

Bacterial Small RNA Regulators

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ABSTRACT Small regulatory RNAs can modify the activity of proteins and the stability and translation of mRNAs. They have now been found in a wide range of organisms, and can play previously unsuspected critical regulatory roles. The bacterial small RNAs include two major classes. The largest family (with at least 20 members in *Escherichia coli* K12) acts by basepairing with target mRNAs to modify mRNA translation or stability; this class of RNAs also uses an RNA chaperone protein, Hfq. DsrA is the best-studied example of this family of RNAs. It has been shown to positively regulate translation of the transcription factor RpoS by opening an inhibitory hairpin in the mRNA, and to negatively regulate translation of *hns* by pairing just beyond the translation initiation codon. The class of RNAs that modify activity of proteins is exemplified by CsrB and CsrC of *E. coli*, two RNAs that bind to and inhibit CsrA, a protein translational regulator. Homologs of CsrA and related regulatory RNAs have been implicated in the regulation of gluconeogenesis, biofilm formation, and virulence factor expression in plant and human pathogens.

KEYWORDS CsrA, CsrB, DsrA, Hfq, RpoS

INTRODUCTION

Small RNAs that do not encode polypeptides have recently seized the attention of many researchers working in both prokaryotic and eukaryotic organisms. The eukaryotic microRNAs have been found to act on translation, regulating specific genes during development, and interfering RNAs have been shown to both have naturally important roles in silencing genes and to provide an extremely useful genetic tool (McManus & Sharp, 2003). In prokaryotes, small RNAs made from the opposite strand from their targets and therefore able to basepair extensively with their target mRNA have been described and studied as regulators of plasmid and phage functions for many years. More recent recognition of the importance of trans-acting small RNAs in regulation of bacterial gene expression, including genes involved in bacterial pathogenesis, has extended interest in these molecules, and has led to genome-wide searches for these small RNAs in *E. coli*, and, more recently, in other organisms as well. More than 200 have been predicted by various approaches; of these, the expression of 60 small RNAs has been confirmed by Northern blotting in *E. coli* K12. What do all these small RNAs do, and how do they do it? In this review, we focus on two of the most extensively studied examples, one each of two major types of non-coding regulatory RNAs.

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The first type acts by basepairing with one or more target messenger RNAs; the outcome of pairing can be activation or inhibition of translation, and/or activation or inhibition of mRNA degradation. Of the currently recognized small RNAs, at least one third and probably more act in this fashion. Many, if not all, of these also bind to and use the RNA chaperone Hfq. As an example of such a small RNA, we discuss in detail DsrA, an *E. coli* non-coding RNA with a significant role in the stimulation of translation of the stationary phase sigma factor, RpoS, as well as other cellular roles. For valuable information on the details of interactions of small RNAs with targets, the reader is recommended to consult recent reviews on the mechanism of action of plasmid-encoded small RNAs (Brantl, 2002; Gerdes *et al.*, 1997; Wagner & Brantl, 1998). These differ from the bacterial RNAs discussed here primarily because the plasmid RNAs are encoded on the opposite strand of their target messages, leading to extensive complementarity; the bacterial RNA genes discussed here are generally far from the genes for their targets.

The second type of non-coding RNA acts by interaction not with a message, but with a protein. CsrA is an RNA binding protein that acts as a translational regulator in *E. coli*; homologs play roles in virulence in *Erwinia* species, and Pseudomonads as well. The activity of CsrA is at least in part modulated by interactions with non-coding RNAs that titrate CsrA away from its mRNA targets. CsrB and CsrC have been identified as such regulatory RNAs in *E. coli*, and homologs exist in many other organisms.

TRANS-ACTING BASEPAIRING RNAs

The small RNA DsrA will be discussed as the paradigm for small RNAs (sRNAs) that basepair with their targets. DsrA is one of the first sRNAs of this class to have been identified and has been one of the most thoroughly studied. DsrA positively regulates translation of the stationary phase sigma factor RpoS and negatively regulates translation of the histone-like protein HNS. Another small RNA, RprA, also acts positively on translation of its only known target, *rpoS*. However, most other basepairing small RNAs act negatively. The only other example of a regulatory RNA with both positive and negative effects is RNAlII of *S. aureus* (Morfeldt *et al.*, 1995) involved in virulence regulation; this RNA is considerably larger than DsrA, but may act similarly in terms of pairing to targets to either

stimulate or inhibit translation; it also contains a short translated region. DsrA exerts a broad effect on gene expression because both of its known targets, RpoS and HNS, are themselves global regulators; as a result, the indirect effects of DsrA via effects on these global regulators are extensive. Although the biological effects of DsrA may be particularly broad, what we know about the mechanism of action of DsrA has many similarities to the many other pairing RNAs that act negatively. These RNAs all require the RNA chaperone protein Hfq for activity, and their binding to and dependence upon Hfq defines the class of regulatory RNAs represented by DsrA. The wealth of information available about DsrA is unmatched for any of the other RNAs and therefore presents a model of successes and obstacles that research on other small RNAs may face.

Discovery of DsrA

DsrA was discovered in this laboratory accidentally during studies on capsule regulation. The transcription of genes involved in synthesis of colanic acid capsule (*cps* genes) in *E. coli* requires the RcsC/YojN (RcsD)/RcsB phosphorelay, in which RcsB is a DNA-binding response regulator (Stout & Gottesman, 1991; Takeda *et al.*, 2001). In addition, a second DNA binding protein, the unstable protein RcsA, is needed for stimulation of transcription of the *cps* genes. A multi-copy plasmid encoding RcsA increased capsule synthesis, as expected because RcsA is normally limiting for *cps* gene expression. More surprisingly, when the *rcaA* gene on the plasmid was inactivated by transposon insertion, it still stimulated *cps* gene expression; stimulation required an intact *rcaA* gene in the chromosome (Brill *et al.*, 1988). This was originally interpreted as suggesting that the plasmid was titrating a negative regulator of *rcaA* synthesis. However, subsequent experiments demonstrated that the active portion of the plasmid was not upstream of *rcaA* but downstream. Darren Sledjeski investigated this further and found that the relevant portion of the plasmid encoded a short RNA with no obvious open reading frames. Transcription of the RNA was necessary to stimulate capsule synthesis and mutations in either the promoter of the RNA or in the Rho-independent terminator abolished function (Sledjeski & Gottesman, 1995). The RNA was named DsrA (downstream from RcsA).

Why did DsrA overproduction from a plasmid turn up capsule expression? Experiments on the regulation

of *rcaA* suggested that *rcaA* transcription is negatively regulated by the pleiotropic regulatory protein HNS. In *hns* mutants, *rcaA* transcription is increased, which is sufficient to make cells mucoid. DsrA overproduction resulted in an HNS mutant phenotype, relieving repression of the *rcaA* promoter, as well as other HNS target promoters (Sledjeski & Gottesman, 1995). The mechanism of the DsrA effect on HNS is discussed further below.

Although overproduction of DsrA clearly had an effect on HNS-regulated genes, it was difficult to discern an effect of deleting the chromosomal copy of *dsrA* on capsule or other HNS targets (Sledjeski *et al.*, 1996). However, a reporter fusion with another gene present on the original plasmid, called *dsrB*, was stimulated by a multicopy *dsrA* plasmid, and mutations in *dsrA* had a significant effect on the expression of the *dsrB-lacZ* transcriptional fusion, particularly at low temperatures (Sledjeski *et al.*, 1996). While the function of DsrB was (and remains) unknown, the pattern of its expression was striking; transcription increased significantly when cells entered stationary phase, a sign that *dsrB* transcription might be dependent upon the stationary phase sigma factor RpoS. Because capsule is made preferentially at low temperature, we examined the effect of DsrA at low temperatures and found that RpoS levels increased at low temperature, an increase that was fully dependent on *dsrA* (Sledjeski *et al.*, 1996). The increase was demonstrated to be due to increased RpoS translation. Because RpoS levels increased at temperatures of 30°C and below, even in exponential phase when RpoS levels are usually low, this suggested a critical role for DsrA and RpoS at low temperatures. These observations were the basis for the future studies on DsrA action that led to the models discussed below.

Structure and Function

DsrA is a non-coding 85 nucleotide-long small regulatory RNA. The gene sequence is very highly conserved (approximately 90% conservation) among closely related Gram-negative organisms such as *Salmonella*, *Shigella*, & *Klebsiella*. In fact, variations in the sequence of the *Klebsiella* DsrA, compared to that of *E. coli*, occur either in regions predicted to be part of single-stranded loops or, simultaneously, in opposing nucleotides in a predicted stem resulting in the maintenance of an intact stem structure. This is a clear indication of the importance of structures in the RNA, consistent with a

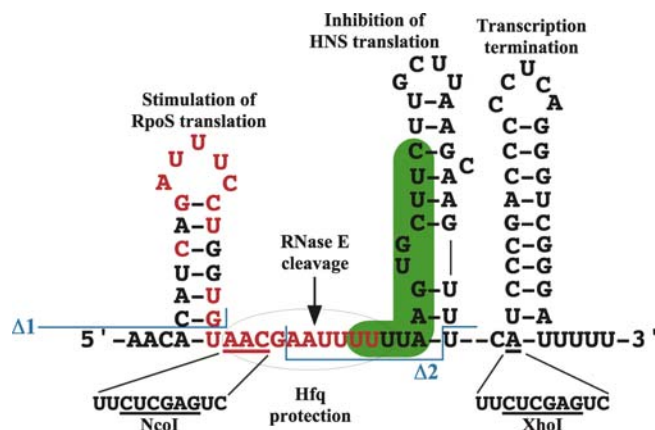


FIGURE 1 Structure of DsrA. The secondary structure of DsrA is that found in studies of *in vitro* nuclease sensitivity and hydroxyl-radical cleavage, as well as Hfq protection (Brescia *et al.*, 2003; Lease & Belfort, 2000a; Lease & Woodson, 2004). Nucleotides in red are capable of basepairing with the *rpoS* leader (Majdalani *et al.*, 1998); nucleotides highlighted with green are capable of pairing with *hns* (Lease *et al.*, 1998). The RNase E cleavage site is as found by (Moll *et al.*, 2003a). The $\Delta 1$, $\Delta 2$, NcoI and XhoI mutations are as described in (Majdalani *et al.*, 1998).

direct role for the RNA. RNA folding programs such as MFOLD predict a number of conformations for this RNA, but two energetically favored folds shape the RNA into a three stem-loop structure (Sledjeski & Gottesman, 1995). While the initial stem-loop (SLI) and the terminator stem-loop (SLIII) are the same in both predictions, the middle (SLII) originally predicted was not the structure found in a nuclease footprinting analysis of *in vitro* synthesized RNA (Lease & Belfort, 2000a) (Figure 1).

