Review

New insights into small RNA-dependent translational regulation in prokaryotes

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Bacterial small RNAs (sRNAs) typically repress translation of target mRNAs by pairing directly to the ribosome-binding site (RBS) and competing with initiating ribosomes, an event that is often followed by rapid mRNA decay. In recent years, however, many examples of translation-repressing sRNAs pairing outside the RBS have been described. In this review, we focus on newly characterized mechanisms that explain how a sRNA can modulate translation by binding outside of the RBS and discuss new insights into the events following translation repression. These new mechanisms broaden current perspectives of sRNA pairing sites on mRNA targets and demonstrate how the interplay between sRNAs, mRNA structures, and protein partners can contribute to post-transcriptional regulation.

Small RNAs in bacteria

When they were first characterized in 1984, bacterial sRNAs were the first example of a *trans*-acting regulator controlling translation of specific mRNAs through an antisense mechanism [1]. Since this seminal discovery, sRNA-based regulation has been shown to play major roles in a wide range of organisms, from bacteria to humans. In bacteria such as *Escherichia coli*, where more than 80 sRNAs have been identified [2], sRNAs have been shown to help cells adjust to environmental pressures by modulating the expression of key proteins. Bacterial sRNAs usually base pair with target mRNAs in the vicinity of the RBS to repress their translation and stimulate their rapid decay. By contrast, several examples of sRNAs activating the translation of mRNAs have also been characterized [3–6]. These sRNAs also pair in the 5′-untranslated region (UTR) of their target mRNAs to disrupt inhibitory secondary structures.

In recent years, a large number of studies have uncovered many new putative targets for sRNAs [7–10]. Most of these mRNAs are thought to be regulated by the typical RBS pairing mechanism described above. However, it is now becoming obvious that some sRNAs regulate their targets by alternative mechanisms that involve pairing outside the RBS, therefore excluding a mechanism that relies on direct competition with initiating ribosomes.

It is interesting to note that, in metazoans and plants, a large class of small non-coding RNAs termed miRNAs has also emerged as key players in post-transcriptional gene regulation. Similar to bacterial sRNAs, miRNAs mediate translation repression and mRNA decay. However, unlike their prokaryotic counterparts, they do so by pairing mainly in the 3′-UTR of their target mRNAs. The mechanism of action of miRNAs has been described in recent reviews [11–13].

In this review, we focus mainly on these new mechanisms of action of bacterial sRNAs, with a special emphasis on the recent discoveries that have uncovered new ways by which sRNAs repress translation. We also discuss new insights into the events that follow translation repression. Aside from a few exceptions, this review is limited to work performed on the enterobacteria *E. coli* and *Salmonella*.

Canonical model for sRNA-mediated gene regulation

Translation initiation in bacteria

During protein synthesis, translation initiation is the most rate-limiting and highly regulated step [14]. The canonical model for prokaryotic translation initiation involves mRNA recognition by the 30S subunit of the ribosome, which is mediated by RNA–RNA base-pairing interactions. The 3′-terminal sequence of the 16S rRNA AUCACCUCCUUA (termed antiSD) base-pairs with the purine-rich Shine-Dalgarno (SD) sequence of mRNA [15]. The antiSD–SD base-pairing directs the initiation codon to the P site of the 30S ribosomal subunit. Following the arrival of initiator tRNA, the so-called 30S initiation complex is then stabilized by codon–anticodon interactions. Because formation of the initiation complex is highly dependent on RNA–RNA interactions between mRNA, 16S rRNA, and initiator tRNA, any RNA structure that prevents these interactions will negatively affect the translation initiation rate [16]. Accordingly, bacteria have developed regulatory mechanisms, such as RNA thermosensors [17], riboswitches [18,19], and sRNAs [1], that depend on the occlusion of RNA sequences critical for translation initiation.

