=========== REVIEW ===========

Bacterial Small Regulatory RNAs and Hfq Protein

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Abstract—Small regulatory RNA (sRNA) is a unique noncoding RNA involved in regulation of gene expression in both eukaryotic and bacterial cells. This short review discusses examples of positive and negative translation regulation by sRNAs in bacteria and participation of Hfq in these processes. The importance of structure investigation of nucleotide−protein and RNA−protein complexes for designing a model of Hfq interaction with both mRNA and sRNA simultaneously is demonstrated.

After the discovery of the fact that the main genetic information is encoded by chromosomal DNA (described in [1, 2]) and development of an approach for its sequencing by Sanger and colleagues [3], DNA sequences were determined for different organisms including humans − the Human Genome Project was started in 1990 and accomplished in 2003 [4, 5]. Genome deciphering showed that not all DNA encodes proteins or ribosomal and transfer RNAs. Even before, "noncoding" regions were found in bacterial DNA. It was proposed that they do not perform any important functions in cells and are rather ballast or "junk" DNA [6]. Later, it was shown that "junk" DNA is transcribed and encodes regulatory RNAs and mobile transgenomic elements − transposons [7]. Both of these play important roles in all living organisms. In the present review, a small portion of the "junk" DNA products will be considered, e.g. regulatory RNAs and their interactions with Hfq protein, a global regulator of gene expression in Gram-negative bacteria.

DISCOVERY OF REGULATORY RNAs

In bacteria, the first regulatory RNAs were found in non-chromosomal genetic elements, namely in the plasmid ColE1 (RNAI) [8] and in the transposon *Tn*10 [9].

They were referred to as small regulatory RNAs (sRNAs) or noncoding RNAs (ncRNAs). Later, in one of the most studied organisms, *Escherichia coli*, it was shown that in many cases, regulation of mRNA translation by small regulatory RNAs is mediated by Hfq protein.

The presence of regulatory RNAs in eukaryotes was demonstrated in 1993. The eukaryotic regulatory RNA (miRNA, microRNA) *lin-4* was discovered during study of the nematode *Caenorhabditis elegans*. This RNA was shown not to encode a protein, and it negatively regulated *lin-14* mRNA translation due to partial complementary pairing with seven repeats present in the 3′-untranslated region of *lin-14* mRNA [10]. Eukaryotic regulatory RNAs are now classified by length − long noncoding RNAs (lncRNAs), by action mechanism − small interfering RNAs (siRNAs), by cellular localization − small nucleolar and nuclear RNAs (snoRNAs and snRNAs) and outside the cell − exRNAs, by size − microRNAs (miRNAs), and by interaction mediators − piRNAs that act together with piwi proteins.

Regulatory RNAs in Archaea were predicted by bioinformatics methods only in 2002. In 2009, the first discovered archaeal regulatory RNA, Göl from *Methanosarcina mazei*, was found (reviewed in detail in [11] and [12]). It turned out that some sRNAs in Archaea contain short regions of open reading frames (ORFs) and are able to combine functions of messenger and regulatory RNAs [13].

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ROLE OF SMALL RNAs IN BACTERIA

Within the last 30 years, more than 100 small regulatory RNAs have been discovered and studied in bacteria. From 80 to 100 regulatory RNAs were found in *E. coli* [14, 15]. Their studies demonstrated that regulation of bacterial vital functions is not performed solely by proteins. Most interesting was the fact that in humans and animal bacterial pathogens, small regulatory RNAs participate in their virulence and drug resistance [16]. Now the most studied small regulatory RNAs are those of Gram-negative bacteria, where the RNA translation regulation system includes such components as RNase E, degradosomes, and Hfq protein. In Gram-positive bacteria, RNase E is absent and Hfq protein does not participate as much in mRNA translation regulation. It is possible that other, not yet studied mechanisms of regulation of mRNA translation exist in Gram-positive bacteria. We will review the interaction of sRNAs with mRNAs and Hfq protein in Gram-negative bacteria.