How does the RNA function? As mentioned above, multicopy expression of DsrA causes both increased expression of a *cps-lacZ* fusion, via down-regulation of HNS amounts or activity, and increased translation of RpoS. Deletion analysis and point mutations in *dsrA* demonstrated that these two activities could be separated (Majdalani *et al.*, 1998). RNAs with deletions and rearrangements of SLI could still down-regulate HNS function, but had lost the ability to regulate RpoS; RNAs with deletion of sequences overlapping parts of SLII still retained some activity for RpoS but were unable to down-regulate HNS (Majdalani *et al.*, 1998) (see $\Delta 1$ and $\Delta 2$, Figure 1).

At the time this deletion analysis was being done, another study on the role of the Hfq protein in the regulation of RpoS translation was being carried out in Tom Elliott's laboratory (Brown & Elliott, 1997). Hfq mutants had been found to make very low levels of RpoS

(Brown & Elliott, 1996; Muffler *et al.*, 1996). Brown and Elliott selected revertants of an *hfq* mutant that expressed an RpoS-LacZ translational reporter at a higher level in the absence of Hfq and mapped in the fusion. Their mutants defined a hairpin in the sequence upstream of the *rpoS* coding region; a stretch of sequence more than 60 nucleotides upstream paired with the ribosome binding region, inhibiting RpoS translation (Brown and Elliott, 1997) (Figure 2A). When the hairpin was present, RpoS translation was dependent upon Hfq and environmental signals.

After closer inspection, DsrA was found to carry a stretch of nucleotides that is complementary to the up-

stream region of the RpoS message. Mutations in these nucleotides abolished DsrA activity on RpoS while compensatory mutations in the *rpoS* leader RNA restored activity to the mutant; this provided proof that the sRNA must pair directly with its target mRNA to act *in vivo* (Majdalani *et al.*, 1998). This pairing of DsrA with the *rpoS* mRNA leads to a stimulation of translation. While the details of activation are not yet clear, the end result is. When the hairpin loop that occludes the RpoS ribosome binding site is intact, there is low translation (Brown & Elliott, 1997) (Figure 2A). Sequences in DsrA that are complementary to the upstream part of the leader hairpin presumably lead to freeing the

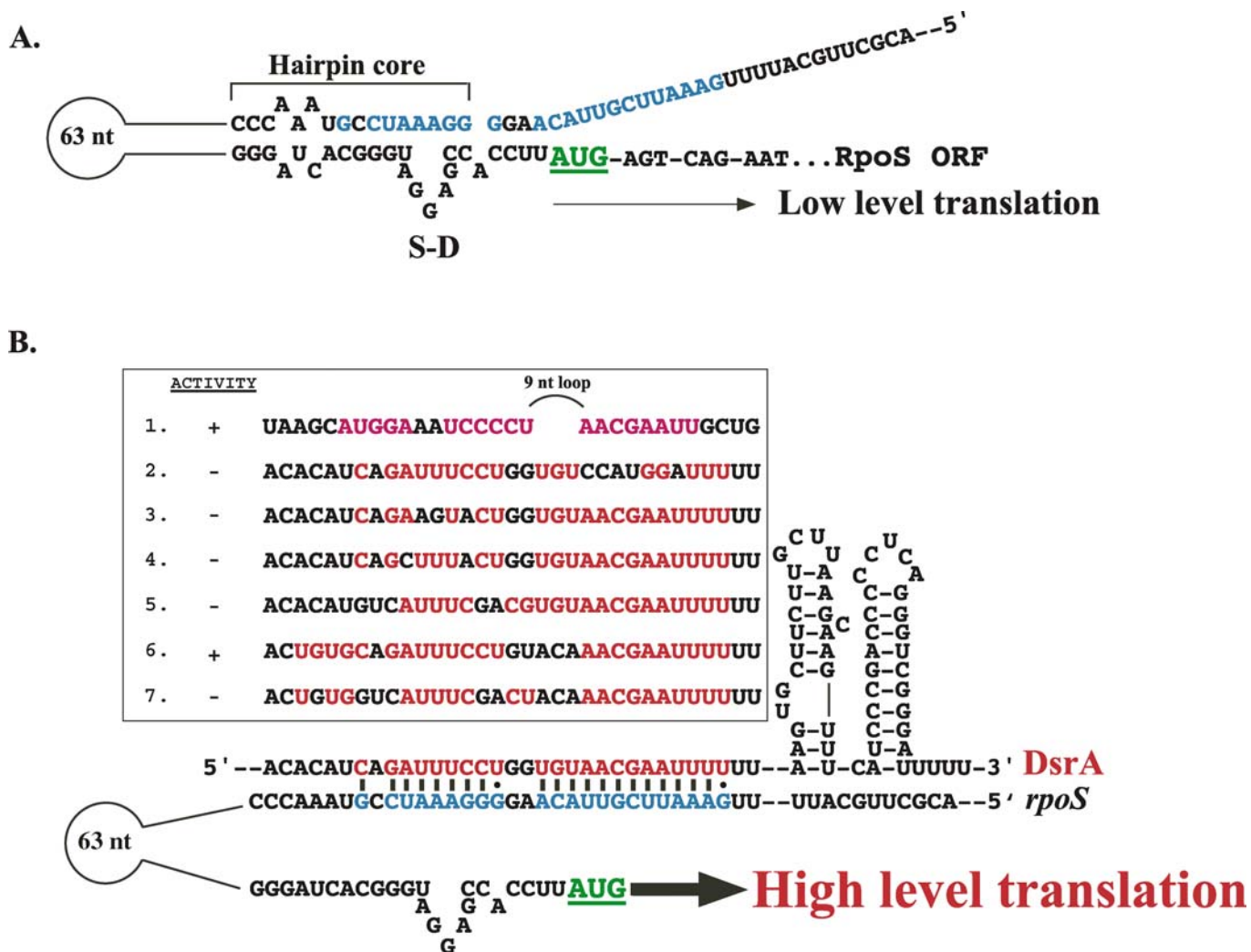


FIGURE 2 Stimulation of RpoS translation by DsrA. **A.** Structure of inhibitory hairpin, based on (Brown & Elliott, 1997). Large AUG is first codon of *rpoS* gene. **B.** Pairing of DsrA, RprA, and derivatives of DsrA with *rpoS* upstream RNA. Pairing prediction for wild-type DsrA is from (Majdalani *et al.*, 1998). RprA and mutant derivatives of DsrA are shown boxed, with predicted pairing in red and their activity for expression of an RpoS-LacZ fusion shown to the left. Derivatives shown: 1. Wild-type RprA (Majdalani *et al.*, 2002). 2-7, derivatives of DsrA (see Figure 1). 2. NcoI mutation (Majdalani *et al.*, 1998); 3. pDDS216, an unpublished variant from D. Sledjeski and N. Majdalani; 4. A-C loop mutations; the AUUUC sequence of the loop for the first stem-loop was changed to CUUUA, unpublished, N. Majdalani; 5. Inversion of top of the first stem (Majdalani *et al.*, 1998); 6. Inversion of bottom of the first stem (Majdalani *et al.*, 1998); 7. Inversion of all of the first stem (Majdalani *et al.*, 1998).

rpoS ribosome-binding site (Figure 2B). The net result is a significant increase in RpoS translation and a significant accumulation of the RpoS protein (Majdalani *et al.*, 1998). Figure 2B summarizes some of the mutant forms of DsrA that have been tested and their effects on pairing. These results, as well as comparisons of the pairing of a second *rpoS*-stimulatory small RNA, RprA, suggest that there are at least two critical regions for pairing to the *rpoS* message (Majdalani *et al.*, 1998; Majdalani *et al.*, 2002). One is 5' to the *rpoS* hairpin, and may provide an initial interaction site with DsrA that will be accessible even when the hairpin is present; the second region is the core of the hairpin, particularly the region that pairs to either side of the ribosome binding site loop (Figure 2A). This region is also close to the binding site for the RNA chaperone Hfq (see below), and whether pairing here occurs before or after Hfq interactions is unclear.