Translation repression by sRNAs

Most sRNAs that have been characterized so far pair directly to the SD sequence and/or the initiation codon

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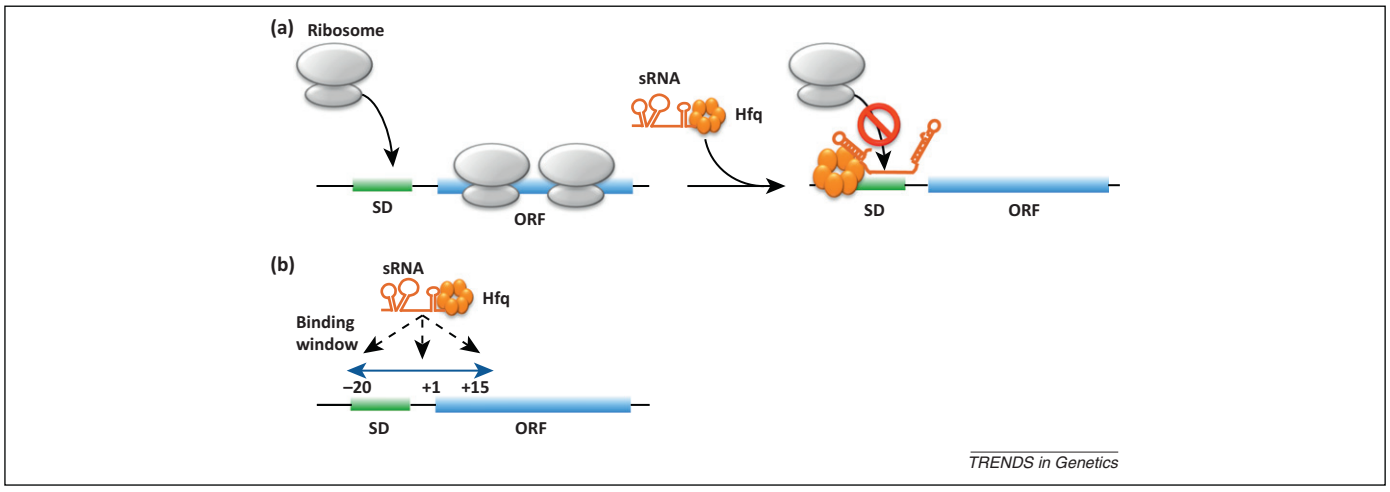

Figure 1. Canonical repression of translation by small RNAs (sRNAs). **(a)** The sRNA, with the help of host factor Qβ (Hfq), blocks translation initiation by directly pairing with the Shine-Dalgarno (SD) sequence in the 5′-untranslated region (UTR) of the target mRNA. **(b)** Binding window of a sRNA blocking translation initiation. The sRNA can pair with a target mRNA anywhere between nucleotides –20 and +15, relative to the first codon. The blue double arrow represents the boundaries of the 30S subunit-binding region. Abbreviation: ORF, open reading frame.

of their target mRNA to repress translation (Figure 1a). This pairing shields translation initiation signals and prevents the binding of the initiating 30S ribosomal subunit. Recently, a 'five codon window' in which sRNAs can efficiently inhibit translation initiation has been identified, meaning that pairing in the upstream part of the open reading frame (ORF) can efficiently inhibit the 30S ribosomal subunit binding to mRNA [20]. This finding is consistent with the previously made observations that the physical boundaries of the RBS extend from nucleotides –20 to +15 relative to the first nucleotide of the start codon (Figure 1b) [21,22]. Although the possibility has not been addressed specifically, it is believed that a sRNA bound up to 20 nucleotides upstream of the initiation codon could also repress translation initiation.

