

The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation

Marc Folichon, Véronique Arluison, Olivier Pellegrini, Eric Huntzinger¹,
Philippe Régnier and Eliane Hajnsdorf*

UPR CNRS No. 9073, Conventionnée avec l'Université Paris 7–Denis Diderot, Institut de Biologie Physico-Chimique, 75005 Paris, France and ¹UPR CNRS No. 9002, Institut de Biologie Moléculaire et Cellulaire, 67084 Strasbourg Cedex, France

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ABSTRACT

The Hfq protein, which shares sequence and structural homology with the Sm and Lsm proteins, binds to various RNAs, primarily recognizing AU-rich single-stranded regions. In this paper, we study the ability of the *Escherichia coli* Hfq protein to bind to a polyadenylated fragment of *rpsO* mRNA. Hfq exhibits a high specificity for a 100-nucleotide RNA harboring 18 3'-terminal A-residues. Structural analysis of the adenylated RNA–Hfq complex and gel shift assays revealed the presence of two Hfq binding sites. Hfq binds primarily to the poly(A) tail, and to a lesser extent a U-rich sequence in a single-stranded region located between two hairpin structures. The oligo(A) tail and the interhelical region are sensitive to 3'–5' exoribonucleases and RNase E hydrolysis, respectively, *in vivo*. *In vitro* assays demonstrate that Hfq protects poly(A) tails from exonucleolytic degradation by both PNPase and RNase II. In addition, RNase E processing, which occurred close to the U-rich sequence, is impaired by the presence of Hfq. These data suggest that Hfq modulates the sensitivity of RNA to ribonucleases in the cell.

INTRODUCTION

The degradation of messenger RNA in *Escherichia coli* is carried out by a combination of endoribonucleolytic cleavages and 3' to 5' degradation, primarily by polynucleotide phosphorylase (PNPase) and RNase II. It is widely accepted that mRNA decay is initiated by a series of endonucleolytic cleavages catalysed by RNase E, or occasionally by RNase III, followed by processive exonucleolytic degradation of the message. The *rpsO* mRNA, which encodes ribosomal protein S15, has been used as a model for the study of mRNA decay. An RNase E cleavage located 10 nucleotides downstream of the coding sequence (M2 site) initiates the mRNA decay by removing the 3' stabilizing terminal hairpin that protects the mRNA against the attack of exonucleases (1). The body of the messenger is then further degraded by the combined actions of

poly(A) polymerase I (PAP I), polynucleotide phosphorylase (PNPase) and RNase II (2,3). Poly(A) tails were detected at the termini of nascent terminated mRNA, and at the end of decay intermediates produced by endonucleolytic and exonucleolytic digestion (4). Poly(A) tails promote decay of *rpsO* mRNA and of other structured RNAs by providing an unstructured 3' end, to which 3' exonucleases bind [for a review see (5)]. The degradation is controlled by RNA binding proteins (6), RNA helicases (7), ribosomes (8,9) and secondary structures (10–12).

The pleiotropic phenotype of *hfq*⁻ strains shows that the Hfq protein acts on different pathways of *E.coli* metabolism (13,14). Hfq has been reported to be involved in the regulation of iron content (15,16), the expression of the σ^S subunit of RNA polymerase upon entry into stationary phase, the response to hyperosmolarity and low temperature (17–19), mRNA stability at slow growth-rate (20) and stationary phase (21), and in controlling the activity of regulatory sRNAs under stress conditions (see below). Hfq is known to stabilize some RNAs (22) and to target others for degradation (15,21,23). In the case of the *ompA* messenger, Hfq interferes with ribosome binding and unmasks an RNase E cleavage site, destabilizing the transcript (12). On the other hand, Hfq stimulates processivity of poly(A) polymerase I (24). We have shown that Hfq affects the position, frequency and length of oligo(A) tails at the 3' end of *rpsO* mRNA *in vivo* (25). However, the failure to detect a direct interaction between Hfq and poly(A) polymerase I (M.Folichon, V.Arluison and E.Hajnsdorf, unpublished results) led us to the notion that the formation of a poly(A)–Hfq complex may stimulate poly(A) elongation by PAP I.

Hfq affects gene expression either by interacting directly with mRNA and/or by promoting annealing between regulatory RNAs that it directs to target messengers. The propensity of Hfq to bind single-stranded A–U-rich sequences (26) is exemplified by the direct interactions between Hfq and the OxyS, DsrA and Spot42 sRNAs (22,27,28). Hfq somehow facilitates the annealing of these riboregulators with their target mRNAs, namely the *rpoS*, *fhlA* and *galK* mRNAs, respectively. On the other hand, in the case of Q β RNA, Hfq facilitates the access of replicase, presumably by melting its 3' end (29,30). Similarly, Hfq binding weakens base-pairing in stem loops of OxyS sRNA (31).

*To whom correspondence should be addressed. Tel: +33 1 58 41 51 26; Fax: +33 1 58 41 50 20; Email: eliane.hajnsdorf@ibpc.fr

In an attempt to understand the role of Hfq in the polyadenylation of prokaryotic mRNA, we have compared the interaction between Hfq and a polyadenylated or a non-polyadenylated mRNA fragment. This fragment corresponds to the 3' end of the *rpsO* transcript, which has been shown to be polyadenylated both *in vivo* and *in vitro* (4,24,32). Measurements of dissociation constants show that Hfq exhibits a high affinity for *rpsO* mRNA and binds more steadily to molecules harbouring poly(A) tails. Consistently, Hfq binds preferentially to poly(A) tails and interacts with an internal site located between the two hairpins of the polyadenylated RNA at higher concentrations. Moreover, we show that the poly(A) tail and the internal binding site are protected by Hfq from ribonucleases, namely PNPase, RNase II and RNase E, which are known to trigger the degradation of this mRNA in the cell (2,32,33). Our findings raise the possibility that the high affinity of Hfq for polyadenylated RNA may play a general role in RNA maturation and degradation.