These results demonstrated that direct pairing of DsrA with the *rpoS* mRNA was necessary for DsrA action. Does DsrA also regulate HNS by pairing? In initial experiments (Sledjeski & Gottesman, 1995), the levels of HNS protein were not found to decrease significantly upon overexpression of DsrA, suggesting a post-translational role for DsrA. However, later studies by Lease and coworkers found a two to threefold decrease in HNS protein upon DsrA overproduction, and computational analysis suggested that SLII, shown to be necessary for HNS regulation, had the potential to pair with a portion of the *hns* mRNA for a 13 nt stretch just beyond the start of translation (Lease *et al.*, 1998). Indeed, five point mutations in SLII caused DsrA to lose its anti-HNS activity; compensatory mutations in the *hns* mRNA restored it, confirming that this pairing is required for the inhibition of HNS translation (Lease *et al.*, 1998). Further pairing between DsrA and the 3' end of the *hns* mRNA is predicted *in silico*, but this pairing, as well as potential pairings to several other candidate DsrA targets, have not been tested experimentally (Lease & Belfort, 2000a). It is intriguing and thus far unexplained that a relatively moderate decrease in HNS levels has a dramatic effect on HNS activity.

Role of Hfq in DsrA Function

The demonstration that both DsrA and the Hfq protein were necessary for high levels of RpoS translation suggested that Hfq might act to help DsrA function. This has proven to be the case. Hfq was first found as

an *E. coli* protein that is required for the RNA phage Q β to replicate *in vitro*. Hfq binds phage RNA tightly and destabilizes a secondary structure at the 3' end of the phage RNA (Franze de Fernandez *et al.*, 1968). More recently, Hfq was shown to form a multimeric ring; its structure is very similar to that of eukaryotic splicing proteins of the Sm family (Sauter *et al.*, 2003; Schumacher *et al.*, 2002).

The involvement of Hfq in RpoS translation and its ability to bind RNAs suggested that it might be involved in DsrA action. In addition, Hfq was found to bind another small regulatory RNA, OxyS, which negatively regulates *rpoS* (Zhang *et al.*, 1998). Sledjeski and coworkers tested the *in vivo* role of Hfq on the activity of DsrA and it was immediately clear that Hfq was important in the DsrA-mediated regulation of both RpoS and HNS (Sledjeski *et al.*, 2001). In the absence of Hfq, the effects of DsrA were more than 30-fold reduced on RpoS and eightfold reduced for the HNS-sensitive *rcsA* promoter. Furthermore, DsrA RNA made from the chromosomal copy of the gene was unstable in an *hfq* mutant background. However, the requirement for Hfq was not absolute. Sledjeski and coworkers detected a weak but clear DsrA-dependent, Hfq-independent activation of RpoS-LacZ when DsrA was overproduced from a multicopy plasmid. The stability of over-expressed, plasmid encoded DsrA was independent of Hfq (Sledjeski *et al.*, 2001). The authors surmised that the abundant, plasmid-encoded DsrA was only partially folding into the correct conformation and activating RpoS-LacZ, while the rest was folding or aggregating into a nuclease-resistant but inactive conformation (Sledjeski *et al.*, 2001). Nonetheless, these results suggested that Hfq played the role of a chaperone protein that would either help fold the small RNA into a nuclease-resistant active form and/or directly hinder the access of the nuclease by binding to DsrA.

This pattern of dependence upon Hfq for RNA stability and activity has been found with many other small regulatory RNAs (Møller *et al.*, 2002a). About a third of the known small RNAs isolated in *E. coli* are bound by Hfq, and binding to Hfq can be used to identify new small RNAs (Zhang *et al.*, 2003). In every case that has been studied, small RNAs that bind to Hfq act on mRNAs by pairing with specific mRNAs. *In vitro*, Hfq has been shown to bind to small RNAs and stimulate their pairing to target mRNAs, supporting a role for Hfq beyond stabilization of the small RNAs. The binding site on the small RNAs was defined as a single-stranded

A-U rich stretch, followed by a stem (Moll *et al.*, 2003b; Møller *et al.*, 2002a; Zhang *et al.*, 2002).

A number of studies have investigated whether Hfq can change RNA structure; the results suggest that it can, but does not necessarily do this in all cases. *sodB* mRNA, a target of the small RNA RyhB, is remodeled by Hfq binding to an A-U rich sequence; this remodeling frees a region that can pair with RyhB (Geissmann & Touati, 2004). Hfq, like another RNA chaperone, StpA, could help promote splicing of a T4 phage gene, suggesting an effect on the structure of the splicing RNA, although whether this was a direct or indirect effect was not examined (Moll *et al.*, 2003b). In *in vitro* experiments, the nuclease cleavage pattern of *ompA* mRNA was changed in the presence of Hfq, again suggesting the possibility that Hfq modulates RNA structures (Moll *et al.*, 2003b). In the case of DsrA and *rpoS*, no major change in RNA structure has been detected upon Hfq binding (Brescia *et al.*, 2003; Lease & Woodson, 2004). The tightest binding of Hfq to the *rpoS* target mRNA is within the 63 nt RNA between the upstream inhibitory stem of the hairpin and the ribosome binding site (Lease & Woodson, 2004). Binding to *hms* mRNA has not yet been reported. Therefore, there is thus far no evidence that Hfq helps DsrA function by changing the availability of the pairing regions.

Hfq helps stabilize DsrA *in vivo*, presumably by binding to it and protecting it from RNase E attack. The recognition site for RNase E is similar to that for Hfq, a single-stranded AU-rich region. Cells defective in RNase E show higher levels of DsrA (Moll *et al.*, 2003a); a similar sensitivity to RNase E was found for RyhB RNA in the absence of Hfq (Massé *et al.*, 2003; Moll *et al.*, 2003a). Competition between Hfq and RNase E has been found in other situations as well (Zhang *et al.*, 2003). *In vitro*, Hfq binds DsrA in gel retardation assays (Brescia *et al.*, 2003; Lease and Woodson, 2004; Sledjeski *et al.*, 2001). Both nuclease protection and competition experiments using unlabeled DsrA domains to compete with the radiolabeled full-length molecule place the Hfq binding site at a single-stranded region between stems I and II of DsrA (Brescia *et al.*, 2003; Lease & Woodson, 2004) (Figure 1). This same region was the site of RNase E cleavage *in vitro*; Hfq was able to interfere with this cleavage (Moll *et al.*, 2003a).

Is stabilization the only role for Hfq in DsrA function? This seems unlikely, given its stimulatory role in pairing of other small RNAs and their targets (Møller

et al., 2002a; Zhang *et al.*, 2002). One possible role of Hfq would be to bring together the regulatory RNA and its target message by binding both. That would imply either that a single Hfq hexamer ring can bind two different RNAs at once, or that interactions between Hfq hexamers, each binding a different RNA, can occur. The co-crystal structure of the *S. aureus* Hfq protein and a small AU-rich RNA oligonucleotide indicated that the RNA was wrapped in the central core of the hexamer (Schumacher *et al.*, 2002). This may be the primary RNA binding site on Hfq. However, studies on DsrA binding to Hfq have suggested the possibility of a second binding site for RNA. Using polyU or polyA chains in a competition assay with DsrA, Brescia *et al.* noticed that polyU competes with DsrA for binding, while polyA caused a supershift of the Hfq-DsrA complex, suggesting two distinct RNA binding sites (Brescia *et al.*, 2003). Further analysis of these binding sites was performed using point mutations that altered specific amino acids that were either highly conserved or represented potential points of protein-RNA interaction in the crystal structure (Mickulecky *et al.*, 2004). Three sets of mutations were made. A set of mutants in critical residues in the central cavity had the most dramatic effects on DsrA binding, consistent with this acting as the primary binding site for DsrA. A second set of mutants on the proximal face of the hexamer affected Hfq stability, possibly by affecting Hfq assembly. The third set of mutants, in the distal part of the Hfq structure, had effects on the binding of polyA chains, consistent with their previous proposal that Hfq indeed has two distinct binding sites. Surprisingly though, none of these mutants were defective in *in vitro* binding of *rpoS* mRNA, and only mutants in the central cavity were significantly defective for DsrA activity *in vivo* (Mickulecky *et al.*, 2004). It is worth noting that Hfq has been shown to have a role in regulating polyA tailing of mRNAs, a possible small RNA-independent function for the polyA binding site (Mohanty *et al.*, 2004); (Hajnsdorf & Regnier, 2000).

Can a role for Hfq in helping to bring DsrA and *rpoS* RNA together be demonstrated directly? Thus far, *in vitro* studies have failed to show a strong requirement for Hfq. When both DsrA and either *hms* or *rpoS* mRNA are present together *in vitro*, they can anneal in the absence of Hfq, resulting in the expected changes in nuclease or hydroxy-radical sensitivity of both RNAs (Lease & Belfort, 2000b; Lease & Woodson, 2004); (Espinosa, submitted). The rearrangements in the *rpoS*

mRNA result in the ribosome binding site becoming more accessible to single-strand nucleases, presumably reflecting accessibility to ribosomes (Lease & Woodson, 2004), as expected from genetic experiments with DsrA (Majdalani *et al.*, 1998). In fact, ribosome accessibility *in vitro* was shown to increase when an antisense oligonucleotide to the inhibitory hairpin of the *rpoS* 5' UTR was present (Worhunsky *et al.*, 2003). Addition of Hfq to *rpoS* 5' UTR and DsrA had only very mild effects on the association of these RNAs (Lease and Woodson, 2004). Annealing of *rpoS* 5' UTR and DsrA interfered with the primary Hfq binding site on DsrA, suggesting that Hfq may be displaced after annealing (Lease & Woodson, 2004). Although ternary complexes with Hfq, DsrA, and *rpoS* leader RNA can be isolated, different studies under somewhat different experimental conditions find Hfq bound to either a site nearer the 3' end of DsrA (Espinosa *et al.*, submitted) and/or to the *rpoS* 5' UTR (Lease & Woodson, 2004).