sRNA-induced mRNA degradation

In most cases, translational repression of a target mRNA by a sRNA results in active and rapid mRNA degradation [23–26], a process that is reminiscent of what is observed in eukaryotic cells for certain miRNA-mediated gene repression [27]. In *E. coli*, this degradation is achieved through the recruitment of the single strand-specific endoribonuclease E (RNase E). An additional player involved is host factor Qβ (Hfq) (Box 1), which is an RNA chaperone that has been described to stabilize sRNAs *in vivo* [28,29] and to facilitate pairing to mRNA targets *in vitro* [29–31]. Thus, it is generally thought that Hfq is essential for the activity of many sRNAs. Importantly, it has been shown that Hfq is able to interact with the unstructured C-terminal region of RNase E, linking sRNA-mediated gene regulation to mRNA degradation [25,26]. Importantly, the C-terminal region of RNase E is also used as a scaffold for the assembly of the RNA degradosome, a machinery dedicated to mRNA degradation in *E. coli* [32]. RNase E is thought to perform the rate-limiting initial cleavage that is followed by rapid and complete mRNA degradation. Even though translation repression and mRNA decay are often observed following sRNA expression, the former is sufficient to achieve gene silencing. Indeed, it was shown a few years ago that sRNAs still repress the translation of target mRNA in the absence of RNase E activity [33]. It is believed that mRNA decay is achieved, following translation silencing, to eliminate translationally inactive mRNA and render repression irreversible.

The fact that many target mRNAs are rapidly degraded following sRNA expression has been extensively used as a tool to identify new sRNA targets. The method consists of monitoring the levels of cellular mRNAs shortly after induction (pulse-expression) of the regulatory sRNA using genomic methods, such as microarray analysis and RNA sequencing [7,8,10,34,35]. Because they are observed only a few minutes after sRNA expression (generally <10 min), these changes in mRNA levels are believed to be the result of the direct action of the sRNA. Interestingly, this type of approach also permits the identification of positive mRNA targets, whose activated translation is often associated with increased mRNA stability and levels [3,6].

Box 1. RNA chaperone Hfq

The Hfq protein was initially characterized as a host factor necessary for the replication of the bacteriophage Qβ [63], thus its name Hfq (host factor Qβ). Hfq forms a hexameric ring comprising six identical units of 11.2 kDa. Hfq is a member of the Sm protein family [64], members of which are also found in eukaryotes and Archaea, where they play key roles in mRNA splicing and decay [65].

In prokaryotes, Hfq has been shown to play crucial roles in the action of a large class of bacterial sRNAs [66]. First, Hfq was shown to stabilize sRNAs presumably by protecting them against the action of RNases [28,29]. Hfq is also thought to act as an RNA chaperone that facilitates sRNA–mRNA interactions [29–31,67]. Additionally, Hfq has been directly implicated in translation repression of an mRNA by competing with initiating ribosomes when recruited to the RBS region by a sRNA [48]. Hfq is also implicated in sRNA-induced mRNA degradation. Because it interacts with both the sRNA and RNase E, Hfq is believed to recruit the degradation machinery to the targeted mRNA [23,25,26].

Independently of its role in sRNA-mediated gene regulation, Hfq is also involved in polyadenylation-dependent mRNA decay [68–70], Rho-dependent transcription termination [71], and, finally, transposition [72]. Lastly, Hfq has been shown to repress translation initiation of its own mRNA [73].

Alternate models of translation repression

sRNAs competing with a ribosome standby site

The first example of translation repression by a non-canonical mechanism was described as occurring through the action of a *cis*-acting antisense RNA, which does not rely on Hfq for activity [36]. In this study, the authors showed that the antisense RNA IstR-1 represses the translation of the *tisB* mRNA, which encodes a protein involved in a toxin–antitoxin system, by binding to a ribosome standby site located approximately 100 nucleotides upstream of the RBS (Figure 2a). Ribosome standby sites are single-stranded regions located in the 5′-UTR of mRNAs in which the RBS is sequestered by secondary structures [37–39]. In such cases, the 30S ribosomal subunit can bind to a standby site, waiting to relocate to the RBS region and initiate translation during a transient opening of the inhibitory structure. Although such a mechanism of gene regulation could potentially be widespread among bacteria, no other cases of translational repression through occlusion of a standby site by a sRNA have been characterized so far. It is an appealing hypothesis to explain the high translation rate of those mRNAs whose RBS region is predicted to be sequestered in secondary structures. However, the existence of ribosome standby sites is mainly based on indirect evidence from studies in *E. coli*, and direct observation of the 30S ribosomal subunit on a standby site is still lacking.