Small regulatory RNAs are synthesized in cells in response to such cellular stresses as glucose or iron starvation, oxidative stress, radiation, unfavorable temperature, etc. The effect of small RNAs on translational activity of mRNAs is provided by mechanisms described below. To regulate translation, small regulatory RNAs utilize their partial complementarity to mRNA regions, due to which RNA duplexes are formed that cannot be read by the bacterial translational apparatus. Hence, because of the coupling between transcription and translation of bacterial mRNAs, translation stop causes termination of mRNA transcription.

Information encoded by the mRNA nucleotide sequence to amino acid sequence of protein molecules in all living organisms is translated by a macromolecular ribonucleoprotein complex − the ribosome. In prokaryotic cells, chromosomal DNA is not separated from the cytoplasm by the nuclear membrane as it is in eukaryotes. For this reason, messenger RNA becomes available to ribosomes immediately after its transcription from DNA. Thus, in bacteria, transcription and translation occur simultaneously, in other words, they are coupled. One of the forms of small regulatory RNA-mediated negative regulation of mRNA translation occurs by means of uncoupling of transcription and translation.

Accumulating in a cell, sRNA can specifically inhibit translation of a messenger RNA by forming a duplex due to partial complementarity of nucleotide sequences. In most cases, small RNA is complementary to the ribosome-binding site (RBS) on mRNA. Thus, duplex formation between a small RNA and a messenger RNA shields a region of a messenger RNA RBS to which the ribosome normally binds. As a result, the ribosome does not bind to a messenger RNA. Inhibition of translation initiation occurs, leading to transcription termina-

tion by Rho-dependent or Rho-independent mechanisms, depending on mRNA type. Irreversibility of translation−transcription processes is achieved by the enzymatic decay of a messenger RNA. RNA decay is initiated by RNase III, which cleaves mRNA−sRNA duplexes, or by RNase E that cleaves single-stranded RNA regions that are not protected by ribosomes. Cleavage of the inhibited mRNA is completed by the degradosome.

Small regulatory RNAs can regulate transcription even if they are complementary to any coding sequence of a messenger RNA (not necessarily the RBS). In this case, translation inhibition at the elongation stage occurs because of formation of a duplex between sRNA and its target mRNA, which cannot be translated by the ribosome. Then, uncoupling of transcription−translation occurs, resulting in messenger RNA degradation by the mechanism described in the previous paragraph.

Positive translation regulation by means of small regulatory RNAs can be direct or mediated by RNA mimicry. Direct positive regulation is realized by providing the ribosomes access to the start codon or by modifying the process of mRNA editing that increases its stability. Indirect positive regulation occurs when sRNA mimics mRNA, which dilutes the regulated RNA pool; thus, the amount of cleaved RNA decreases [17].

Examples of negative translation regulation. Regulation of *rpoS* messenger RNA translation is now most extensively studied. The *rpoS* gene encodes an alternative σ-factor of RNA-polymerase. It is induced under cellular stresses (starvation, acidic or basic pH, osmotic shock, stationary phase) and triggers expression of genes encoding stress-response proteins. When cellular stresses are absent, translation of *rpoS* mRNA is auto-inhibited due to an internal hairpin structure at the 5′-untranslated region near the start codon.

Negative translation regulation of the RpoS σ-factor is mediated by sRNA OxyS. This was the first regulatory RNA discovered, by Gisela Storz in 1985 during a study of an effect of hydrogen peroxide on processes in cells. Storz found that after hydrogen peroxide treatment, a 109-nt OxyS RNA is accumulated in cells [18]. Later, this regulatory RNA was shown to accumulate in cells under oxidative stress. It inhibits translation of messenger RNAs *rpoS* and *fhlA*, and some others, by blocking access to the ribosomal binding site [15, 19].