Because these *in vitro* experiments do not provide a clear picture of why Hfq is needed for DsrA function, one possibility is that additional factors present *in vivo* but not *in vitro* increase the dependence upon Hfq. One such additional factor might be the ribosome itself. Worhunsky and coworkers found that DsrA, in the absence of detectable Hfq, binds to the 30S ribosomal subunit with a binding constant similar to that of *rpoS* mRNA (Worhunsky *et al.*, 2003). Binding occurred, albeit less well, with a fragment that corresponded to the first 34 nucleotides (or the 5' end) of DsrA. Furthermore, DsrA binding could not be competed by tRNA or *rpoS* mRNA, suggesting that the 30S subunit may have different binding sites for mRNA and small regulatory RNAs. Such binding to the ribosome would position DsrA to act on the *rpoS* message, freeing the ribosome binding site to immediately bind to the ribosome and begin translation, which in turn stabilizes the opening of the inhibitory hairpin (Worhunsky *et al.*, 2003). Whether this binding to the 30S subunit will prove to be physiologically relevant remains to be seen. Hfq is known to associate with ribosomal protein S1 in promoting replication of the RNA phage Q β (Blumenthal & Carmichael, 1979). Hfq and S1 have also been found to associate with RNA polymerase; in this study, an ATPase activity was found for Hfq, although no catalytic site is obvious from the sequence or crystal structures (Sukhodolets & Garges, 2003). If Hfq either has an ATPase activity or associates with an ATPase,

such an ATPase might help in remodeling the RNAs as they anneal.

Additional Candidates for Modulators of DsrA

Other proteins have also been implicated in DsrA action, although their roles are unclear. The histone-like protein HNS that preferentially binds to curved DNA (Atlung & Ingmer, 1997) was shown to bind RNA as well. *In vivo* assays of DsrA stability in an *hns* mutant indicated that DsrA was more stable than in a wild-type strain (DsrA half-life, 18 min); after an initial rapid degradation (half-life, 14 min), it had a half-life of >60 min (Brescia *et al.*, 2004). This effect of an *hns* mutant in stabilizing DsrA was even more evident in the absence of Hfq. In an *hfq* mutant, DsrA becomes unstable, with a half-life of 2.5 min; in the *hns hfq* double mutant, DsrA has a half-life of 34 min (Brescia *et al.*, 2004). Whether this stable DsrA is active was not determined, and because *hns* mutants have pleiotropic effects, it is not clear whether the effects are direct or indirect. In support of a direct interaction of HNS and DsrA, Brescia and coworkers demonstrated *in vitro* that HNS specifically bound DsrA (Brescia *et al.*, 2004). In parallel to the *in vivo* observations, low to intermediate concentrations of HNS enhanced RNase I single-strand attack on DsrA *in vitro*. However, high levels of HNS protected DsrA (Brescia *et al.*, 2004). If HNS does directly interact with DsrA, we would expect an effect *in vivo* on one or more of the DsrA activities. We have found that *hns* mutants have increased levels of RpoS, but resulting from stabilization of the protein, not increased translation (Zhou & Gottesman, in preparation).

Another protein that seems to bind to DsrA *in vitro* is the histone-like protein HU. This protein binds to DNA and double-stranded RNA without any sequence specificity yet seems to recognize similar structural features in DNA and RNA (Balandina *et al.*, 2002). Using gel retardation assays, Rouviere-Yaniv and coworkers demonstrated that HU bound full-length DsrA quite well. Using truncations of DsrA, the authors determined that HU was in fact binding to a region of duplex RNA preceded or followed by a region of single-strandedness. These 3' or 5' overhangs are important for HU binding to DsrA. HU also stimulates RpoS translation (Balandina *et al.*, 2001), leading the authors to suggest that HU may play a physiologically significant role for DsrA function *in vivo* (Balandina *et al.*, 2002).

Model for DsrA Action

Although the roles of ribosome, HNS, HU, or ribosomal proteins are not yet clear, the basic picture that we are left with is consistent with (and expands) the model derived from *in vivo* experiments (Majdalani *et al.*, 1998). DsrA is synthesized at low temperatures (see below), and binds Hfq with high affinity. A site on the *rpoS* 5' UTR is also bound by Hfq. Possibly, interactions between Hfq oligomers or binding of both RNAs to different sites on one Hfq oligomer help bring the RNAs together, enhancing pairing. Because there is no evidence that the specificity of pairing can be provided by Hfq, it seems possible that the initial pairing is stabilized by these Hfq interactions, rather than initiated by Hfq interactions. Hfq may then cycle on or off DsrA and the target mRNA, to be used again, and, in some cases, to reveal RNase E cleavage sites. While DsrA functions as a positive regulator on *rpoS*, many other small RNAs also depend upon Hfq, but lead to rapid degradation of their target messages; in those cases, displacement of Hfq would be important to allow access of the ribonuclease. It is possible that the relatively extensive base-pairing of DsrA and *rpoS* represents one of the least Hfq-dependent small RNA targets, while for other pairs of sRNA and mRNA, Hfq stimulation of pairing will be more critical *in vitro* as well as *in vivo*. This would be consistent with the observation of some Hfq-independent DsrA activity *in vivo* (Sledjeski *et al.*, 2001). Whether DsrA is normally limiting, and, if so, how its use is partitioned between *rpoS*, *hns*, and possible other targets has not been explored at all.

Regulation of DsrA Synthesis and Activity

Perhaps the most difficult aspect of working with small RNAs is assigning them a physiological role. No small regulatory RNA has yet been found to be essential in *E. coli*. *hfq* mutants of *E. coli*, defective in stability and activity of many small RNAs, are also viable, albeit slow-growing. Thus, the small RNAs may act in many cases as regulators under stress conditions or in developmental pathways, but not necessarily as critical components of vegetative growth. Consistent with a role under specific conditions, synthesis of most of these trans-acting regulatory RNAs is tightly regulated. Therefore, it is critical to find the environmental and/or physiological signals that lead to their expression to understand when the

RNA is likely to be important. Even when conditions of expression are well understood, defining the range of targets poses difficulties. The state of our information on these two issues is discussed, both generally and with respect to DsrA specifically.

DsrA was first defined by its stimulation of capsule synthesis. Because capsule synthesis occurs at low temperature, the effect of DsrA on RpoS expression at low temperature was investigated. This led to two major findings: RpoS levels increase significantly at low temperature, even in exponential phase, and this increase is completely dependent upon DsrA (Sledjeski *et al.*, 1996). DsrA stimulation of RpoS at low, but not high, temperatures might reflect differences in synthesis, stability, and/or activity as a function of temperature. While additional effects on DsrA activity have not been ruled out, DsrA RNA accumulated at low temperature. Both synthesis and degradation were found to contribute to the higher DsrA levels (Repoila & Gottesman, 2001).

Tests of the *dsrA* promoter fused to the *lacZ* reporter gene indicated that the promoter was more active at 25°C than at 37°C or 42°C, in comparison to the *lacUV5* and λ pL promoters, which were generally most active at 37°C and only slightly less active at 42°C and 25°C. Only the region from -35 to +1 was necessary for the temperature regulation (Repoila & Gottesman, 2001).

Promoter elements in addition to those required for temperature regulation were also identified. Promoter activity at all temperatures is enhanced by an UP element, present in promoters that contain the region from -46 to +1. Previous work had demonstrated that the transcriptional regulator LeuO decreases RpoS synthesis when overexpressed, and acts by decreasing DsrA synthesis, in a temperature-independent manner (Klauck *et al.*, 1997). LeuO action was localized to a region from -64 to -46 relative to the start of transcription of *dsrA* (Repoila & Gottesman, 2001). Whether LeuO at normal levels ever has an important role in regulating DsrA is not known, but *leuO* mutants have no obvious decline in *dsrA* promoter activity.

An in-depth analysis of the *dsrA* promoter was done using chimeric constructs between various regions of the *dsrA* and the *lacUV5* promoters. The data suggest that the *dsrA* promoter becomes inactive at higher temperatures rather than being induced at low temperature. Analysis of the regions within the promoter necessary for temperature regulation suggest that temperature

regulation is complex. However, the most critical element appeared to be the unusual -10 region, TAAGGT (rather than the consensus TATAAT sequence). Single nucleotide changes to bring this sequence closer to consensus (TAAAGT or TAAGAT) or to otherwise change the GG sequence (TAACCT) all led to promoters able to act at high as well as low temperatures. This -10 is absolutely dependent upon a spacer of 17 nt for activity even at low temperature; many but not all changes in sequence of the spacer are tolerated. Finally, the distance between the -10 box and the start of transcription is also unusually short and, in conjunction with this -10 box, confers temperature dependency to the promoter. While these data do not completely eliminate the possibility of a protein factor being required for temperature regulation of the promoter, they are most consistent with a scenario where the geometry and supercoiling of the promoter DNA are responsible for low temperature activity. The regulatory information for DsrA synthesis, and therefore for RpoS synthesis, is thus thought to be intrinsic to the cellular environment that affects RNA polymerase interaction with this promoter.

