. Hfq protected several Gs (Fig. 2B, lanes 2 and 3; Fig. 2C) within the –70 to +20 region from T1 cleavage. In particular, the G of the AUG start codon, as well as the Gs of the *ompA* SD sequence, were protected by a 50-fold molar excess of Hfq (Fig. 2B, lane 2; Fig. 2C).

These changes in the accessibility of nucleotides in the *ompA* RBS in the presence of Hfq explain why Hfq inhibits the formation of the 30S translation-initiation complex (Vytvytska *et al.*, 2000; see Fig. 3B, lane 4). As suggested by structural data from the *Staphylococcus aureus* Hfq protein (Schumacher *et al.*, 2002), binding of Hfq to A/U rich sequences could trigger RNA unwinding at the target site and at surrounding RNA structures, which would permit new RNA–RNA interactions. We have recently shown that the A/U-rich sequence between hp2 and the SD sequence of *ompA* mRNA (Fig. 2C) is involved in Hfq binding (Vytvytska *et al.*, 2000). The changes in the nuclease accessibility of bases upstream and downstream of hp2 (Fig. 2A and B), and downstream from the A/U sequence, are consistent with the proposed model of Hfq action (Schumacher *et al.*, 2002); that is, Hfq binding can bring about the formation of new RNA–RNA interactions, including those that have a negative effect on the formation of stable translation-initiation complexes on *ompA* mRNA.

Block of 30S binding after proteolysis of Hfq

In contrast to RNA-binding proteins, RNA chaperones are defined by their transient action and by their dispensability for maintenance of the structural changes induced in RNA by their binding. To test whether Hfq fulfils these criteria, the toeprinting experiments illustrated schematically in Fig. 3A were carried out using *ompA* mRNA. Briefly, because a translation initiation complex bound to an mRNA can block the progress of reverse transcriptase from a downstream primer, a stop signal (toeprint signal) is generated. When compared to the intensity of the readthrough signal, obtained from all mRNAs

in the population to which no ribosomes are bound, the toeprint signal provides a measure for the efficiency of ternary-complex formation at a given start codon (Hartz *et al.*, 1988). As shown in Fig. 3Aa, in the first experiment, Hfq was added at a 90-fold molar excess to *ompA180* mRNA, to which either primer Avall (*ompA180*–Avall mRNA) or primer Y17 (*ompA180*–Y17 mRNA) were annealed, and incubated for 10 min at 37 °C. In these conditions, formation of the translation initiation complex was found to be inhibited (Fig. 3Aa; Fig. 3B, lane 4).

In the second experiment (Fig. 3Ab), Hfq was first incubated with *ompA180*–Avall mRNA for 10 min at 37 °C and then proteolytically digested with protease K. This was verified by removal of an aliquot from the reaction mixture, followed by western blotting with anti-Hfq antibodies (Fig. 3C, lane 2). Next, *ompA180*–Y17 mRNA (used as an internal control) and phenylmethylsulphonylfluoride (PMSF) were added. 30S ribosomes and tRNA^{Met} were included in the reaction mixture, and after a 10-min incubation at 37 °C, the primers were extended using reverse transcriptase. As shown in Fig. 3B (lane 7), the proteolytic removal of Hfq did not restore translation-initiation-complex formation on *ompA180*–Avall mRNA, whereas the experimental conditions did not significantly affect 30S initiation on the *ompA180*–Y17 mRNA internal control (Fig. 3Ab; Fig. 3B, lane 7). In addition, the primer extension signal obtained for *ompA180*–Avall mRNA showed that its integrity remained unaffected during the course of the experiment. The lack of translation-initiation-complex formation on *ompA180*–Avall mRNA under the conditions outlined in Fig. 3Ab can be explained by the inaccessibility of the G of the AUG start codon to RNase T1 after proteolytic removal of Hfq (Fig. 2A, lane 11).