Since then, tens of new regulatory RNAs have been discovered, and mechanisms of their action have been investigated. One of these RNAs is RyhB RNA. The mechanism of its action is interesting because of the involvement of the additional specific iron metabolism protein Fur. Fur is a repressor of translation of six mRNAs that encode iron-binding proteins in *E. coli* [20]. Except for inhibition of translation of these mRNAs, they are degraded by RNase E upon binding of Hfq protein and sRNA RyhB [21].

Synthesis of *ptsG* mRNA encoding a membrane subunit of the glucose transporter of the phosphoenolpyruvate phosphotransferase system (EIICB^{Glc}) is regulated similarly [22]. Under sugar-phosphate stress, when glucose 6-phosphate and its derivatives are accumulated in cells, sRNA SgrS is induced with participation of SgrR protein. SgrS RNA, in turn, forms a complex with RNase E, Hfq protein, and a partially complementary *ptsG* mRNA region; this leads to inhibition of initiation the mRNA translation. Then, the mRNA is degraded by RNase E [23-25].

An interesting mechanism of negative translation regulation of polycistronic templates by Hfq and sRNA *chiPQ* exists in *E. coli*. It is known that in bacterial cells transcription and translation of mRNA are coupled and proceed simultaneously. During complementary pairing of sRNA ChiX with its target mRNA *chiPQ*, the ribosomal binding site on the mRNA is hidden, which triggers termination of the mRNA transcription by providing access for transcription terminator Rho. Therefore, the next gene is not transcribed, and the mRNA–sRNA complex is degraded by RNase E [26].

Another mechanism exists for regulation of translation of polycistronic templates with involvement of Hfq and sRNA Spot42 in sugar metabolism. This sRNA is encoded by the *spf* gene, which is inhibited by the complex of receptor protein SRP with cyclic AMP (cAMP). The *galETKM* operon has two overlapping promoters, P1 and P2. The cAMP–CRP complex stimulates transcription from the first promoter and inhibits transcription from the second promoter P2. Promoter 1 drives expression of UDP-galactose epimerase (*galE* gene product) and galactokinase (*galK* gene product). While galactokinase is only required under glucose shortage to cleave galactose, UDP-galactose epimerase plays a role not only under glucose shortage, but it also participates in other cell growth conditions in UDP-glucose synthesis, which is a building block for the cell wall and the capsule.

Spot42 sRNA is partially complementary to the translation initiation region of the *galK* gene, and upon binding, it inhibits *galK* translation by hiding its ribosomal binding site. At the same time, it does not inhibit translation of *galE*, i.e. causes discoordination of expression of the *gal* operon genes [27, 28]. Beside the *gal* operon, Spot42 regulates at least 14 other operons encoding proteins required for assimilation of alternative carbon sources. Some of them are also back regulated by the cAMP–CRP complex [29].

Examples of positive translation regulation. Three sRNAs (DsrA, ArcZ, and RprA) can bind to the 5′-untranslated region of RpoS mRNA, which causes its unwinding and facilitates ribosome binding [28, 30]. The *dsrA* promoter is activated at lower temperatures, below 30°C [31, 32] it activates expression of σ factor RpoS, which in turn activates expression of the *otsA* and *otsB* genes. These genes regulate levels of the disaccharide tre-

halose (mycose), which protects cell at low (about 4°C) temperatures [33]. Furthermore, sRNA DsrA negatively regulates expression of the *hns* gene encoding global transcriptional regulator HNS. It is known that HNS negatively regulates genes responsible for protection of cells against osmotic stress. Hence, DsrA removes negative control by HNS and triggers expression of not only cold shock proteins, but also proteins that protect against osmotic shock. By the example of sRNA DsrA, it was shown that Hfq accelerates formation of DsrA-RpoS by a factor of 30-50 [34] and protects RpoS against degradation [35]. A second sRNA that affects translation levels of *rpoS* mRNA, RprA, was discovered as a suppressor in cells that lack sRNA DsrA. Nevertheless, a signal that activates sRNA RprA is not yet identified [28]. Translation inhibition by a third sRNA (ArcZ) is mediated by the ArcA/ArcB system and occurs under oxygen deficiency in anaerobic conditions, while negative control is removed in the presence of oxygen in the medium [36].