MATERIALS AND METHODS

Protein purification

Hfq was overproduced from strain BL21 (DE3) transformed with pTE607 plasmid (kindly provided by T. Elliott). Cells from induced cultures were resuspended at 4°C in 20 ml buffer containing 20 mmol/l Tris-HCl (pH 7.8), 500 mmol/l NaCl, 10% glycerol (v/v) and 0.1% Triton X-100 (v/v). The suspension was passed through a French press (1200 bar, 20 000 psi) and submitted to centrifugation (30 min at 15 000 g). Imidazole-HCl (pH 7.8) was added to the supernatant to a final concentration of 1 mmol/l. The resultant suspension was applied to a 1 ml Ni²⁺-NTA column (Qiagen). The resin was then sequentially washed with ~15 column volumes of: (i) 20 mmol/l Tris-HCl (pH 7.8) buffer containing 0.3 mol/l NaCl and 20 mmol/l imidazole; and (ii) 50 mmol/l sodium phosphate (pH 6.0) buffer containing 0.3 mol/l NaCl. Hfq was eluted with buffer containing 50 mmol/l sodium phosphate (pH 6.0), 0.3 mol/l NaCl and 250 mmol/l imidazole. Fractions containing Hfq were determined by SDS-PAGE analysis, pooled and heated to 80°C for 15 min. Insoluble material was removed by centrifugation and the supernatant was dialyzed against the buffer containing 50 mmol/l Tris-HCl (pH 7.5), 1 mmol/l EDTA, 50 mmol/l NH₄Cl, 5% glycerol (v/v) and 0.1% Triton X-100 (v/v). The protein was kept at 4°C.

RNase II was overproduced from BL21 (DE3) strain transformed with plasmid GC100, (kindly provided by G. Mackie) and purified as described previously (34).

Protein concentrations were determined by using the Bradford protein assay with bovine serum albumin as a standard. Hfq concentration was calculated on the basis of the monomer form.

PNPase was a gift from C. Portier and F. Allemand, and RNase E, purified as described previously (35), was a gift from A.J. Carpousis.

RNA preparation and labelling

The 97RNA, referred to as the *ΔrpsO* mRNA, corresponds to the last 97 nucleotides of the *rpsO* transcript, with a GGG

sequence at the 5' end. This RNA and its polyadenylated derivative were synthesized by T7 RNA polymerase as described previously (24). An extended polyadenylated *ΔrpsO* mRNA was transcribed using a PCR-amplified template, using the following primers: 5'-TAATACGACTCACT-ATAGGGAGACGTAGCACGTTACACC (upstream primer) and 5'-GATCCCGGGATCCACCACCAT₁₈GAAAAAAGG-GGCCACTCAGG (downstream primer). Underlined nucleotides correspond to the T7 RNA polymerase promoter. Transcription reactions were carried out either as described previously, yielding uniformly labelled RNA with high specific activity (24), or according to Ambion's MEGAscript protocol and with [α -³²P]UTP as tracer, except that 20 mmol/l guanosine was added to allow direct 5'-labelling. Radiolabelled RNAs were gel purified, eluted and resuspended in water. The percentage of radioactivity incorporated was determined and used to calculate RNA concentrations. 5' end labelling was performed with [γ -³²P]ATP and T4 polynucleotide kinase. Labelled RNAs were separated from unincorporated nucleotides using a ProbQuant G-50 Micro column (Pharmacia Biotech).

Heterogeneous poly(A) (Sigma) was separated on denaturing polyacrylamide gels. Bands were located by comparing with radioactive RNA markers. The gel was cut into horizontal strips and poly(A) RNA was eluted. Precipitated poly(A) was dissolved in water. Its length was determined by analysing 5'-end-labelled aliquots on sequencing gel along with RNA markers of known lengths and alkaline hydrolyzed poly(A). Due to the resolution of the preparative gel, the poly(A) size fractions contained different size distributions. Values are estimated mean values of each fraction. Poly(A) concentration was determined by spectrophotometry with the extinction coefficient taken from the Pharmacia catalogue.

Gel shift assays

Radiolabelled RNAs (5 pmol/l) were titrated against Hfq protein (15 pmol/l to 2 nmol/l, expressed as monomers) in 50 μ l buffer containing 10 mmol/l Tris-HCl (pH 8), 1 mmol/l EDTA, 80 mmol/l NaCl, 1% glycerol (v/v), 0.01% dodecyl maltoside (v/v) at 37°C for 30 min. Reactions were loaded onto running native gels (27) and quantified using a PhosphorImager (Molecular Dynamics). The apparent dissociation constant (K_d) was determined for Hfq-*rpsO* RNA complexes, assuming that complex formation obeys a simple bimolecular equilibrium and the concentration of the labelled RNA is negligible compared to Hfq concentration. In practice, the reactions were performed with protein in excess over RNA, and thus the free Hfq concentration changed little during RNA binding. Therefore, the results were plotted using the equation:

$$Q = [\text{Hfq}] / (K_d + [\text{Hfq}])$$

Q represents the fraction of labelled RNA bound to Hfq. The apparent K_d values were calculated from at least three independent experiments. The percentage of free and complexed RNAs in each sample were deduced by counting each band and normalizing to the total radioactivity in each lane.