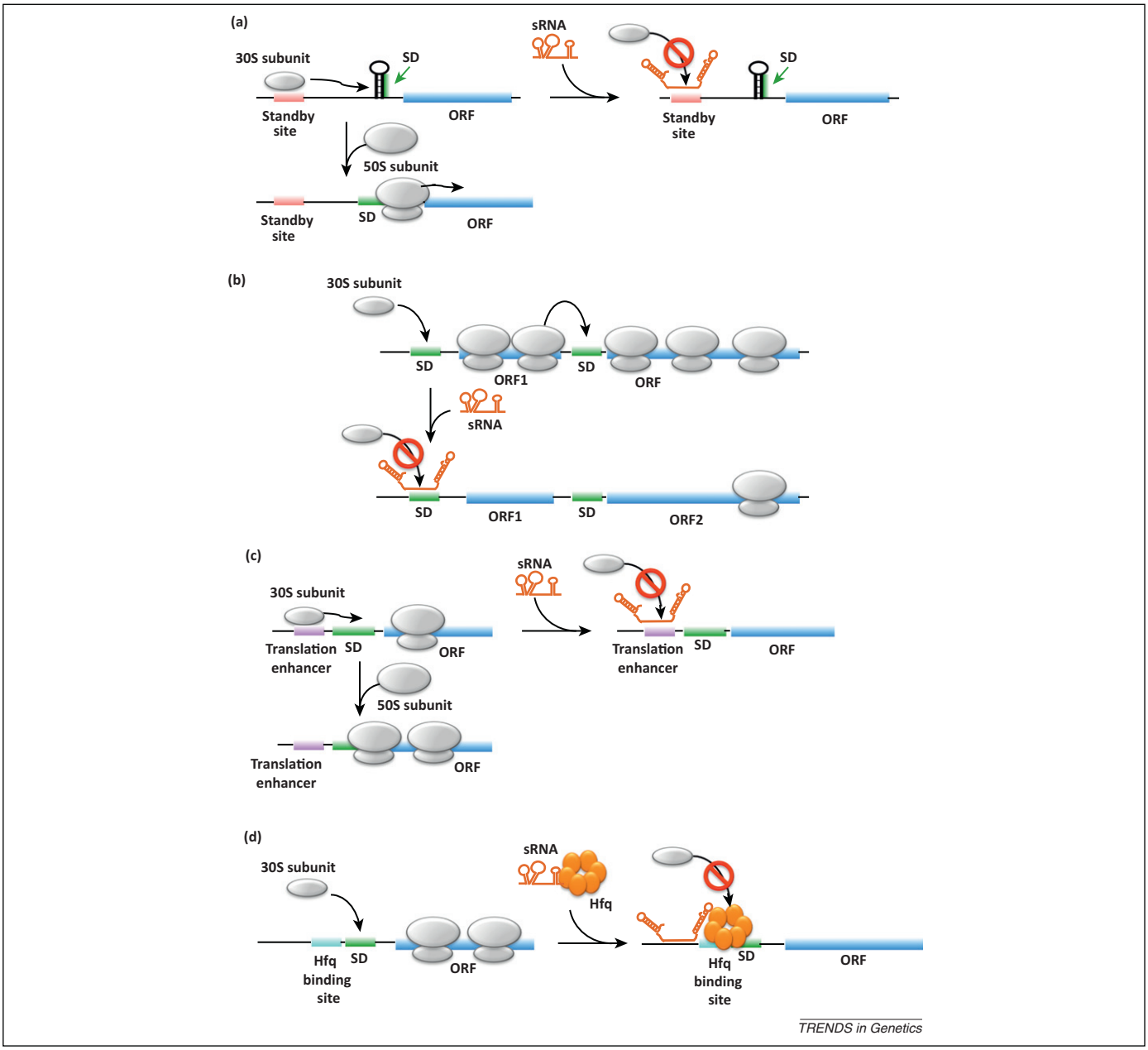

Figure 2. Non-canonical repression of translation by small RNAS (sRNAs). **(a)** The presence of secondary structures in the ribosome-binding site (RBS) region of target mRNAs prevents the binding of the 30S subunit to the Shine-Dalgarno (SD) sequence. The 30S subunit binds to the upstream standby site until the inhibitory RNA structure is resolved, which then leads to translation initiation. In the presence of the sRNA, which blocks access to the standby site, the binding of the 30S subunit to either the standby site or the SD is prohibited and translation is repressed. **(b)** In the case of translation coupling of a polycistronic mRNA, the sRNA pairing with the SD of the leader open reading frame (ORF; upstream) negatively affects the translation of the second ORF. **(c)** The sRNA pairing with a translation enhancer element (red), such as a C/A-rich region in the 5′-UTR of an mRNA, represses the translation initiation by restricting the access of ribosomes. **(d)** The inhibition of translation is caused by the sRNA-dependent recruitment of the RNA chaperone host factor Qβ (Hfq) to an A/U-rich site located close to the SD.

Targeting of a leader ORF

In prokaryotes, translation coupling between adjacent ORFs has been known for many years as an efficient strategy for cells to maintain equimolar quantities of the various proteins translated from a polycistron [40,41]. This mechanism occurs when the stop codon of the first cistron is in close proximity to the start codon of the second cistron. Thus, translation of the first cistron increases the local concentration of ribosomes near the RBS of the second cistron and expedites the initiation of its translation [41]. In addition, ribosomes translating the upstream cistron will relieve inhibitory structures in the RBS of the downstream cistron via the helicase activity of the elongating ribosome. Together, these mechanisms will facilitate the translation of the second cistron following that of the first one.