ROLE OF Hfq PROTEIN IN FUNCTIONING OF SMALL RNAs

Translation regulation of bacterial mRNAs by antisense RNAs is well documented for plasmids, phages, and transposons. In these cases, a regulatory RNA (sRNA) and a target RNA are encoded in the same chromosomal locus but in opposite directions. Hence, regulatory RNA is fully complementary to an mRNA region. Here, Hfq protein is not required for functioning sRNA [37]. Hfq is necessary for other types of regulatory RNAs that show imperfect complementarity to the target RNAs, the so-called *trans*-encoded RNAs. These RNAs are expressed "*in trans*", i.e. genes encoding these RNAs are located far from genes encoding their target messenger RNAs. It was shown that Hfq protein accelerates duplex formation between partially complementary RNAs [38-42]. Acceleration of duplex formation can occur both due to local increase in RNA concentration in the vicinity of Hfq protein during their interaction, and due to the ability of Hfq to unwind double-stranded RNAs [28, 43, 44]. Furthermore, it is also known that Hfq protects some short-lived sRNAs from degradation by RNase E (e.g. DsrA, Spot 42, and RyhB) [41, 45, 46].

In addition to regulation of mRNA translation by means of regulatory RNAs, Hfq can directly change mRNA translation levels by affecting mRNA half-life. Destabilization of mRNA in the presence of Hfq is best studied for mRNA *ompA*. This mRNA encodes OmpA, a major protein of the outer membrane of *E. coli*. Stability of this mRNA is inversely proportional to the cell growth rate. The half-life of mRNA is determined by the 5′-untranslated region, which contains a stabilizing hairpin structure and a site for RNase E cleavage. In fast growing cells, ribosomes upon binding to the 5′-untranslated

region protect it against degradation by RNase E. In slow growing cells (in the stationary phase), Hfq protein accumulates, which competes with ribosomes for the 5'-untranslated region of mRNA, impedes ribosome binding, and this results in mRNA degradation due to uncovering of the RNase E binding site [46-48].

Hfq has three RNA-binding sites that upon polymerization form two main binding regions — a U-rich RNA-binding site on the proximal side of a toroid and a region for interaction with poly(A) stretches of RNAs on the distal side. Several structures of Hfq complexes with short RNA-oligomers (PDBID 4HT8, 4HT9, 3QSU, 3RER, 3AHU, 3HSB, 3GIB, 1KQ2) have been determined, and a low resolution structure of Hfq in complex with sRNA RydC form *Salmonella* sp. was obtained (PDB ID 4V2S) [49].

Structures of complexes of Hfq with oligo(U) RNA fragments revealed details of interaction of U-rich RNAs with the distal surface of the protein near the central pore of a hexamer [50, 51]. These are structures of Hfq from *Staphylococcus aureus* in complex with AU_5G RNA [52], Hfq from *Salmonella typhimurium* in complex with U_6 RNA [53], and Hfq from *E. coli* in complex with AU_6A RNA [54].

A uracil-binding site is formed by amino acid residues of two adjacent subunits of the hexamer. Upon binding to the protein, uracil nucleobase stacks to a benzene ring of Phe42, while the O2 and O4 atoms of uracil form hydrogen bonds with atoms of side chains of Gln8 and Gln41, correspondingly [53]. It is worth mentioning that there are certain differences between the three structures of Hfq complexes with U-rich RNAs. In the complex of Hfq from *S. typhimurium*, the asymmetric unit contains one monomer of the protein and one uridine with symmetrical phosphate residues. Moreover, free oxygens of the phosphates do not form bonds with the protein, being directed toward the central pore [53]. In the structure of the complex Hfq *S. aureus* with AU_5G RNA, free oxygens of a phosphate group are faced away from the central pore and form hydrogen bonds with the NH_2-group of His56 [52].