Chemical and enzymatic probing

Hfq-polyadenylated *ΔrpsO* mRNA complex formation was carried out with 10⁵ c.p.m. of 5'-labelled RNA in the presence

of 1 μg *E. coli* tRNA in 50 mmol/l HEPES–NaOH (pH 7.7), 1 mmol/l Mg–acetate, 80 mmol/l sodium acetate for 15 min at 37°C. The chemical reaction was performed in 10 μl in the presence of 16 mmol/l lead acetate (Merck) at 20°C for 5 min. The reactions were stopped by adjusting the sample to 50 mmol/l EDTA. RNase V1 (Pharmacia Biotech) (5×10^{-5} U) and RNase T2 (Sigma and Ambion) (0.02 U) were used for enzymatic hydrolysis. Incubation controls in the absence of the probes were carried out in parallel. All samples were extracted with phenol/chloroform and precipitated with ethanol. Pellets were counted, resuspended in 8 mol/l urea, 0.025% bromophenol blue (w/v) and 0.025% xylene cyanol blue (w/v), to load identical amounts of radioactivity on the sequencing gel. Cleavage positions were identified by running RNase T1 (Ambion) (indicated on the right side of panel C) and alkaline ladders of the same 5'-end-labelled RNA, performed under denaturing conditions, in parallel (36).

Assays for RNase II activity were performed in a 10 μl reaction volume containing 10^5 c.p.m. 5'-labeled RNA (~100 fmol) and 1 μg of *E. coli* tRNA in reaction buffer containing 50 mmol/l HEPES–NaOH (pH 7.7), 1 mmol/l Mg–acetate, 80 mmol/l sodium acetate, in the absence and in the presence of 1 $\mu\text{mol/l}$ Hfq, and samples were incubated at 37°C for 15 min. Next, 0.2 pmol RNase II was added, and samples were withdrawn at various times and quenched by phenol extraction. After precipitation, samples were resuspended in 8 mol/l urea, 0.1% xylene cyanol (w/v) and 0.1% bromophenol blue (w/v). Normalized amounts of RNA were separated on 8% sequencing gels and visualized using a PhosphorImager (Molecular Dynamics).

PNPase reactions were as for RNase II reactions, except that the buffer was supplemented with 10 mmol/l K_2HPO_4 and 2 pmol PNPase were added to initiate the enzymatic assay. The formation of a Hfq-polyadenylated $\Delta rpsO$ mRNA complex under these buffer conditions was monitored by band shift assay.

RNase E digestions were carried out in buffer containing 100 mmol/l NaCl, 10 mmol/l Tris–HCl (pH 7.5), 1% glycerol (v/v), 1 mmol/l MgCl_2 , 0.1% Triton (v/v), 0.2 mmol/l DTT, 1 μg of *E. coli* tRNA and 50 ng of a degradosome preparation for 5 min at 37°C.

RESULTS

Equilibrium dissociation constant measurement of complex formation by mobility shift assay

Polyadenylated and non-polyadenylated RNAs were synthesized to examine the contribution of a poly(A) tail to the binding of Hfq to RNA. We used an RNA fragment corresponding to the 3' end of the *rpsO* transcript, which was shown to be polyadenylated both *in vivo* and *in vitro* (4,24,32). An 18-A tail was added to the 3' end of this RNA for two reasons. First, it corresponds to the minimum size of the oligo(A) tails at the 3' end of this fragment (ranging from 20 to 35 As) required to detect Hfq stimulation of poly(A) tail elongation by poly(A) polymerase I (24). Secondly, it fits the size of the tails found at the end of total RNA, which can be up to 50 nucleotides in length (37,38). The RNA, referred to as $\Delta rpsO$ mRNA, corresponds to the last 97 nucleotides of the *rpsO* primary transcript, and is predicted to contain two

secondary structures separated by a single-stranded region (see Fig. 2 below). This RNA and its adenylated derivative containing an 18-residue poly(A) tail [$\Delta rpsO\text{-(A)}_{18}$] were incubated with increasing quantities of Hfq and of His₆-Hfq. Overexpressed His₆-Hfq had the same binding capacity as overexpressed native Hfq (data not shown), indicating that the histidine tag at the C-terminus of the protein does not perturb the RNA binding capacity of the protein. The tagged protein was used throughout this study, and is referred to as Hfq.

$\Delta rpsO$ or $\Delta rpsO\text{-(A)}_{18}$ RNAs were incubated with increasing concentrations of Hfq protein. The formation of complexes was analysed by mobility shift assay. Figure 1 shows that Hfq binds both non-polyadenylated and polyadenylated RNA. In both cases, two complexes were observed (referred to as Ia and IIa, and Ib and IIb in Fig. 1A), the second ones (IIa and IIb) forming at higher protein concentrations. The equilibrium dissociation constants (K_d) were deduced from the strongest binding site (Ia and Ib) for both $\Delta rpsO$ and $\Delta rpsO\text{-(A)}_{18}$ mRNAs, assuming a bimolecular model. The apparent binding affinity of Hfq for the adenylated RNA (K_d of 90 ± 16 pM) is 15 times higher than that for the non-polyadenylated RNA (1.4 ± 0.1 nM). It is likely that the binding of the protein to the poly(A) tail accounted for this difference. To address this point, we incubated Hfq with poly(A) RNA under the same conditions. We performed the titration experiment with poly(A)₁₁₅, which has the same length as the polyadenylated RNA, $\Delta rpsO\text{-(A)}_{18}$. The mobility shift assay revealed the appearance of distinct intermediary complexes, which presumably correspond to the sequential filling of binding sites as the protein concentration is increased (Fig. 1A). These data suggest that Hfq binds non-cooperatively to the poly(A)₁₁₅. A similar Hfq concentration is required to detect binding to either poly(A)₁₁₅ or $\Delta rpsO\text{-(A)}_{18}$ RNA (Fig. 1A, lanes 13–14 and 24–25, respectively). Thus the formation of complex Ib with $\Delta rpsO\text{-(A)}_{18}$ is likely to be due to the binding of the protein to the polyadenylated extremity of the RNA. This result also implies that the $\Delta rpsO$ mRNA does not affect the binding of Hfq to its poly(A) tail and that a tail of 18 As is sufficient for efficient Hfq binding (24). Moreover, the fact that similar Hfq concentrations were required for the formation of complex Ia with non-polyadenylated $\Delta rpsO$ RNA (Fig. 1A, lane 7) and complex IIb with polyadenylated $\Delta rpsO\text{-(A)}_{18}$ RNA (Fig. 1A, lane 22), suggests that this latter complex corresponds to the interaction of the protein with an internal region of $\Delta rpsO$ RNA. We cannot determine, however, if complex IIa on Figure 1A results from sequential binding of Hfq to two sites on $\Delta rpsO$ RNA or if protein–protein interactions account for the appearance of complex IIa (39).