Notably, several studies report the use of translation coupling to alter gene expression via the action of sRNAs (Figure 2b). The first characterized case involved the iron-responsive sRNA RyhB. RyhB has been reported to repress translation indirectly of the Fur protein by inhibiting the translation of an upstream small ORF, termed *uof* (upstream of *fur*) [42]. It has been shown that the 28 codon-long ORF of *uof* overlaps with the beginning of the *fur* ORF, allowing translation coupling between both ORFs. Because the SD sequence of the *fur* mRNA is sequestered by a stem-loop structure preventing efficient ribosome binding, translating ribosomes originating from *uof* are thought to allow efficient *fur* translation by unfolding this stem loop structure. In addition, a recent study has shown that, in *Pseudomonas aeruginosa*, the sRNA PhrS activates the translation of an mRNA involved in quorum sensing, namely *pqsR*, by activating an upstream short ORF [43].

Another report identified an additional example of a sRNA targeting a leader peptide. These authors showed that GcvB sRNA represses translation of *thrL* [10], a 63-nucleotide ORF whose expression regulates the transcription of the downstream *thrABC* polycistron by an attenuation mechanism (Box 2). Although the effect of GcvB on the *thrABC* operon transcription was not directly addressed, these results suggest for the first time that a sRNA modulates transcription of an operon.

Box 2. Transcription attenuation in bacteria

Transcription attenuators are RNA sequences present in the 5′-UTR or a leader peptide of a gene that can fold into two mutually exclusive structures: a Rho-independent transcription termination structure and an anti-terminator structure. Rho-dependent transcription terminators are characterized by a stable G/C-rich stem-loop structure followed by a stretch of unpaired Us that destabilizes the RNA polymerase, causing its dissociation from DNA. Transcription attenuation was first discovered by studying amino-acid operons leader RNA, such as the tryptophan [74] and the threonine operons [75]. In these cases, the sensor element is a short leader peptide (such as ThrL) that is enriched in codons for a specific amino acid. When there is plenty of the corresponding amino acid in the cell, ribosomes will translate the leader peptide and disrupt the formation of the anti-terminator structure, causing transcription termination.