The latest structure of the complex of Hfq with U-rich RNAs [54] demonstrated that RNA binding to the proximal side of a toroid might be labile. A region of DsrA RNA containing the AU_6A sequence was utilized for crystallization. It turned out that in the obtained structure not all nucleotides are localized in the uridine-binding pocket: two of six uridines (U30 and U33) do not contact the protein and face away from the protein surface. One uridine (U29) was bound close to but not exactly in the pocket where it should have formed stack with Phe42. The sugar-phosphate backbone of RNA is directed either toward the central cavity (as in Hfq complexed with the U_6 RNA) and away from the central cavity (as in Hfq complexed with the AU_5G RNA).

The structure of Hfq from *E. coli* with a 15-nt oligo(A) RNA was first determined in 2009 [55]. It showed that oligo(A) binds to the distal surface of the protein by means of a repeated tripartite motif referred to as the A-R-E RNA binding motif. Initially, it was proposed that the A-site corresponds to a specific adenine-binding pocket, the R-site corresponds to a purine-binding pocket, while the E-site is a non-discriminating nucleotide-binding site, since the nucleobase placed in this site does not interact with atoms of the protein [55]. However, later it was demonstrated that, in fact, the adenosine specificity site is the R-site [54, 56]. At the same time, another purine, guanine, does not bind to Hfq either in the R-site or in the A-site; but cytidine can bind to the "purine specificity" R-site [53, 57].

The distal A-binding site of *E. coli* Hfq is situated between adjacent subunits of the Hfq toroid. Upon RNA binding to the protein, hydrogen bonds are formed between Gln33 and the N7 and N6 atoms of the adenine base, the Gln53 residue and N1 atom of the adenine base, and the Lys31 residue and phosphate group of the adenosine. In addition, a hydrophobic contact arises between Leu32 and the adenine base [55].

The binding of RNA to the protein R-site is realized through stacking interactions between Tyr25 and the adenine base and through hydrophobic interactions of the latter with Leu26, Ile30, and Leu32. Change of Tyr25 to alanine causes 100-fold decrease in affinity between RNA and Hfq [58]. Hydrophobic interactions are supplemented with formation of hydrogen bonds between the side chain of Gln52 and the N6 atom of the adenine base, between Thr61 and N1 of the adenine base, and between Asn28 and the N3 and Nδ atoms of the adenine base [55].

In Gram-negative bacteria, binding of A-rich oligo RNAs occurs almost identically. Simultaneous binding of AU_6A and A_7 in a complex with the *E. coli* protein revealed that formation of the triple complex does not affect the position of RNA or the protein structure [59].

However, nothing was known about structure of RNA/Hfq complexes from Gram-positive bacteria. In Gram-positive bacteria, Hfq does not play such an important role, e.g. *hfq* gene knockout in *S. aureus* and *Listeria monocytogenes* does not change the phenotype of these bacteria, partially because of the absence of RNase E [37]. The first structure of Hfq from the Gram-positive bacterium *Bacillus subtilis* in complex with $(AG)_3A$ RNA was determined in 2011 [60]. It turned out that binding of A-rich RNAs to the surface of Hfq in Gram-positive bacteria differs from that in Gram-negative bacteria. *Bacillus subtilis* Hfq does not bind AU_5G RNA. It has less affinity to A_{18} RNA as compared to Hfq proteins from Gram-negative bacteria, but it specifically binds AG-repeat-containing RNA. Instead of three binding pockets (A-R-E), only two are present, which were referred to as the A-L motif. The adenine base binds to the protein surface at the A-pocket, which is analogous to the R-pocket in Gram-negative bacteria. Binding occurs by means of

stacking interactions of adenine with two phenylalanines of the protein, Phe24 and Phe29. Binding is further stabilized by hydrogen bonds between adenine and the Ser60 and Thr61 protein residues. The guanine of the $(AG)_3A$ RNA in complex with Hfq forms hydrogen bonds with Arg32, which is a strictly conserved amino acid residue among Hfq proteins in Gram-positive bacteria [60].