Enzymatic and chemical probing of polyadenylated RNA complexed to Hfq

Chemical and enzymatic probing was used to determine where Hfq binds on the polyadenylated RNA and whether Hfq binding causes conformational changes in the polyadenylated $\Delta rpsO$ mRNA. The polyadenylated RNA, free or bound to Hfq, was submitted to partial enzymatic hydrolysis with RNase V1 (specific for double-stranded regions) and RNase T2 (specific for unpaired nucleotides with a preference for adenines). The RNA was also subjected to Pb(II)-induced cleavages, which has proven to be exquisitely sensitive to

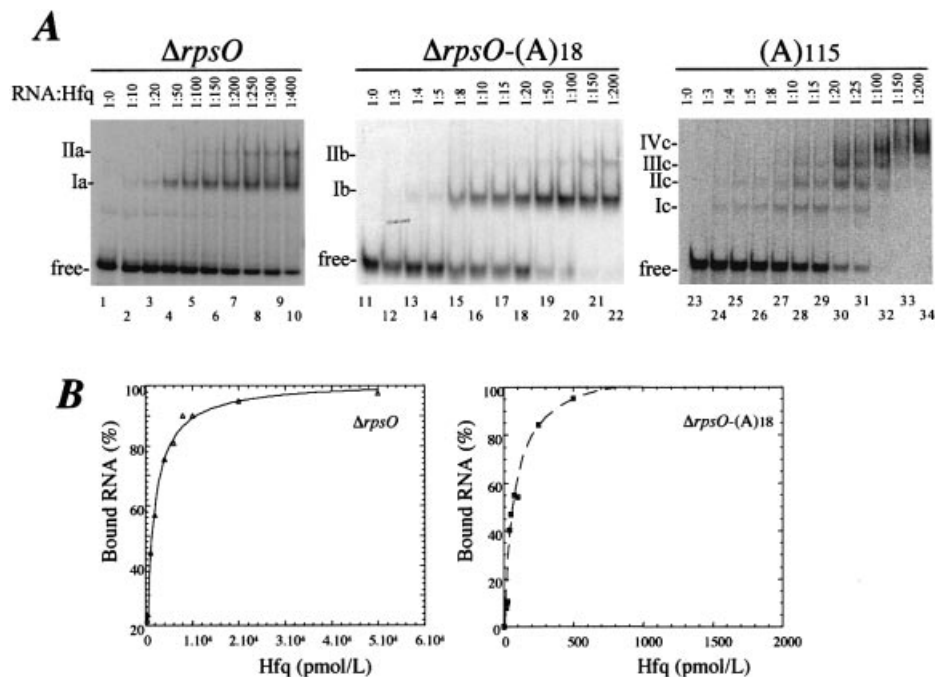


Figure 1. Affinity of Hfq for *ΔrpsO* mRNA. (A) Non-polyadenylated *ΔrpsO* mRNA, polyadenylated *ΔrpsO* mRNA fragments [*ΔrpsO*-(A)₁₈] and poly(A)₁₁₅ were incubated with various concentrations of Hfq. Five pmol/l of 5'-end-labelled non-polyadenylated *ΔrpsO* mRNA (lanes 1–10), uniformly labelled polyadenylated *ΔrpsO* mRNA (lanes 11–22) and 5' labelled poly(A)₁₁₅ (lanes 23–34) were mixed with increasing concentrations of Hfq, ranging from 15 to 2000 pmol/l. RNA:Hfq molar ratios are indicated at the top. Complexes were separated on native polyacrylamide gels as described in Materials and methods. (B) The data were plotted using KALEIDAGRAPH 3.0.4 (Abelbeck Software, Reading, PA) and the generated curves were fitted by non-linear least squares regression assuming a bimolecular model such that the K_d values represent the protein concentration at half-maximal RNA binding. The data show one representative experiment of three performed to calculate the K_d value.

subtle structural variations and which induces cleavages, mainly in interhelical regions, loops and bulged nucleotides. The experimental results and a schematic view of the secondary structure of the RNA are shown in Figure 2.

The data correlate well with the existence of the two hairpin structures (helix 1 and the terminator stem-loop, t1) connected by a single-stranded interhelical region (connecting region shown in Fig. 2A, D and E). Both helices are cleaved by RNase V1, whereas the single-stranded and loop regions are cleaved by both RNase T2 and Pb(II). The presence of several Pb(II) cleavages in helix I (C26–C28/G32–G34) and in helix II (A72–A73) suggests that these two regions are metastable. Finally, the presence of concomitant RNase V1 cleavages (at positions 53–54, 58 and 64) and single-stranded specific cleavages in region 53–60 suggests the existence of an equilibrium between unpaired and paired residues, resulting in the formation of alternative conformers. Indeed potential base pairings may occur either between residues GAGA6 and UCUU60 or between residues AUUC58 and GAGU66.