To verify these results, the experiment shown in Fig. 3Ab was repeated, except that after proteolysis of Hfq, *ompA180*–Avall mRNA was incubated for 3 min at 80 °C (see Fig. 3Ac). This was done to resolve the RNA conformation inhibitory to 30S binding. As expected, this restored formation of the 30S initiation complex (Fig. 3B, lane 8). These toeprinting studies showed that the Hfq-induced structural changes in *ompA* RNA, which impede 30S binding (Fig. 3B, lane 4), are maintained after removal of the protein. Thus, from these experiments and from the results shown in Figs 1 and 2, Hfq fulfils all the criteria for it to be classed as a genuine RNA chaperone.

METHODS

In vivo splicing assays. *In vivo* splicing assays were carried out using the *E. coli* *hfq* mutant strain AM111F⁺, with genotype $\Delta(\arg-lac)U196\ araD139\ rpsL150\ ptsF25\ flbB5301\ rbsR\ deoC\ relA1\ hfq1::\Omega(F'proAB, lacI^q\ \Delta M15\ Tn10)$ (Muffler *et al.*, 1996). Bacteria were transformed with either pAZ205 (a low copy-number pACYC184 derivative carrying the *E. coli* *stpA* gene under control of *lacpo*; Zhang *et al.*, 1995), pDLE102 (a low copy-number pACYC184 derivative, carrying the *E. coli* *hfq* gene under the control of *lacpo*; Sonnleitner *et al.*, 2002), pACYC184, pTZ18U–*td*ΔP6–2 (a high copy-number pUC18 derivative carrying the wild-type *td* gene under the control of *lacpo*; Galloway-Salvo *et al.*, 1990) or pTZ18U–*td*KS82 (a high copy-number pUC18 derivative carrying a mutation in exon 1 of *td*, under the control of *lacpo*; Clodi *et al.*, 1999) or combinations thereof, as described in the legend to Fig. 1. Total cellular RNA isolation and the poisoned primer reaction were both carried out as described by Clodi *et al.* (1999).

Fig. 3 | Assaying the RNA chaperone activity of Hfq on *ompA* mRNA by toeprinting. An outline of the toeprinting experiments is shown in (A). The 'A' denotes the start codon of *ompA* messenger RNA. (Aa) Hfq was added in a 90-fold molar excess to *ompA*180–AvaII RNA and *ompA*180–Y17 RNA. Translation initiation on *ompA*180 mRNA was inhibited, as shown in B, lane 4. (Ab) In a parallel experiment Hfq was digested by adding protease K to the *ompA*180–AvaII mRNA/Hfq mixture. *ompA*180–Y17 RNA (internal control) and phenylmethylsulfonylfluoride (PMSF) were then added to the reaction mixture. Last, 30S ribosomal subunits and tRNA^{Met} were added and, after a 10 min of incubation at 37 °C, the toeprinting reaction was performed. (Ac) *ompA*180–AvaII mRNA was heated to 80 °C after proteolytic digestion of Hfq. (B) Toeprinting assays using *ompA*180 mRNA in the absence of Hfq (lanes 3, 5 and 6), in the presence of Hfq (lane 4), or after proteolytic digestion of Hfq (lanes, 7 and 8). Lanes 1 and 2 show primer extension using *ompA*180–AvaII RNA and *ompA*180–Y17 RNA, respectively. Lanes 3 and 4 show toeprinting assays carried out using an equimolar concentration (4 nM each) of both *ompA*180–AvaII and *ompA*180–Y17 RNAs in the absence (lane 3) and presence (lane 4) of Hfq. The primer extension signals (marked by squares) and the toeprint signals (marked by circles) obtained with *ompA*180–AvaII RNA (filled symbols), *ompA*180–Y17 RNA (open symbols) are schematically shown below the autoradiograph, and indicated on the right of the autoradiograph of the 8% polyacrylamide–urea gel. Lane 7 shows a toeprinting experiment in which *ompA*180–AvaII mRNA was incubated with Hfq, which was then removed by protease K. The *ompA*180–Y17 RNA annealing mix was added after protease K treatment and prior to the addition of PMSF, 30S subunits and tRNA^{Met}. Lane 8 shows the same experiment as lane 7, except that the RNA was heated to 80 °C after digestion of Hfq. Lanes 5 and 6, assays were performed as shown in (Ab) and (Ac), respectively, except that Hfq was omitted. The final concentration of each mRNA was 4 nM. 30S subunits and tRNA^{Met} were added in a 100-fold and 400-fold excess, respectively, to the mRNAs. When included, Hfq was added at a 90-fold molar excess over the *ompA*180–AvaII and *ompA*180–Y17 mRNAs. (C) Hfq degradation by protease K confirmed by western blotting. Four-fifths of the reaction mixture was removed from toeprinting assay reactions. Samples were analysed by western blot analysis with anti-Hfq antibodies. Lane 1, 10 pmol of purified Hfq protein was loaded on the gel (control). Lanes 2, 3 and 4, immunodetection of Hfq from the reactions analysed in (B), lanes 7, 8 and 4, respectively.