Targets for DsrA Action

The initial targets of DsrA were determined as a result of the serendipitous observations on the phenotypes of overproduction of this RNA as discussed earlier. Increased capsule synthesis was traced to the anti-HNS activity of DsrA, and the increase in expression of a fusion to a neighboring gene as cells entered stationary phase was traced to the positive effect of DsrA on RpoS translation (Sledjeski & Gottesman, 1995; Sledjeski *et al.*, 1996). Are these the only targets of DsrA, and, if not, how can one identify others? This is a general problem faced in the analysis of the many other small RNAs that act by pairing to mRNAs.

Two general approaches, each with its own problems, may define targets for a given small regulatory RNA. The first approach is to use computational means to predict potential pairing between the small RNA and other RNAs in the cell. In general, the region of pairing between the small RNA and its target can be anywhere from 7 to 20 nucleotides; whereas 15 to 20 nucleotide matches may be possible to find computationally, shorter matches are much more difficult. Programs used for such searches also do not usually recognize a G-U pairing even though this pairing occurs in RNA.

While this problem can be addressed, some small RNAs (such as OxyS on *fhfA* and RprA on *rpoS*, as well as DsrA on *bms*) have two regions of pairing with their target, separated by stretches of unpaired nucleotides on either the small RNA or the target message (Argaman & Altuvia, 2000; Lease & Belfort, 2000a; Majdalani *et al.*, 2002). Nevertheless, Lease and colleagues used this approach to predict several potential targets (Lease *et al.*, 1998), most of which have not yet been confirmed experimentally. Computational approaches have also been useful in finding targets for RyhB (Massé & Gottesman, 2002) and Spot 42 (Møller *et al.*, 2002b), and are likely to improve as we learn more about the behavior of these small RNAs.

The second general approach to identifying targets is to look for the outcome of small RNA expression. This can be done most globally using either microarrays or proteomic approaches. Microarrays can be useful in identifying potential direct targets if the target mRNA is expressed under the growth condition to be tested and if the small RNA leads to rapid target mRNA degradation, as is true for a number of small RNAs. For instance, RyhB expression causes a rapid decrease in target mRNA abundance by 5- to 10-fold (Massé & Gottesman, 2002), but in other cases the changes in target mRNAs may be less or absent.

In the case of DsrA, while *bms* mRNA is destabilized when DsrA is expressed, the effects are not as dramatic as those for RyhB (Lease & Belfort, 2000b). In addition, because DsrA regulates RpoS and HNS, both themselves global regulators, detecting what is a direct and what is an indirect effect of DsrA can be particularly daunting. A recent publication by Lease and colleagues reporting a role of DsrA in acid resistance illustrates the complexity of interpreting array data and distinguishing direct from indirect DsrA targets (Lease *et al.*, 2004). The authors used microarrays to look at the profile of *E. coli* transcripts upon overproduction of DsrA; two genes whose expression was significantly elevated, *hdeA* and *hdeB*, as well as a number of other genes less dramatically affected, such as *gadA* and *gadX*, are involved in acid resistance. A *dsrA* mutant affected in its ability to pair with the *bms* mRNA did not show the increase in these transcripts, consistent with a role for DsrA and not other plasmid sequences.

Finally, a *dsrA* mutant was much more sensitive to acid treatment than a wild-type strain. Both of the major known DsrA targets have an effect on acid resistance,

with HNS acting negatively and RpoS acting positively (see Waterman & Small, 2003), so this result would not be unexpected. Consistent with an indirect effect, both the *gad* genes and *hde* genes are known to be regulated by RpoS and/or HNS (Arnqvist *et al.*, 1994; Waterman & Small, 2003). These genes are also part of a complex regulatory network (Masuda & Church, 2003). Thus, it is quite possible that the effects of DsrA on the acid resistance genes are indirect. Nonetheless, the results do demonstrate a role for DsrA, direct and/or indirect, in acid resistance.

Given that DsrA is already known to directly regulate two genes (*bms* and *rpoS*) in a direction consistent with increased acid resistance, does it also have other direct targets with a role in acid resistance? To answer this, it is necessary to sort out direct from indirect effects. While this is not simple, it is possible. For instance, in the case of DsrA effects on acid resistance, Lease and coworkers found that a plasmid expressing a mutant form of DsrA unable to pair with *bms* and shown previously, under other conditions, to have decreased stimulation of RpoS, partially complements the acid-sensitive phenotype of the *dsrA* mutant, which led them to suggest targets other than RpoS and HNS were involved (Lease *et al.*, 2004). Such specificity mutants have the potential to define direct versus indirect effects. No measurements of either RpoS or HNS levels were carried out in these strains to conclusively determine whether the mutant DsrA is affecting these proteins under the acid shock conditions. It is possible, for instance, that even the mutant DsrA, in combination with other environmental cues from low acid, stimulates RpoS and/or represses HNS sufficiently to complement. In a somewhat parallel case, osmotic shock induction of RpoS is dependent upon DsrA at 37°C, although *dsrA* mutants have very little effect on basal levels of RpoS at 37°C, suggesting that even sub-optimal levels of this small RNA may function effectively under some conditions (Majdalani *et al.*, 2001). A particularly useful experiment would have been to determine if the *bms* allele that restores pairing with the mutant *dsrA* would restore full complementation of acid resistance (and restore induction of the acid resistance genes). If so, this would provide evidence that the acid sensitivity was a result of the anti-HNS phenotype of DsrA; if instead complementation was not restored, the role of *bms* could be ruled out. Fully understanding how DsrA (and other small RNAs) acts will require sorting out such direct and indirect targets.

Although arrays are particularly useful when small RNAs affect the level of their target mRNAs, other approaches can be useful because they are independent of the exact mechanism of action. Studying growth under various conditions and/or the expression of reporter fusions under conditions of over-expression or absence of the small RNA has been productive in a number of cases, although they too are not guaranteed to give direct rather than indirect targets. The identification of a phenotype allows further genetic experiments to work back to the direct targets. The inability of cells overproducing RyhB to grow on succinate (Massé & Gottesman, 2002) and the inability of cells overproducing SgrS to grow on glucose (Vanderpool and Gottesman, 2004) were critical clues to their function, and in fact provided direct targets for these small RNAs.

Physiological Roles of DsrA and Other Regulatory RNAs in Regulation of RpoS

What does DsrA do? DsrA is expressed best at low temperatures (around 25°C). Promoter studies confirmed that the promoter is active at low temperatures and decreases in activity at higher temperatures. Furthermore, the expression of RpoS is DsrA-dependent at 25°C. All this would suggest that DsrA is more important for growth at low temperature or under other conditions that might have similar effects on the *dsrA* promoter. From these data we can start to form an image of the physiological role of DsrA: cold temperatures are perceived as a stressful event for the bacterium and it has to respond accordingly. This predicts that among the genes activated by RpoS and/or repressed by HNS will be genes important for low temperature survival or growth. One such set of genes are those necessary for the production of trehalose, a disaccharide that seems to protect bacterial cells against cold damage. The *otsA* and *otsB* mRNA levels show a marked increase when cells are grown at 4°C. This leads to an accumulation of trehalose in those cells and results in an increased viability at low temperature (Kandror *et al.*, 2002). Trehalose synthesis is dependent on RpoS. While Kandror and coworkers did not test the DsrA dependence of this synthesis, RpoS expression at low temperature is dependent on DsrA (Sledjeski *et al.*, 1996), so we would predict that *dsrA* mutants should be defective in trehalose synthesis and therefore in cold resistance. Whether other

RpoS-dependent genes are also needed at low temperature is not known.

The effects of DsrA on HNS and HNS-regulated genes have been observed when DsrA is overproduced. Whether this regulation is seen under physiologically relevant conditions is not yet known. Other postulated targets for DsrA (Lease *et al.*, 1998) also remain to be explored, although preliminary data from microarrays (Lease *et al.*, 2004) as well as other data from this lab supports a role in regulation of the *rbs* operon (J. Wright, Y. Zhou, & S. Gottesman, unpublished observations). The ability of a small RNA like DsrA to regulate multiple targets provides a novel mechanism of coordination of gene expression, independent of the transcriptional regulation of the individual targets. If this coordination is important, we would predict that the conditions which require DsrA-dependent RpoS translation would also require or be helped by the regulation of other DsrA targets. DsrA is not the only small RNA that regulates RpoS translation. A *dsrA* mutant has decreased activity of an RpoS-LacZ reporter fusion, a fact that was used to screen for suppressors of the *dsrA* null mutation. The multicopy screen yielded RprA, a second small RNA that is capable of stimulating RpoS translation by pairing to similar regions in the *rpoS* 5' UTR as DsrA (Figure 2B) (Majdalani *et al.*, 2001; Majdalani *et al.*, 2002). However, since cells do not become mucoid when overexpressing RprA, we conclude that RprA does not have the same additional targets (*bms*) as DsrA. RprA also differs from DsrA in when it is made. It is under the tight regulation of the RcsC/RcsD/RcsB phosphorelay, which is activated by thus-far undefined stresses to the cell surface (Majdalani *et al.*, 2002). Presumably, yet other RpoS targets are important for dealing with this stress. OxyS, another small RNA, negatively regulates RpoS translation, possibly by competing for Hfq (Zhang *et al.*, 1998). Finally, yet other small RNAs may stimulate RpoS. Two other small RNAs detected in a global search in *E. coli* were able to stimulate expression of an RpoS-LacZ reporter and are currently under study (Wassarman *et al.*, 2001). These small RNAs share with RprA and DsrA the ability to pair with the *rpoS* 5' UTR, but share little else, possibly reflecting their activities on other targets, presumably important under the specific conditions of synthesis of that small RNA. This pattern of multiple small RNAs acting on a single target is certainly not unique. In *V. cholerae* and other *Vibrio* species, four small RNAs are an essential part of the pathway for signaling in response to quorum sens-

ing; in this case, at least part of the regulatory signals for making the small RNAs are the same, and only deletion of all four RNAs abolishes the downstream effect (Lenz *et al.*, 2004). In *P. aeruginosa*, tandem small RNAs are part of the response to iron limitation (Wilderman *et al.*, 2004). Nonetheless, the diversity of small RNAs regulating *rpoS* stands as a special case.