The addition of increasing concentrations of Hfq induced a strong reduction in the number of cleavages in the poly(A) tail region by Pb(II) and RNase T2 hydrolysis. Moreover, the RNase V1 cleavages at position 99–102 were strongly reduced by Hfq binding. These data indicate that the poly(A) tail is the major binding site of Hfq. Higher Hfq concentrations caused a significant reduction in Pb(II) cleavages in the U-rich sequence U66–U68. Hfq, concomitantly, prevented cleavages at positions U53–A54, C58 and A64 by RNase V1. Since Pb(II) cleavages were not affected by Hfq binding in region

55–60, a more open conformation of residues C52–U60 may be favoured. Subtle rearrangements induced by Hfq binding are also supported by the fact that weak, but reproducibly enhanced, cleavages by RNase V1 (at G46) and Pb(II) (at C52 and U53) were observed at the bottom of helix 1.

In summary, the results suggest that Hfq binds strongly to the poly(A) tail and to a lesser extent to the U-rich interhelical region, and induces subtle changes in the structure of the polyadenylated RNA.

Hfq binding protects poly(A) from RNase II- and PNPase-mediated decay

We next used RNase II, an exoribonuclease of 3' to 5' polarity that degrades single-stranded polyribonucleotides, to examine the effect of Hfq binding to the poly(A) tail. The *rpsO* mRNA fragment used as a substrate (see above) contained a tail of 18 As downstream of the stable terminator stem-loop, which protects the upstream RNA from attack by exonucleases. We confirm here that degradation of this transcript by RNase II is arrested by the terminator structure and show that Hfq protects the poly(A) tail from digestion by RNase II.

RNase II degraded the polyadenylated substrate in a two-step process. An initial rapid shortening from the poly(A) tail yielded a set of stalled intermediates (Fig. 3A, first three lanes) followed by a slower progressive shortening of the intermediates. These data suggest that the first step is processive, while the second step is distributive, i.e. RNase II dissociates from its substrate after the removal of each nucleotide. Carrying out the RNase II digestion with five times less enzyme confirms