SPECIFICATION OF THE INTERACTION MODEL OF Hfq PROTEIN WITH MESSENGER AND REGULATORY RNAs

A complete model of Hfq–RNA interaction was missing for a long time due to the impossibility to obtain stable complexes of the protein with full length regulatory and messenger RNAs. To build models of interaction of Hfq protein with regulatory RNAs [61-63], data from the structures of Hfq complexed with short RNA fragments or with single nucleotides was used. It turned out that since Hfq protein binds single-stranded RNAs, it is capable of binding single nucleotides in the RNA-binding sites [64]. Complexes of the protein with UTP, CTP, and ATP that were obtained revealed structural details of the interaction of uridine in the Hfq protein lateral site whose existence was shown biochemically [65], and demonstrating the ability of Hfq to bind cytidines in both general RNA-interaction sites (Fig. 1). It is worth mentioning that, despite all efforts, complexes of the protein with guanine were not obtained. This is in agreement with biochemical data showing low affinity of Hfq to guanine-containing RNA sequences [64].

Many regulatory RNAs that interact with Hfq have polyuridylic sequences at both the 3′- and 5′-ends. It is assumed that Hfq protein first recognizes a U-rich sequence at the 3′-end of sRNA via the proximal site [66], then the 5′-end of sRNA binds to the repeated lateral binding sites of the protein [62] (Fig. 2). The uracils contacting the lateral binding site may be responsible for mRNA interaction [67]. The outcome of our work provided the initial model. We suggest that not less than four-five successive uridines should be present at the sRNA 3′-end. They could be partially replaced by cytosines. A sequence containing uridines in every 4th or 5th position should be present at the 5′-end for interaction with the Hfq lateral sites [64]. The data obtained were used later to build a model of interaction of Hfq with *rpoS* sRNA [63], and then were confirmed by the structure of the *E. coli* Hfq in complex with RydC sRNA from *Salmonella* sp. [49] (Fig. 3).

An important role of small regulatory RNAs in regulation of biosynthesis in bacterial cells is now beyond doubt. An interesting feature of these processes is the participation of the bacterial protein Hfq that acts as an RNA chaperone and promotes interaction of sRNAs with mRNA targets. The unique feature of this hexameric protein is the presence of three RNA-interacting sites on its surface: one uridine-specific site, a second – adenine-specific site, and an additional site that is required for realization of interaction of two RNA molecules. The regulation of protein synthesis with participation of sRNAs and Hfq protein in different bacteria is extensively studied, and we still expect many interesting discoveries.
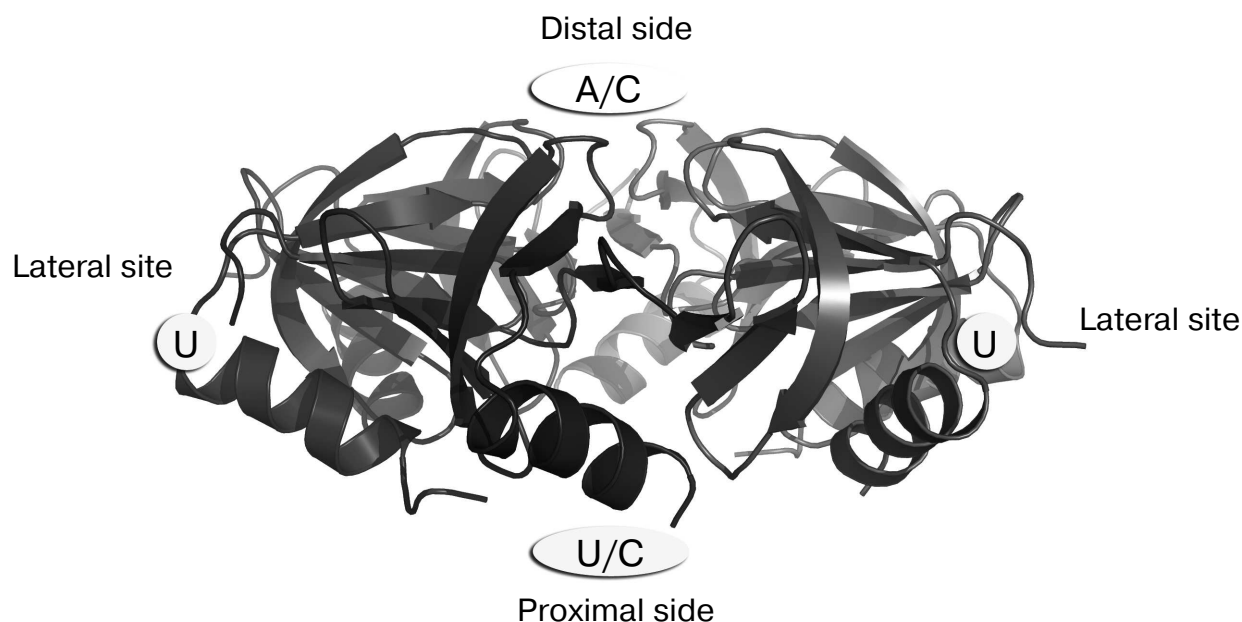