Future Directions and Unanswered Questions

While DsrA is among the best-studied of the small RNAs, there is still much we do not understand. What is required for efficient competition between DsrA and sequences around the *rpoS* binding site for binding to the inhibitory stem of the *rpoS* message? The importance of a few pairing regions have been tested, but the contribution of others, if understood, might help in evolving more general rules for the pairing of other regulatory RNAs with their target mRNAs. One example of how a more detailed study of DsrA can be useful is a recent study aimed at perfecting RNA regulators for biotechnology uses (Isaacs *et al.*, 2004).

Where did DsrA and related regulatory RNAs come from? Are they recently evolved regulators? DsrA itself is conserved in closely related organisms, as is RprA. It is not yet clear whether RpoS, which is quite widespread in bacteria, is regulated by comparable mechanisms in some less closely-related organisms.

PROTEIN-BINDING RNAs: CsrB/CsrC/CsrA

A class of untranslated RNA molecules that function in an Hfq-independent fashion is exemplified by the CsrB/C RNAs of the carbon storage regulatory system of *E. coli*. Rather than mediating their effects through base-pairing with other RNA molecules, these RNAs function by binding to the global regulatory protein CsrA and inhibiting its activity (Romeo, 1998). Other members of this family of regulatory RNAs include homologs of CsrB/C in other organisms that also bind to and inhibit the function of CsrA-like proteins. Another regulatory RNA that modulates the function of a protein is the 6S RNA, which forms a structure that may mimic an open promoter complex that is recognized and bound by the RNA polymerase holoenzyme containing the vegetative sigma factor, σ^{70} (Wassarman & Storz, 2000).

Function of CsrA-Like Proteins

Because the primary function of non-coding RNAs of the CsrB/CsrC family is to inhibit the activity of their target CsrA-like proteins, the physiological processes that are controlled by these regulatory proteins in various organisms must first be considered. A global regulator of carbon metabolism, CsrA (Carbon Storage Regulator), was first identified by transposon mutagenesis followed by screening for mutants with altered glycogen accumulation phenotypes (Romeo *et al.*, 1993). A mutant strain with greater than 20-fold higher levels of glycogen than wild-type was identified and the transposon insertion was mapped to a small open reading frame (ORF) at approximately 60 minutes on the *E. coli* chromosome, between the *alaS* and *serV* genes. This ORF encoded a 61-aa polypeptide that was designated CsrA (Romeo *et al.*, 1993). CsrA mutants display a pleiotropic phenotype, with alterations in gluconeogenesis (Sabnis *et al.*, 1995) and glycogen accumulation, a larger cell size and altered cell surface properties (Romeo *et al.*, 1993). CsrA mutants are non-motile (Wei *et al.*, 2001) and show increased adherence properties and the tendency to form biofilms (Jackson *et al.*, 2002; Romeo *et al.*, 1993).

CsrA functions as a negative regulator of genes involved in gluconeogenesis and glycogen biosynthesis and catabolism (Liu *et al.*, 1995; Romeo *et al.*, 1993; Yang *et al.*, 1996). The mechanism of negative regulation has been examined in two cases. For the *glgC* gene, encoding a glycogen biosynthetic enzyme, CsrA binds to two sites in the 5' region of the message; one site overlaps the ribosome binding site, and the other is positioned 25 nt upstream of the ribosome binding site within a short hairpin structure (Baker *et al.*, 2002). Binding of CsrA to these two sites within the *glgC* leader sequence blocks ribosome binding and translation of the message, and also results in destabilization of the *glgC* mRNA (Liu *et al.*, 1995). Another target of CsrA negative regulation is the *cstA* mRNA, which encodes a peptide transporter induced by carbon starvation (Schultz & Matin, 1991). CsrA has been found to bind to the *cstA* mRNA leader region and block ribosome access (Dubey *et al.*, 2003). The effect on *cstA* mRNA stability as a result of CsrA binding and translational inhibition has not been examined. Cells lacking CsrA express higher levels of activity of several enzymes required for gluconeogenesis, including fructose-1-6-bisphosphatase, phosphoenolpyruvate

synthase, and phosphoglucomutase (Sabnis *et al.*, 1995), suggesting that these are additional targets of CsrA negative regulation. However, it is currently unclear whether this effect is mediated directly through CsrA binding to the mRNAs encoding these enzymes.

CsrA-dependent positive regulation of target genes has been reported, but the mechanism has been examined in only one case. The finding that *csrA* mutants were non-motile (Wei *et al.*, 2001) suggested that CsrA may be required for positive regulation of genes involved in flagellar biosynthesis. Expression of the *flhDC* genes, encoding the master regulators of flagellar biosynthesis, was found to be directly stimulated by CsrA, using FlhDC'-LacZ translational fusions, and coupled *in vitro* transcription-translation of *flhDC* in extracts with or without CsrA. Analyses of the *flhDC* mRNA showed that this message was three- to four-fold more abundant in wild-type cells compared with *csrA* mutant cells, consistent with the observation of an approximately three-fold decreased *flhDC* mRNA half-life in the mutant cells. RNA gel mobility shift assays showed specific binding of CsrA to the *flhDC* 5'-leader sequence, suggesting that stabilization of *flhDC* mRNA is indeed a direct consequence of CsrA recognition and binding of this transcript (Wei *et al.*, 2001). Other positively regulated targets included some glycolytic enzymes, including phosphoenolpyruvate carboxykinase, glucose-6-phosphate isomerase, and triose phosphate isomerase; their activity was shown to be stimulated by CsrA but the mechanism is unknown (Romeo *et al.*, 1993; Sabnis *et al.*, 1995).

Similar regulators have been identified and studied in a number of other gram-negative bacteria. The enterobacterium *Erwinia carotovora* subspecies *carotovora* (*Ecc*) is a plant pathogen that causes soft-rot disease in a variety of plants. Various extracellular enzymes produced by the bacterium in response to quorum-sensing signals (N-acyl homoserine lactones, AHLs) are required for the pathogenesis of the organism (Collmer & Keen, 1986). In a genetic screen for transposon mutants that produced these virulence factors in a cell-density independent fashion, the *rsmA* (Repressor of Secondary Metabolites) gene was identified. Cells lacking RsmA produced the extracellular enzymes required for *Ecc* pathogenesis in the absence of AHLs and produced a more severe soft-rot disease than wild-type *rsmA*⁺ bacteria (Chatterjee *et al.*, 1995). Sequencing of the *rsmA* locus revealed that the 61 aa *Ecc* RsmA protein was 95% identical to the *E. coli* CsrA protein. In fact,

heterologous complementation studies showed that the *Ecc rsmA* gene could complement an *E. coli csrA* mutant strain to restore glycogen accumulation to wild-type levels (Cui *et al.*, 1995), indicating that RsmA and CsrA were functionally interchangeable. While the level of homology between *E. coli* CsrA and *Ecc* RsmA suggests that the mechanism of RsmA action on its target genes may be similar to that of CsrA, this has not been conclusively demonstrated. However, in one study, it was found that mRNA levels of an *Ecc* gene *brpN_{Ecc}*, which encodes a molecule that elicits a hypersensitive reaction in plant hosts, were greatly increased in an *rsmA* mutant compared with the wild-type strain (Cui *et al.*, 1996), consistent with the idea that RsmA negatively modulates the expression or stability of its target transcripts.

In *Pseudomonas aeruginosa*, an opportunistic human pathogen, RsmA was identified by homology to CsrA of *E. coli* and RsmA of *Ecc* (Pessi *et al.*, 2001). The *P. aeruginosa* RsmA is a 61 aa protein sharing 92% identity with CsrA of *E. coli*, and can functionally complement an *E. coli csrA* mutant for the glycogen accumulation phenotype (Pessi *et al.*, 2001). Similar to the role of RsmA in *Ecc*, in *P. aeruginosa*, RsmA overexpression reduced the production of secreted virulence factors by more than 80%, suggesting that RsmA may negatively regulate the genes encoding these factors. Further, the levels of AHLs in *P. aeruginosa*, known to be involved in positive control of virulence factors, were also reduced by RsmA overexpression. However, addition of exogenous AHLs to RsmA-overexpressing cells failed to restore production of these factors, suggesting that RsmA also has a direct role in regulating virulence gene expression (Pessi *et al.*, 2001). In addition to these negative regulatory effects, RsmA has also been reported to positively regulate expression of rhamnolipids, which are required for swarming motility in *P. aeruginosa* (Heurlier *et al.*, 2004). In *Pseudomonas fluorescens* CHA0, a plant symbiont, the RsmA protein has also been shown to influence production of secreted products and secondary metabolites (Blumer *et al.*, 1999; Heeb *et al.*, 2002). To date, none of these regulatory effects have been demonstrated to be the result of a direct interaction between *Pseudomonas* RsmA and mRNA transcripts. Recently, a second CsrA-like protein that works in the same pathways, RsmE, was also identified in *P. fluorescens* (Reimann *et al.*, 2004).