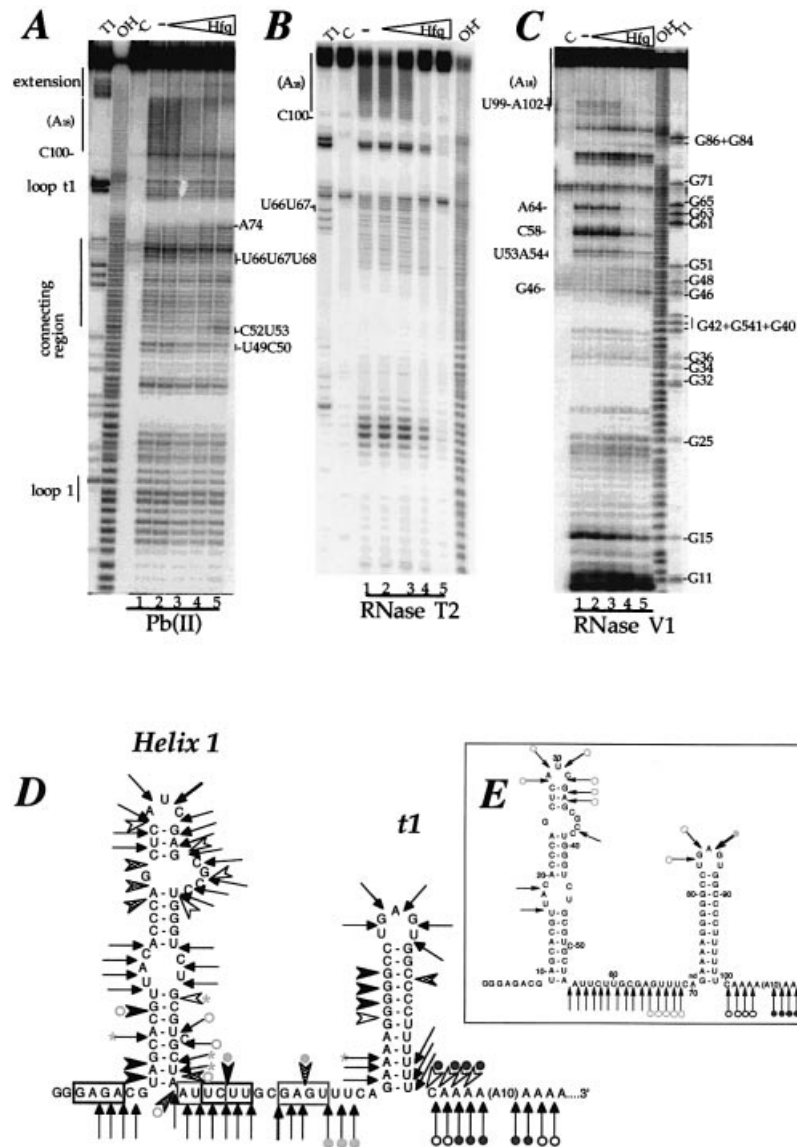


Figure 2. Enzymatic and chemical probing of polyadenylated $\Delta rpsO$ -(A)₁₈ mRNA, free and complexed to Hfq. (A) Chemical probing of $\Delta rpsO$ -(A)₁₈ with Pb(II). (B) Enzymatic probing with RNase T2. (C) Enzymatic probing with RNase V1. 5'-end-labelled polyadenylated $\Delta rpsO$ -(A)₁₈ mRNA with (A) or without a UGGUGGUGGAUCCCGGAUC 3'-extension [In (B) and (C) the extension is not shown in the secondary structure]. Pb probing was also performed on the RNA lacking the 3' extension. The extension did not alter the cleavage pattern (results not shown). RNase V1, free (lanes C, 1) or bound with increasing amounts of Hfq protein: 10 nmol/l (lanes 2), 100 nmol/l (lanes 3), 1 μ mol/l (lanes 4) and 10 μ mol/l (lanes 5). Samples were treated with RNase T2, RNase V1 or Pb(II), under conditions where statistically less than one cleavage per molecule takes place (lanes 1–5). Lanes T1 and OH⁻ correspond to the sequencing products generated by RNase T1 cleavages of the same RNA (G-specific cleavage) and an alkaline hydrolysis ladder under denaturing conditions, respectively. (D and E) Summary of the probing results of three independent experiments, represented on the secondary structure of polyadenylated $\Delta rpsO$ mRNA. (D) Enzymatic cleavages by RNase V1: strong (filled arrowheads), moderate (grey arrowheads) and low (open arrowheads). Pb²⁺ cleavages: strong (bold-tailed arrows), moderate (thin-tailed arrows) and low (dashed-tailed arrows). Enhancement of cleavage is represented with an asterisk. Potential base-pairings that may occur between GAGA6 and UCUU60, and AUUC58 and GAGU66, are boxed in black and grey, respectively. (E) Enzymatic cleavages by RNase T2: strong (bold-tailed arrows) and moderate (thin-tailed arrows). Positions that became protected in the complex are denoted with filled circles for strong protection and open circles for weaker protection. Dark grey and light grey symbols reflect modification of cleavage intensity observed at low and high Hfq concentrations, respectively. Higher Hfq concentrations are required in footprinting experiments than in gel retardation assays because of tRNA addition.

this two-step reaction (data not shown). The processive step of RNase II degradation generates intermediates with 3' tails of approximately eight A-residues 3' to the stable hairpin structure ending at C100 (Fig. 3A), in agreement with Coburn *et al.* (40). Then, RNase II generates stable intermediates distributively, with 3' ends close to the base of the

stem-loop structure. A comparison with a non-polyadenylated *rpsO* substrate used as a marker indicated that RNase II hydrolysis of the $\Delta rpsO$ -(A)₁₈ RNA stopped three A-residues downstream of the secondary structure under these conditions (data not shown). In the presence of 1 μ mol/l Hfq, 80% of the substrate was protected against RNase II degradation (Fig. 3A

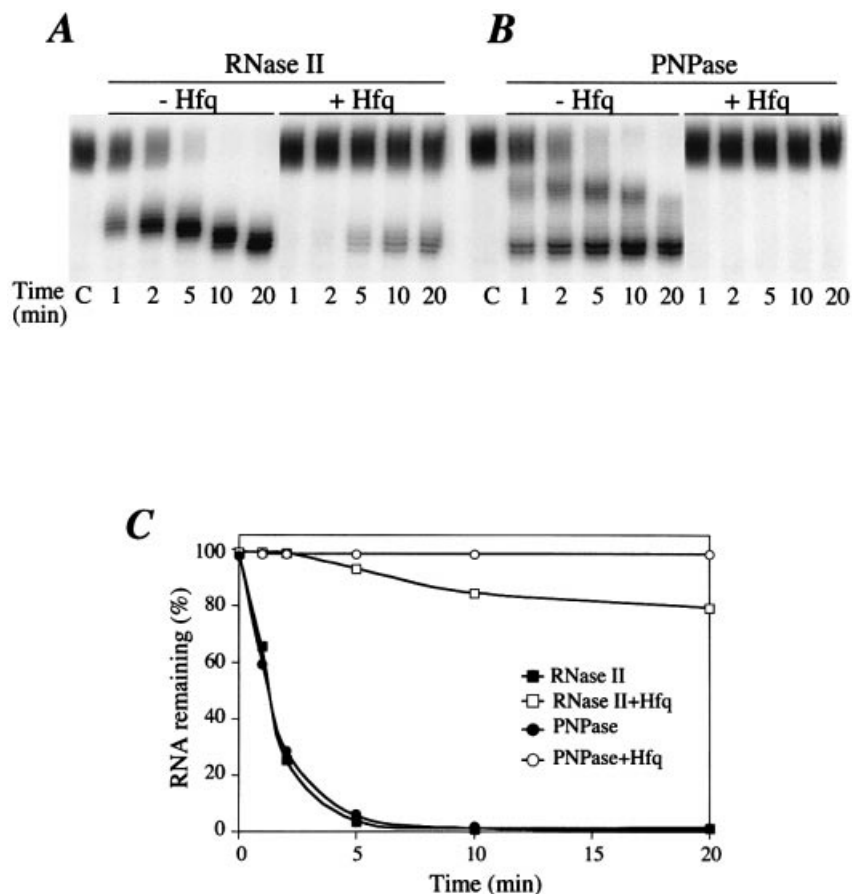


Figure 3. Hfq impedes the poly(A) shortening activity of PNPase and RNase II. Digestion of 5'-labelled polyadenylated $\Delta rpsO$ mRNA in the absence (-Hfq) and presence of 1 $\mu\text{mol/l}$ Hfq (+Hfq) was performed as described in Materials and methods. Aliquots were removed at 1, 2, 5, 10 and 20 min digestion. 'C' indicates the non-digested sample. (A) Digestion with 0.2 pmol RNase II and (B) with 2 pmol PNPase. We chose conditions in which each enzyme exhibits comparable exoribonuclease activity and in which the activity was limited. Lanes with sequence ladders and non-polyadenylated mRNA are not shown. (C) Quantification of experiments shown in (A) and (B).

and C). We have calculated that Hfq concentration ranges from 8 to 20 $\mu\text{mol/l}$, depending on physiological conditions (41). This suggests that Hfq binding protects poly(A) tailed RNA by preventing the access of RNase II to the 3' extremity of the RNA. Surprisingly, the few molecules that are still attacked by RNase II are shortened up to residues A4 and A5 of the oligo(A) tail, suggesting that processive degradation stops closer to the t1 hairpin. This is in agreement with the footprinting experiments, which suggest that Hfq binding weakens base-pairing at the bottom of the hairpin.