Fig. 1. Nucleotide specificity and arrangement of RNA-binding sites on the surface of Hfq hexamers.
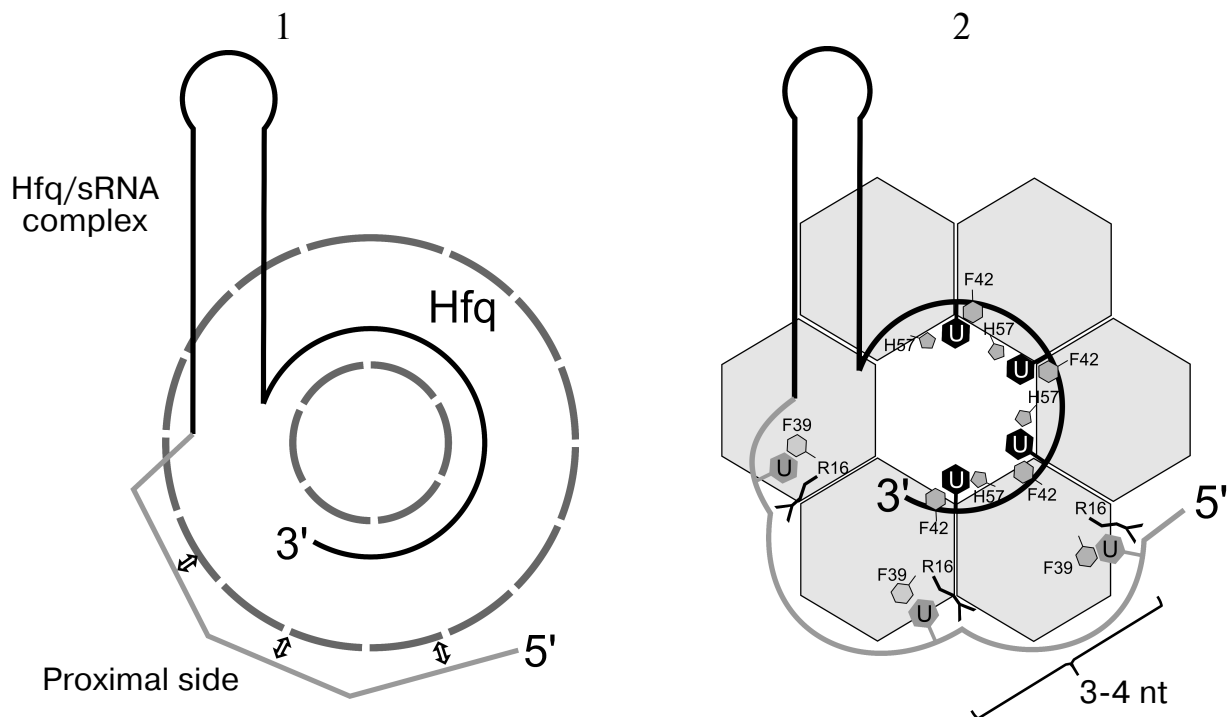
Fig. 2. Proposed model for arrangement of small regulatory RNAs on the Hfq protein surface. 1) Initial model based on mutagenesis data [62]. 2) Model for sRNA binding specified based on our structural data regarding UTP position on the Hfq surface. For the protein binding, the sRNA should have at the 3′-end four or five successive uridines, which could be partially replaced by cytidines. At the 5′-end, uridine should be present in every 3-4 nt.
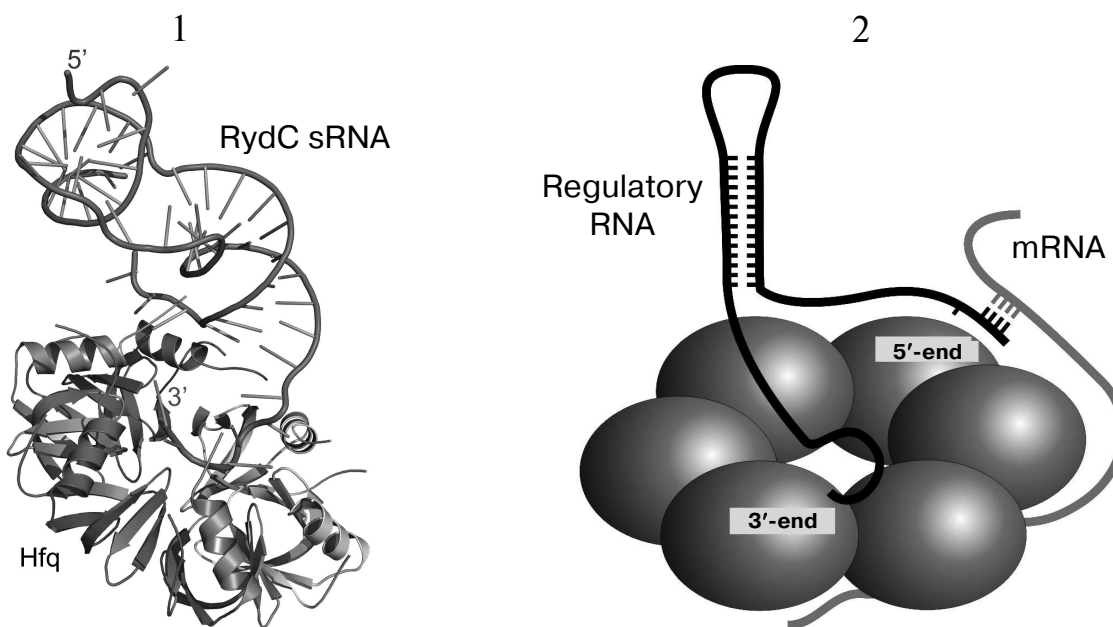

Fig. 3. 1) Structure of RydC sRNA–Hfq complex (HDB ID 4V2S). The Hfq hexamer is shown as a cartoon model, RydC sRNA is shown as a ribbon with indicated base directions (sticks). The 5′- and 3′-ends of sRNA are denoted. The 3′-end of RydC sRNA forms a network of contacts with amino acid residues of the proximal protein surface at the central part of the hexamer. Conserved uridines U23/24 and U46/47 of the sRNA interact with the lateral protein region. The 5′-end of RydC RNA forms additional contacts with the lateral part of the hexamer of the symmetrically bound Hfq protein molecule (not shown). 2) Proposed model for interaction of RydC sRNA and *cfa* mRNA from *Salmonella* sp. on the Hfq protein surface (a simplified scheme from [49]). The 3′-end of RydC sRNA (black) interacts with the protein central pore on the Hfq proximal surface. The *cfa* mRNA (gray) is associated with the distal Hfq surface and forms a duplex with the 5′-end of RydC (concurrently bound to Hfq) at the lateral protein surface. It has been suggested that the *C*-terminal part of Hfq directed towards a contact between the two RNAs and stabilizes their interaction.

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