CsrA/RsmA functional homologs have been identified in a variety of other bacterial species (White *et al.*,

1996), and their global regulatory roles are just beginning to be elucidated. In *Salmonella enterica* serovar Typhimurium, CsrA is involved in regulation of genes required for invasion of eukaryotic cells (Altier *et al.*, 2000). In the intracellular pathogen *Legionella pneumophila*, CsrA was found to repress traits involved in transmission, including motility and pigment production (Fettes *et al.*, 2001; Molofsky & Swanson, 2003). Other preliminary reports have identified and examined the physiological role of CsrA homologs in the bacteria *Helicobacter pylori* (Barnard *et al.*, 2004), *Proteus mirabilis* (Liaw *et al.*, 2003), and *Serratia marsescens* (Ang *et al.*, 2001). Furthermore, when the *E. coli* CsrA protein is used as a query in a BLAST search at the NCBI, CsrA homologs are found in a broad range of bacterial species, including but not limited to *Yersinia* species, *Photobacterium luminescens*, *Shewanella oneidensis*, *Azotobacter vinelandii*, *Vibrio* species, *Xanthomonas campestris*, *Coxiella burnetii*, and *Haemophilus influenzae*.

Regulatory RNAs That Act on CsrA Family Proteins

Non-coding RNA molecules have been found to modulate the activity of CsrA/RsmA proteins; they appear to work by binding to the CsrA proteins, in competition with the mRNA targets that the proteins work on. We consider the available data on the mode of action in the next section. CsrB was the first RNA of this class to be described, and it was discovered fortuitously by virtue of its strong interaction with purified CsrA protein (Liu *et al.*, 1997). Other CsrB-like RNAs were found as multicopy suppressors of CsrA overproduction phenotypes; these include CsrC of *E. coli* (Weilbacher *et al.*, 2002), PrrB of *P. fluorescens* F113 (Aarons *et al.*, 2000), and RsmB (formerly designated *aepH* locus) of *Ecc* (Liu *et al.*, 1998; Murata *et al.*, 1994). Two regulatory RNAs that control RsmA activity in *P. fluorescens* CHA0 were designated RsmZ and RsmY. RsmZ was identified by a direct search for RNAs bound to the *P. fluorescens* RsmA protein (Heeb *et al.*, 2002). RsmY was identified computationally (Valverde *et al.*, 2003) in *P. fluorescens* and *P. aeruginosa* by searching for a locus homologous to one from *Pseudomonas syringae* (Rowley *et al.*, 1993) that had characteristics similar to other CsrB-like riboregulators. Likewise, RsmZ of *P. aeruginosa* was identified based on its conserved sequence and genomic location compared with PrrB of *P. fluorescens* (Heurlier *et al.*, 2004). For the purposes of this review, the properties of these RNA

(Liu *et al.*, 1997). The gene encoding CsrB was identified by cDNA synthesis, cloning and sequencing. The *csrB* gene is located at 64 minutes on the *E. coli* chromosome between the *syd* and *yqcC* genes, more than 100 kb away from *csrA*, and encodes a 366-nt molecule with no significant open reading frames. Within the *csrB* coding region, eighteen copies of a repetitive sequence (consensus 5'-CAGGA{U/C/A}G-3') were observed, and when the structure of the CsrB molecule was predicted, the majority of these consensus sequences occurred in single stranded regions of the molecule, predominantly in the loops of predicted stem-loop structures. This finding, combined with the 18:1 CsrA:CsrB stoichiometry, led to the hypothesis that these repeated sequences constituted the CsrA binding site (Liu *et al.*, 1997). Consistent with this idea, similar sequences in the *glgC* leader region were shown to be required for CsrA binding and translational repression (Baker *et al.*, 2002).

A second RNA molecule involved in the regulation of CsrA activity was identified in a genetic screen for plasmids carrying genomic regions that increased glycogen accumulation in *E. coli* cells (Weilbacher *et al.*, 2002). This RNA was called CsrC. The *csrC* gene encodes a 245 nt RNA molecule and is located between the *yihA* and *yihI* genes, far from either *csrA* or *csrB*. Putative σ^{70} -like promoter elements and a Rho-independent transcriptional terminator were identified upstream and at the 3'-end of the *csrC* coding sequence, respectively. Like CsrB, the CsrC molecule contained repetitive sequences similar to those found in CsrB and predicted to constitute CsrA binding sites; however, in CsrC there were only nine repeats (instead of 18 as in CsrB).

Although CsrA-like proteins are readily identified in many sequenced bacterial genomes using BLAST analysis, it has not been possible in many cases to identify CsrB/C homologs by nucleotide sequence similarity. While it is possible that CsrA-like proteins in some organisms are regulated by mechanisms not mediated by non-coding RNAs, the more likely explanation is that the nucleotide sequence of these RNAs has not been conserved in evolution while the structure and function has. Several examples supporting this theory of conserved structure and mechanism for CsrB/C-like RNAs will be briefly described.

The *Ecc* RsmB RNA exists as two different species in the cell: a long 479 nt full-length species (RsmB) and a 258 nt species ('RsmB) that comprises the 3' end of full-length RsmB (Liu *et al.*, 1998). 'RsmB is processed from

full-length RsmB and is the more abundant species *in vivo*. This 3' RsmB region was both necessary and sufficient to antagonize RsmA activity in *Ecc* and CsrA activity in *E. coli* (Liu *et al.*, 1998). The overall level of nucleotide sequence similarity between RsmB and CsrB is low. However, RsmB does contain repeated sequences similar to those proposed to be involved in CsrA binding in *E. coli*, which may account for the ability of *Ecc rsmB* to complement *E. coli csrB* mutants. The role of RsmB processing in the regulation of RsmB activity in *Ecc* has not yet been examined.

The regulatory RNA molecule PrrB, in *P. fluorescens* F113, bears little sequence similarity to *E. coli* CsrB and CsrC. The PrrB molecule is only 133 nt long (Aarons *et al.*, 2000), considerably smaller than the CsrB, CsrC, and *Ecc* RsmB molecules. However, secondary structure predictions showed a PrrB structure with eight stem-loops containing nine imperfect 5'-AGGA-3' repeats predominantly in the loops or other unpaired regions of the RNA, a configuration strikingly similar to that predicted for CsrB and CsrC.

The RsmZ RNA of *P. fluorescens* CHA0 was initially found using an experiment to identify RNAs bound to the *P. fluorescens* CHA0 RsmA protein (Heeb *et al.*, 2002). Cloning and sequencing of the *rsmZ* gene revealed that it shared 72% identity with PrrB of *P. fluorescens* F113 and was also localized to the same genomic region, between the *rpoS* and *fdxA* genes. Like PrrB, RsmZ of *P. fluorescens* CHA0 showed very little sequence similarity to *E. coli* CsrB or *Ecc* RsmB; however, the conserved stem-loop structure with repetitive sequence motifs was present. The RsmZ RNA exists *in vivo* as a full-length 127 nt species and a 92 nt species lacking the 3' terminator region. The contribution of each of these molecules to RsmZ function has not been described. The second RsmA activity-modulating RNA in *P. fluorescens* CHA0 is RsmY, which was identified by computational prediction (Valverde *et al.*, 2003). In *P. fluorescens* CHA0, the *rsmY* gene was found to be located in an intergenic region between a putative acyl-CoA dehydrogenase (homologous to PA0508 in *P. aeruginosa*) and a putative LysR-like transcriptional regulator (homologous to PA0528 in *P. aeruginosa*). The *rsmY* gene is also found in this genomic location in other sequenced *Pseudomonas* species. The RsmY RNA is 118 nt and like RsmZ, is predicted to contain stem-loop secondary structures with characteristic repeat sequences in the loops and unpaired regions of the molecule (Valverde *et al.*, 2003).

As in the other *Pseudomonas* species, the *P. aeruginosa* *rsmZ* gene was identified in the *rpoS-fdxA* intergenic region (Heurlier *et al.*, 2004). While RsmZ sequence conservation among pseudomonads is only ~45%, the *P. aeruginosa* RsmZ also shows the conserved predicted structure and presence of repeated sequences in unpaired regions of the RNA.

Mechanism of CsrB/CsrC Action

Several lines of evidence point to a CsrA binding and titration mechanism for CsrB/CsrC activity (Figure 3B). First, as described above, CsrB/C-like RNAs have been identified by virtue of their binding to CsrA homologs (Heeb *et al.*, 2002; Liu *et al.*, 1997). Romeo and coworkers found that each CsrB RNA was capable of binding 18 CsrA molecules (Liu *et al.*, 1997), consistent with the idea that CsrB has the capacity to titrate CsrA away from mRNA targets. *In vivo* overexpression and mutant studies support this picture. Overexpression of CsrB/C-like RNAs result in phenotypes resembling those of *csrA* mutants. For example, overexpression of CsrC in *E. coli* causes a substantial increase in glycogen levels, similar to what is observed in *csrA* mutants (Weilbacher *et al.*, 2002). Overexpression of RsmZ in *P. aeruginosa* caused a loss of swarming ability, decreased lipase production, and increased pyocyanin production, all phenotypes of *rsmA* mutant cells (Heurlier *et al.*, 2004). Conversely, deletion of CsrB/C-like RNAs usually results in CsrA “hyperactive” phenotypes, although in at least two cases this is only seen when both CsrB/CsrC genes are deleted, suggesting functional redundancy (Valverde *et al.*, 2003; Weilbacher *et al.*, 2002). Finally, *in vitro* experiments examining translation of a CsrA target mRNA, *glgC*, found that RNA-free CsrA preparations were more inhibitory to *glgC* translation than CsrA-CsrB complexes (Liu *et al.*, 1997), suggesting that CsrB competes with mRNAs for CsrA binding. Whether some messages are more susceptible to competition than others is not yet clear.