However, transcription attenuators are present in a wide range of mechanisms that are used to couple the sensing of an environmental change with transcription termination [76]. These include riboswitches, T-boxes (tRNA sensing elements), and transcription attenuation by RNA binding proteins.

Targeting of translation enhancer elements

Although the SD sequence and the initiation codon are critical signals for translation initiation in many bacteria, including *E. coli*, additional elements affect the efficiency of translation initiation. Accordingly, these translation enhancer elements can also be targeted by sRNAs to repress translation (Figure 2c). One of the first reported examples of such a mechanism involves the sRNA GcvB, which is known to regulate various ABC transporters [44]. In addition to its mechanistic role describe above, this sRNA has also recently been shown to regulate many periplasmic proteins by pairing to C/A multimers located in the 5′-UTR of their target mRNAs. When located downstream of the start codon, C/A repeats had been demonstrated to stimulate translation *in vivo* and to facilitate ribosome binding to an mRNA *in vitro* [45]. C/A repeats were later shown to stimulate the translation of mRNA when located inside or upstream of the RBS region [46]. The mechanism by which C/A repeats stimulate translation is unknown, but does not seem to require factors other than those present in the translation initiation complex. Interestingly, it seems that the sRNA GcvB has evolved to target these elements by way of a highly conserved G/U-rich unstructured region [46]. Intriguingly, in the case of the *gltI* target of GcvB, the C/A rich region is located far upstream of the RBS region. However, it still acts as a translation enhancer and so causes a shutdown of translation when base-paired with GcvB. Because C/A-rich regions are present in the 5′-UTR of target mRNAs encoding proteins involved in amino acid uptake and metabolism, it is tempting to speculate that GcvB has evolved to recognize these motifs via its G/U-rich region. In fact, it has been recently reported that GcvB may regulate up to 1% of *Salmonella* genes by targeting a CACAaCAY motif present in the 5′-UTR of target mRNAs [10]. However, it is still not clear whether this motif serves as a translation enhancer in all these mRNAs.

Recently, more sRNAs that target regions far upstream of the RBS have been discovered, but they do not seem to fall into any of the categories described above. For instance, the sRNAs OmrA and OmrB, which downregulate the expression of many outer membrane proteins [35], have been shown to repress translation of the transcriptional regulator CsgD, by targeting a conserved structural element in the 5′-UTR [47]. In addition, the sRNA RybB has been shown to regulate outer membrane protein C (*ompC*) and *ompF* translation by pairing far upstream of the start codon [9]. However, these targeted sites do not seem to be known translational enhancers and so the mechanism by which these sRNAs repress translation remains unknown.

Recruiting a protein to compete with initiating ribosomes

Recently, a new mechanism of sRNA-mediated translation repression that relies on the recruitment of the RNA chaperone Hfq (Figure 2d) has been uncovered [48]. It was found that the sRNA Spot42, which is involved in catabolite repression [7,49], repressed translation of *sdhC*, which encodes part of succinate dehydrogenase, a component of the tricarboxylic acid (TCA) cycle. Spot42 pairs with the *sdhC* leader more than 40 nucleotides upstream of the start codon, a finding that excludes direct competition with

Box 3. S1 ribosomal protein

According to the NCBI protein sequence database, the ribosomal protein S1 is present in all proteobacteria but is absent from Gram-positive bacteria with a low GC content, such as *Bacillus subtilis*. At least in *Escherichia coli*, the S1 protein is an integral part of the translation machinery. S1 has strong RNA binding activity and exhibits a preference for unstructured A/U-rich regions [77]. The S1 protein contains two functional domains: an N-terminal domain that is responsible for the incorporation of S1 to the 30S ribosomal subunit via protein–protein interactions and a C-terminal domain that contains RNA-binding activity [78]. Because of this RNA-binding activity, the S1 protein was shown to be essential for the translation of mRNA lacking a SD element [79]. However, S1 is also necessary for the translation of some mRNAs containing canonical SD sequences. It is believed that the S1 protein may facilitate the binding of the 30S ribosomal subunit to the transcription initiation region (TIR) of an mRNA. In fact, a cryoelecton microscopy study has shown that S1 interacts with the 5′-end region of mRNAs, which would facilitate the SD–antiSD interaction with the ribosome [80]. Given the high occurrence of A/U-rich regions in the 5′-UTR of proteobacteria, it is believed that the S1 protein is essential, or at a minimum is implicated in, the translation of a high proportion of mRNAs [81,82].