Toeprinting assays. Toeprinting assays (Hartz et al., 1988) were carried out using 30S ribosomal subunits and tRNA_i^{Met} with *ompA*180 mRNA. The plasmid pUH100 (Vytvytska et al., 2000) was used as a template for the production of *ompA*180 mRNA, to which either the Avall primer (complementary to the +110 to +87 region downstream of the *ompA* mRNA start codon) or the Y17 primer (complementary to nucleotides +60 to +76 downstream of the start codon) were annealed. Moloney murine leukaemia virus reverse transcriptase was pre-mixed with deoxynucleotide triphosphates before addition to the toeprint assay reaction mixture. When included in the reaction, Hfq was added in a 90:1 molar ratio to the mRNA. Hfq protein was prepared as described by Vytvytska et al. (1998). For removal of Hfq after incubation with *ompA*180–Avall mRNA, 0.01 units of protease K were added for 10 min at 37 °C. PMSF was then added to a final concentration of 2 mM, and the toeprinting reactions were carried out in SB buffer (with 10 mM Mg-acetate) as described in Hartz et al., 1988, with one-fifth of the reaction mixture.

RNase mapping. The Avall primer, labelled at the 5' end with ³²P (Fig. 3B), was annealed to the *ompA*180 runoff transcript of *HindIII*-digested pUH100, as described in Vytvytska et al. (2000). The annealed mixture then was subjected to RNase T1 or RNase CV1 digestion in the presence (90:1 molar ratio of Hfq to mRNA) or absence of Hfq (see Fig. 2A) in SB buffer. The cleavage sites were then mapped by primer extension using avian myeloblastosis virus reverse transcriptase. The concentration of the *ompA*180 mRNA used was 12 nM, and 1 unit of RNase T1 or 0.01 units of RNase CV1 were used.

In addition, RNase T1 mapping was carried out with 12 nM *ompA*180 mRNA labelled at the 5' end with ³²P. Hfq was added to the mRNA in a 50-fold or 90-fold molar excess (see Fig. 2B), and the mixture was incubated for 5 min at 37 °C in SB buffer before addition of 1 unit of RNase T1. After a 3-min incubation at room temperature, the reaction was terminated by ethanol precipitation.

Western blotting. To quantify Hfq and StpA protein levels in the strains used for the *in vivo* RNA-chaperone assay (Fig. 1A, lanes, 1–6), equimolar amounts of total cellular protein, prepared concomitantly with total RNA, were separated on 12% SDS-polyacrylamide gels. Quantitative immunoblotting was carried out using anti-Hfq and anti-StpA antibodies. Visualization of Hfq- and StpA-specific immunocomplexes was carried out using a goat anti-rabbit antibody, labelled with alkaline phosphatase, in a colour reaction using a nitroblue tetrazolium p-toluidine salt (Sonnleitner et al., 2002).

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