Regulation of *csrB/csrC* Expression

Because the proposed function of CsrB/C involves antagonism of CsrA activity, the ratio of CsrA:CsrB/C is likely to be crucial to the regulation of CsrA activity. Thus, it follows that regulation of CsrB/C synthesis and/or stability could be important regulatory checkpoints that dictate the expression of CsrA-regulated

genes. Primer extension analysis to define the 5' end of the CsrB RNA showed that the promoter of the *csrB* gene shows significant similarity to σ^{70} -like promoters (Gudapaty *et al.*, 2001). Northern blot analysis of CsrB levels throughout the growth curve showed that CsrB RNA levels rise modestly (three-fold) as wild-type cells enter stationary phase. Strikingly, in *csrA* mutant strains, CsrB levels were diminished approximately ten-fold compared to the wild-type strain. The decrease in steady-state CsrB levels in *csrA* mutants was not due to altered stability of CsrB in the absence of CsrA protein; the chemical half-life of CsrB was equivalent in wild-type and *csrA* null strains (Gudapaty *et al.*, 2001). *In vivo* and *in vitro* studies provided evidence that CsrA indirectly affects transcription of *csrB*, possibly through post-transcriptional regulation of a transcription factor required for full *csrB* expression; the result is a negative autoregulatory loop in which uncomplexed CsrA increases the level of its negative regulatory RNA.

In *Pseudomonas* species and *Ecc*, a two-component signal transduction system, GacA/GacS, had been implicated in regulation of RsmA targets (Blumer *et al.*, 1999) or directly shown to regulate expression of *rsmB* (Aarons *et al.*, 2000; Cui *et al.*, 2001; Heeb *et al.*, 2002). A homologous two-component system, composed of the BarA sensor kinase and UvrY response regulator, was likewise found to control expression of *csrB* in *E. coli* (Suzuki *et al.*, 2002). In a *csrA* mutant strain with decreased *csrB* expression, *csrB* transcription could be restored with either *csrA* or *uvrY* in trans. In a *uvrY* mutant, on the other hand, *csrB* expression was nearly eliminated and could only be complemented by *uvrY* in trans. *In vitro*, UvrY was also found to activate *csrB* transcription (Suzuki *et al.*, 2002), strongly suggesting a direct role for UvrY in regulation of *csrB*.

Like CsrB, CsrC levels were also diminished in *csrA* and *uvrY* mutant strains (Weilbacher *et al.*, 2002), and as with *csrB* expression, the effect of CsrA appeared to be indirect, while UvrY directly stimulated *csrC* transcription *in vitro*. Interestingly, mutation of either *csrB* or *csrC* resulted in an apparent compensatory increase in expression of the remaining gene (Weilbacher *et al.*, 2002), though the mechanism of this compensation was not determined.

Although these studies have genetically defined factors involved in regulation of *csrB/csrC* expression, the signal causing BarA/UvrY-dependent transcriptional activation of these genes remains unknown. Likewise, the role of CsrA in influencing *csrB/C* transcription

has not been investigated; a reasonable model may be that CsrA positively regulates translation of some as yet unidentified gene involved in this regulatory circuit.

As mentioned above, the involvement of BarA/UvrY in the regulation of *csrB/C* of *E. coli* was first hypothesized based on comparison to regulatory studies in *P. fluorescens* and *Ecc* showing the role of GacAS in regulation of *rsmB* (*Ecc*) and *rsmZ* (*P. fluorescens* CHA0). In these organisms, *gacAS* mutants were found to have phenotypes similar to those of RsmA-overexpressing strains; that is, expression of RsmA-target genes was significantly lower than in wild-type strains (Blumer *et al.*, 1999; Cui *et al.*, 2001; Heeb *et al.*, 2002; Hyytiainen *et al.*, 2001). In *P. fluorescens* CHA0, the GacAS system apparently senses and responds to a non-AHL quorum sensing signal (Heeb *et al.*, 2002), the chemical nature of which remain unknown. Thus, expression of the genes *rsmY* and *rsmZ*, the two RsmA-modulating RNAs in *P. fluorescens* CHA0, increases as a function of cell density, *i.e.*, when the signal accumulates in the medium (Heeb *et al.*, 2002; Valverde *et al.*, 2003). Overexpression of RsmY or RsmZ suppressed the *gacAS* mutation and restored expression of negatively regulated RsmA targets (Heeb *et al.*, 2002; Valverde *et al.*, 2003). In studies examining the role of RsmY 5'-GGA-3' motifs, which were predicted to constitute the core of the RsmA binding site, it was observed that mutant RsmY molecules that bound poorly to RsmA *in vitro* had a significantly reduced half-life *in vivo* (Valverde *et al.*, 2004), suggesting that in *P. fluorescens* CHA0, RsmA binding to RsmY stabilizes this RNA molecule.

In *Ecc*, the regulation of the *rsmB* gene may be more complicated than in *P. fluorescens* and *E. coli*. In addition to positive regulation of *Ecc rsmB* by GacAS, at least two other factors seem to influence transcription of *rsmB* in this organism. A transcriptional repressor Kdgr_{*Ecc*} was shown to repress expression of *rsmB* (Liu *et al.*, 1999), while a 130 aa protein designated RsmC had a positive effect on *rsmB* transcription (Cui *et al.*, 1999). The signals to which these regulators are responding in order to modulate *rsmB* expression have not been elucidated.

Unanswered Questions and Issues

The family of CsrA-related proteins is widespread in bacteria and regulate a variety of important functions. It seems likely that where CsrA-family proteins are found, the regulatory RNAs will be as well. What remains least clear about these regulators are the conditions under

which the regulatory RNAs successfully compete for CsrA. Once a regulatory RNA has bound CsrA, is there a mechanism for dissociation and/or degradation of either RNA or protein? Is this a regulated process?

What is the advantage of redundancy in the regulatory RNAs? By comparison to what we know about pairing RNAs, such redundancy implies possible differences in both regulation of each regulatory RNA and in outcome of having a certain level of one or other RNA. This would suggest the possibility of an as yet unidentified environmental condition that enhances the expression of one regulatory RNA more than the other, coupled with a hierarchy in their ability to compete for CsrA relative to specific targets. The complexity of the possible regulatory networks is even greater when redundant regulatory proteins also exist as they do for *P. fluorescens* (Reimmann *et al.*, 2005). Future work should clarify some of these issues.

Finding Small RNAs/Targets: Past and Future Prospects

DsrA, the small pairing RNA discussed above, was found accidentally during studies of a plasmid that carried the *dsrA* gene. CsrB was found as an RNA that bound to the CsrA protein. A number of other small RNAs were either found by accident or during early searches for highly stable RNA species. More recently, some of the characteristics of these small RNAs have been used in global searches for yet others. The properties that have been useful in such searches are discussed below:

- 1) Non-coding RNAs are frequently conserved between related species, and their genes are found in intergenic regions. A number of searches have used conservation within the intergenic regions as a primary criterion (Rivas *et al.*, 2001; Wassarman *et al.*, 2001). A computer program, QRNA, has been developed to identify new small RNAs; the search parameters incorporate conservation in secondary structure elements (stems) typical of these small RNAs (Rivas *et al.*, 2001). Conservation of small RNAs is sometimes even better than that for the flanking genes, although in some cases, conserved RNAs are found in different contexts in different organisms as well (Hershberg *et al.*, 2003). It is worth noting, however, that conservation rarely extends far beyond genomes with reasonably high overall identity, at

least at the level of small RNA sequence. Thus, *E. coli* small RNA homologs are directly identifiable by homology search in *Salmonella*, *Klebsiella*, *Vibrio* and *Yersinia*, but not in *Pseudomonas*. Therefore, finding the functionally similar small RNAs in more distant species requires other approaches.

- 2) These short RNAs frequently end in rho-independent terminators; the presence of such a structure, in combination with an appropriate promoter (see below) or high conservation (characteristic 1) has successfully been used to identify small RNAs in organisms other than *E. coli* (Lenz *et al.*, 2004; Wilderman *et al.*, 2004).
- 3) The transcription of these small RNAs is highly regulated, frequently from recognizable promoters. In both *Pseudomonas aeruginosa* and *Vibrio cholerae*, searches for promoters of a specific type in combination with other characteristics allowed identification of appropriately regulated small RNAs (Lenz *et al.*, 2004; Wilderman *et al.*, 2004).
- 4) A subset of RNAs, such as DsrA, bind and use the RNA chaperone, Hfq. Immunoprecipitation of Hfq with bound RNAs, followed by characterization of the RNAs on arrays, has been used to identify and characterize *E. coli* regulatory RNAs of this family (Zhang *et al.*, 2003). Since Hfq is well-conserved in other organisms (Sun *et al.*, 2002), this approach should work in them as well, as should parallel approaches with other RNA binding proteins.
- 5) Small RNAs are small. Direct cloning of RNAs shorter than the bulk of tRNAs and mRNAs has been used in both prokaryotes and eukaryotes to identify novel non-coding RNAs (He & Hannon, 2004; Vogel *et al.*, 2003; Kawano *et al.*, 2005).

Now that small RNAs have been recognized in many organisms and methods for finding them have been developed, it seems likely that understanding regulation in the future will mean not only appreciating the importance and mechanism of transcriptional regulators, but will also require understanding what small RNAs are doing to expand, upstage and modulate the efforts of the transcriptional regulatory proteins.

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