initiating ribosomes. None of the mechanisms reviewed above were found to explain the repression mediated by Spot42. However, it was shown that the sRNA-associated RNA chaperone Hfq plays an active role in the translational repression of *sdhC*. This protein had previously been described to stabilize sRNAs *in vivo* and to facilitate sRNA–mRNA interactions *in vitro*. However, its role as a translation repressor had not been described within the context of sRNA-mediated regulation.

It was demonstrated that Hfq is recruited to an A/U-rich stretch in the vicinity of the SD region of *sdhC* and that, in contrast to any previously characterized sRNA-mediated regulations, Hfq competed directly with initiating ribosomes, and not the sRNA itself [48]. Of note, A/U-rich regions have been known to act as translation enhancers because they are recognized by the ribosomal protein S1 (Box 3) [50,51]. This important feature underlined the fact that both S1 ribosomal protein and Hfq are RNA binding proteins that prefer the A/U-rich region. Therefore, it is possible that S1 and Hfq may have antagonistic effects on translation and that Hfq may play an active role in sRNA-mediated translation repression and may add robustness to the action of the sRNA. Furthermore, new data (M.P. Bouchard *et al.*, unpublished) suggest that Hfq is also recruited to additional mRNA targets, following sRNA pairing. Thus, it is possible that Hfq plays a direct role in translation repression in many examples of sRNA-mediated translation repression.

New insights into sRNA-mediated mRNA degradation

To degrade or not to degrade?

As stated above, translation repression by bacterial sRNAs is often followed by rapid decay of the mRNA (nucleolytic repression). However, in some cases, there is an absence of rapid degradation of the mRNA following the block of translation (non-nucleolytic repression) [10,42,48,49,52]. The reasons for this dichotomy are not totally understood, but recent studies have shed some light on this question.

For example, one particularly interesting case is the sRNA-mediated regulation of *sdhCDAB* mRNA. This mRNA was shown to be repressed by three sRNAs that are expressed in response to different physiological conditions: Spot42, RyhB, and RybB [48,53]. Whereas Spot42 is expressed when glucose is present in the medium [49], RyhB and RybB are expressed during iron starvation [53] and cell envelope stress, respectively [54]. Surprisingly, although these three sRNAs were able to repress translation, only RyhB and RybB triggered the rapid decay of *sdhCDAB* mRNA. Spot42 expression only blocked translation but did not activate mRNA decay. These differences in mRNA decay kinetics cannot be explained by intrinsic characteristics of sRNAs. Indeed, Spot42 is known to trigger the degradation of other target mRNA, such as *gltA*, *maeA*, and *sthA* [7], whereas RyhB has been shown to affect *cysE* translation [52] without triggering mRNA degradation [8]. In the case of the *sdhCDAB* mRNA, it is likely that the differences in the kinetics of mRNA decay observed following the action of Spot42 and RyhB is due to their varied effects on translation. Indeed, whereas RyhB represses translation by a tenfold factor, Spot42 only has a 2.5-fold effect [48]. Therefore, it is likely that the residual translating ribosomes protect mRNA from the ribonucleolytic action of RNase E. Furthermore, the initial RNase E cleavage site, which is necessary for rapid mRNA decay following sRNA expression, is located in the *sdhCDAB* coding sequence located more than 210 nucleotides downstream of the start codon (unpublished data). The presence of sRNA-sensitive RNase E cleavage sites in the coding sequence of target mRNAs seems to be recurrent, as suggested by a previous study that revealed similar distal cleavage sites for *sodB*, *fumA*, and *iscS* mRNAs that are responsible for the rapid decay of the mRNA when a sRNA binds to them [24]. There are also data to suggest that such cleavage sites, in the coding region of an mRNA, are normally protected from RNase E by translating ribosomes. Indeed, the protecting action of ribosomes against RNase E action is well established [55]. Conversely, when a sRNA blocks translation, these cleavage sites become available for RNase E action.