We have shown previously that PNPase degradation of polyadenylated *rpsO* transcript is arrested three to five adenine residues downstream of the transcription terminator site *in vivo* (42). *In vitro* assays, using the polyadenylated substrate described above, also revealed a two-step degradation process by PNPase. The first step is characterized by the appearance of an intermediate, seven residues shorter than the initial substrate. This intermediate was further degraded to the final stalling site of PNPase, one or two adenines downstream of C100 (Fig. 3B). The addition of purified Hfq dramatically impeded PNPase digestion of poly(A) tails (Fig. 3B and C). Hfq may either mask the 3' end of the RNA or prevent interactions between the enzyme and its RNA binding site.

Hfq binding decreases RNase E cleavage efficiency

Our data indicate that Hfq also binds to the 14-nucleotide-long sequence lying between the two hairpins of the polyadenylated $\Delta rpsO$ mRNA fragment, albeit with a weaker binding affinity than for the poly(A) sequence. In particular, the GCGAGUUU sequence, containing the M2 RNase E cleavage site (underlined) (1), is protected by Hfq from cleavage by RNase T2 and Pb(II). We performed *in vitro* processing experiments with a degradosome preparation containing RNase E to determine the effect of Hfq binding to this region. We omitted phosphate from the buffer to prevent exonucleolytic degradation of the RNA by PNPase present in the degradosome.

We first showed that most of the full-length RNA was efficiently cleaved using different concentrations of the degradosome, and concomitantly a shorter RNA fragment corresponding to the M2 RNase E cleavage site accumulated. Interestingly, the addition of increasing amounts of Hfq (10 nmol/l, 100 nmol/l, 1 $\mu\text{mol/l}$ and 10 $\mu\text{mol/l}$) progressively reduced the processing of the polyadenylated $\Delta rpsO$ mRNA by RNase E from 89 to 20% (Fig. 4). This was also accompanied by a lesser amount of the RNase E main cleavage product as Hfq concentration increased.

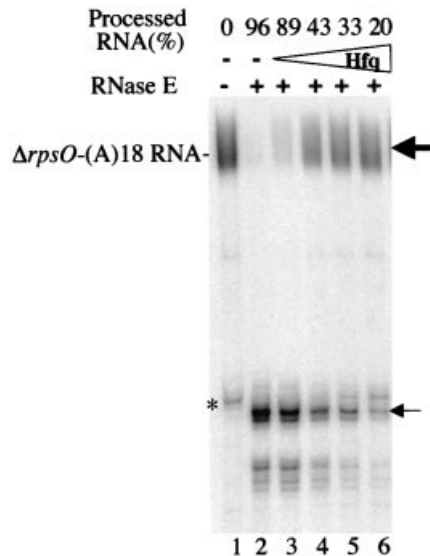


Figure 4. Hfq impedes the endonucleolytic activity of RNase E in the degradosome. 5'-end-labelled polyadenylated $\Delta rpsO$ -(A)₁₈ mRNA (lanes 1) was incubated with 50 ng of the degradosome preparation in the absence (lane 2) and in the presence of 10 nmol/l (lane 3), 100 nmol/l (lane 4), 1 μ mol/l (lane 5) and 10 μ mol/l (lane 6) Hfq. The positions of 5'-labelled RNA and of the cleavage product by RNase E are indicated by the bold and normal arrows, respectively. Positions were determined relative to sequence ladders obtained by alkaline hydrolysis and RNase digestion, and to location of non-polyadenylated mRNA (not shown). The asterisk indicates a contaminant RNA of $\Delta rpsO$ -(A)₁₈ RNA preparation, which is also cleaved by RNase E. The fraction of processed RNA is indicated at the top. This fraction was derived from the ratio of the amount of full-length labelled RNA divided by the total amount of radioactivity in each lane.

Furthermore, the non-adenylated $\Delta rpsO$ RNA was protected by Hfq binding against RNase E hydrolysis, suggesting that binding of Hfq to the poly(A) tail does not contribute to the inhibition of RNase E cleavage (results not shown). Altogether, these results suggest that Hfq binding to the connecting region inhibits RNase E cleavage by masking the GUUU sequence of the cleavage site. However, the Hfq binding effect was less pronounced on RNase E cleavage efficiency than on RNase II or PNPase hydrolysis. This may result from the fact that binding of Hfq to the GUUU sequence is weaker than for the poly(A) tail.

DISCUSSION

This report shows that Hfq forms stable complexes with *rpsO* RNAs and preferentially binds the poly(A) tail of polyadenylated mRNA. A second and weaker binding site is also detected in the single-stranded region just upstream of the transcription terminator hairpin. We further demonstrate that binding of these sites by the Hfq protein protects the polyadenylated *rpsO* RNA from the two 3' to 5' exoribonucleases, RNase II and PNPase, and from cleavage by RNase E.