In our view, a different mechanism than the one described above could also explain the observations that some target mRNAs are rapidly degraded following sRNA pairing, whereas others are not. As mentioned above, recent evidence suggests the existence of rate-limiting distal cleavage sites on *sodB*, *fumA*, and *iscS* mRNAs that are essential for their rapid degradation [24]. New data indicated that it was possible to confer resistance of a target mRNA to sRNA-induced mRNA decay by eliminating this distal cleavage site [24]. Therefore, it is possible that some mRNAs do not possess such cleavage sites and that they are intrinsically resistant to sRNA-mediated mRNA degradation, even though RNase E is recruited by the sRNA.

As mentioned before, the discovery of mRNA targets for a given sRNA is often accomplished by performing pulse-expression experiments that monitor global mRNA levels by genomic methods (microarray analysis and RNA sequencing) following sRNA expression [7,8,34,35]. This approach has been used successfully in several instances. However, the fact that some target mRNAs are not actively degraded following sRNA expression may mask many of

these targets, leading to an underestimation of the number of targets. Evidence for this possibility has been highlighted by a recent study where a combination of bioinformatics and reporter gene fusion was used to identify some mRNA targets regulated solely at the level of translation [10].

Targeting of the coding sequence

An alternative model for sRNA-induced mRNA degradation has recently been proposed that does not require a block of translation for degradation. For instance, it has been reported that the sRNA MicC pairs with the coding sequence of *ompD* mRNA, without seemingly affecting translation, and promotes its decay with the help of RNase E [56]. The pairing site is located 67 nucleotides downstream of the start codon, thus eliminating any competition with initiating ribosomes. The authors showed that MicC induces an RNase E-dependent cleavage in the coding sequence, thereby promoting rapid degradation of *ompD* mRNA. In contrast to the canonical model, where mRNA degradation is regarded as a consequence of translation repression and RNase E action, the regulation of *ompD* by MicC only depends on the action of RNase E. Interestingly, a recent study has shown that MicC is able to direct a RNase E cleavage just a few nucleotides downstream of its pairing site [57]. This action requires that the 5′-end of the sRNA be in a monophopshorylated (5′-P) state rather than the usual triphosphorylated (5′-PPP) state. Monophosphorylated 5′-ends have been known for several years to activate the action of RNase E [58], and a group of enzymes has been shown to catalyze the dephosphorylation of 5′-PPP to 5-P′ [59]. Recent additional reports have further suggested that other mRNAs are regulated by a similar mechanism involving recruitment of the RNase E to the coding sequences of mRNAs [9,60,61].

Concluding remarks

The recent characterization of sRNA-regulated mRNAs has suggested an unexpected array of singular mechanisms in which structures strictly related to mRNAs (and not sRNAs) are involved. Indeed, one common feature among these novel mechanisms is the unsuspected contribution of mRNA features to the mechanisms regulating these targeted mRNAs, such as distal cleavage sites. This interpretation is supported by another description of critical structures specifically adopted by target mRNAs that are prerequisites for sRNA-mediated regulation. For instance, the presence of an Hfq-binding site close to the sRNA pairing site has been shown to be required for selection of a target mRNA [62]. Given the increasingly large number of target mRNAs being discovered, there is good reason to believe that additional key structures intrinsic to these targeted mRNAs will be uncovered in the near future, shedding more light on the understanding of the sRNA-dependent post-transcriptional regulatory mechanisms in prokaryotes.

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