Hfq binds tightly to the poly(A) tail of *rpsO* mRNA

Gel retardation assays demonstrated that Hfq exhibits a high affinity for polyadenylated RNA. The discrepancy with the lower K_d value previously reported (24) probably reflects variation in the proportion of active protein resulting from the purification method or from aggregation of the protein used in early experiments. The high affinity of the Hfq protein for the poly(A)-tailed $\Delta rpsO$ mRNA is of the same order of magnitude as that for poly(A)₁₁₅ (Fig. 1A). The K_d values of Hfq bound to poly(A)-tailed RNA and non-polyadenylated RNA reported here are 90 pM (15 pM if a Hfq hexamer binds the RNA) and 1.4 nM (0.23 nM for the hexamer), respectively. The K_d for poly(A)-tailed *rpsO* RNA is also of the same order of magnitude as the value reported by de Haseth and Uhlenbeck (43) for (Ap)₂₇ (6.25 pM for the hexamer) at a similar salt concentration. With RNA substrates containing significantly fewer adenosine residues, the equilibrium dissociation of both *Staphylococcus aureus* and *E. coli* Hfq proteins with AAAAAAG and A₇, respectively, was only 30.5 nM (44) (V. Arluison, unpublished data). These data suggest that a critical number of adenine residues is required for high binding affinity. This is in agreement with earlier fluorescence titration experiments indicating a saturation point of 14 A-residues per Hfq hexamer (43). On the other hand, the K_d value we found for non-polyadenylated *rpsO* mRNA (0.23 nM for hexameric Hfq) differs from the values measured for Spot42 [20 nM (28)] and DsrA [1.2 μ M (45)]. A comparison of known binding sites (Table 1) suggests Hfq does not recognize a strict RNA consensus. Our data are nevertheless consistent with earlier observations that Hfq binds single-stranded A-U-rich RNA regions (26), either adjacent to hairpin structures [Spot42 (28), *ompA* mRNA (12)] or surrounded by them [DsrA (45), OxyS (27), Spot42 (28)].

Table 1. Comparison of Hfq binding sites with mRNAs, viral RNA and small regulatory RNAs

GUUUC AAAAAAAAAAAAA	<i>ΔrpsO</i> mRNA	Present study
CGUAUUUUGGAUGUAACGAGG GGAUUUGG	<i>ompA</i> mRNA	(12)
AUUUUAG GUUUU	Spot42	(28)
AUAACUAAA	OxyS	(31)
ACCAAUACUAAAAAG AAUAAAUAUCACAAUUACUCUUACG	Q β RNA	(26)
AAUUUUU	DsrA	(45)
PuAU4-6Pu	Sm binding site	

These RNA hairpin structures probably help to constrain the Hfq binding site in a single-stranded conformation.

We propose that two Hfq hexamers bind independently to $\Delta rpsO$ RNA-(A)₁₈; the first strong binding occurs on the poly(A) moiety of the RNA, and the second weaker binding on the U-rich connecting region (Fig. 2). Moreover, the gel-shift assay performed with poly(A)₁₁₅ (Fig. 1A) and previous experiments (43) suggest that Hfq hexamers can sequentially bind every 14 residues and cover long poly(A)-sequence-like 'pearls on a string'. We cannot exclude, however, that Hfq hexamers exhibit several distinct binding sites for RNA. Two sites were indeed described for the *Pyrococcus abyssi* Sm protein (46), and the contribution of these sites differs depending on the RNA sequence. Mutational analysis of *E. coli* Hfq will be required to define the poly(A) binding site more precisely.

The high affinity of Hfq for poly(A) and A-U-rich regions, implies that this abundant protein [between 2500 and 35 000 Hfq molecules per cell (47,48)] can be strongly associated with RNA fragments during exponential growth. However, Hfq has been shown to be a co-factor of the transiently expressed sRNA (22,27,28). This binding would imply either that new Hfq molecules are synthesized or that Hfq is released from short-lived RNAs, or possibly that Hfq is transferred from storage RNA to the transiently expressed sRNAs under particular physiological conditions. It is indeed conceivable that the capacity of Hfq to promote RNA-RNA interactions could allow the protein to be rapidly transferred from a storage RNA, for example poly(A) tails, to a newly synthesized small regulatory RNA, despite the high affinity of the Hfq for RNA. This hypothesis is presently under investigation.

Hfq modulates *rpsO* mRNA degradation

The footprinting data suggest that Hfq destabilizes the bottom of both helices in the $\Delta rpsO$ mRNA. This correlates well with the data showing that RNase II continues the processive degradation of nucleotides to the bottom of the t1 hairpin of the *rpsO* transcript in the presence of Hfq. These data are consistent with the proposed role of Hfq as an RNA chaperone (31,49) and with the idea that Hfq facilitates base pairing between small RNAs and their target mRNAs (28,31). It has also been reported that Hfq affects the orientation of helices of the DsrA sRNA rather than its secondary structure (45). In the case of $\Delta rpsO$ mRNA, we propose that weakening of base pairing at the bottom of the transcription terminator may facilitate the addition of A-residues at the 3' end of the molecule by PAP I (24).

Our data also suggest that Hfq may play a role in the stabilization of RNAs. We showed here that at physiological concentrations, Hfq masks the M2 RNase E site, where RNase E initiates degradation of the *rpsO* transcript (1,33). Since RNase E and Hfq both recognize single-stranded A-U-rich regions of RNA, one can speculate that Hfq may bind the RNase E sites of other RNAs, therefore may modulate processing and degradation of the RNA (50). In contrast, Hfq may also cause degradation of *ompA* mRNA by competing with the binding of the ribosome to the A-U-rich translation initiation site of the *ompA* mRNA and as a result the mRNA is no longer protected from ribonucleases by translating ribosomes (12).

We proposed that Hfq also protects poly(A) tails from degradation by the exoribonucleases PNPase and RNase II. This may explain why poly(A) can be detected in the cell (51). However, there is an apparent paradox; on the one hand Hfq stimulates processive synthesis of poly(A) tails, which promote RNA degradation, and on the other hand Hfq protects these tails from PNPase and RNase II. This property raises the interesting possibility that Hfq prevents poly(A)-dependent degradation of full-length transcripts whose stability is mostly controlled by RNase E and favors poly(A)-dependent degradation of stably structured RNAs devoid of RNase E sites.

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