Annu. Rev. Microbiol. 2004. 58:303–28
doi: 10.1146/annurev.micro.58.030603.123841
First published online as a Review in Advance on June 16, 2004

THE SMALL RNA REGULATORS OF *ESCHERICHIA COLI*: Roles and Mechanisms*

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Key Words Hfq, DsrA, OxyS, RyhB, Spot 42

■ **Abstract** Small noncoding RNAs have been found in all organisms, primarily as regulators of translation and message stability. The most exhaustive searches have taken place in *E. coli*, resulting in identification of more than 50 small RNAs, or 1%–2% of the number of protein-coding genes. One large class of these small RNAs uses the RNA chaperone Hfq; members of this class act by pairing to target messenger RNAs. Among the members of this class are DsrA and RprA, which positively regulate *rpoS* translation, OxyS, which negatively regulates *rpoS* translation and *fhlA* translation, RyhB, which reapportions iron use in the cell by downregulating translation of many genes that encode Fe-containing proteins, and Spot 42, which changes the polarity of translation in the *gal* operon. The promoters of these small RNAs are tightly regulated, frequently as part of well-understood regulons. Lessons learned from the study of small RNAs in *E. coli* can be applied to finding these important regulators in other organisms.

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INTRODUCTION

Escherichia coli is the organism in which researchers first identified and studied regulatory proteins, worked out most metabolic pathways, clearly recognized "regulons" and the concept of global regulatory networks, and first documented regulatory degradation of proteins. Decades of genetics, biochemistry, and, more recently, global analysis of gene expression have been documented for this organism. In the last few years, *E. coli* has again been in the forefront of a new field, the discovery and study of many new regulators, small noncoding RNAs. Small RNA regulators are proving to be multifunctional and have provided explanations for a number of previously mysterious regulatory effects.

Not surprisingly, this sort of regulator is not confined to *E. coli*. Phages and plasmids have long been recognized to use antisense RNA regulators (reviewed in References 21, 102); now these regulators are showing up in all bacterial cells, including pathogens. Quorum sensing in *Vibrio* species has now been shown to depend upon small RNA regulators (54a). In eukaryotic cells, microRNAs and RNAi parallel in many ways the bacterial small RNA regulators, confirming that this level of regulation is widespread and is as central to creating a working organism as are the protein regulators we are used to thinking about (16, 63). The intent of this review is to use what we have learned in studying small RNAs in *E. coli* to deduce some of the general principles of action and roles that these regulatory molecules are likely to play in other microorganisms. The focus is on the small subset of regulatory RNAs that have been most extensively studied. The broadest term for these molecules is noncoding RNA (ncRNA) (89). Because the bacterial noncoding RNAs are generally small, I refer to them here as sRNAs (small RNAs) or small regulatory RNAs.

THE HISTORY AND RANGE OF sRNAs IN *E. coli*

Most regulatory proteins were first identified by mutations that perturbed or abolished the regulation of a particular gene. More recently, genomic analyses have uncovered additional putative regulators, identified by their similarity to known

regulatory proteins. Neither of these approaches has contributed significantly to identification of sRNA regulators in bacteria. None of the sRNAs in *E. coli* was first found by mutation. Failure to identify sRNA regulators by classical genetics may be due to the small target size for mutagenesis, a resistance to inactivation by single nucleotide changes, and activities not always measured in studies with transcriptional reporters. The standard sequence analysis to identify open reading frames (ORFs) in genomes generally leaves the sRNAs unrecognized and unannotated. Therefore, developing ways of identifying novel sRNAs has been an important part of the recent work in this field. sRNAs have been most intensively hunted in *E. coli*, and as a result we have the clearest picture of the range of types and numbers of sRNAs in this organism. Even in *E. coli*, however, new approaches continue to uncover new regulatory sRNAs. Table 1 provides a summary of which sRNAs may be expected in bacterial genomes, grouped by functional class. If the sRNA is included in this table, then it meets two criteria:

1. It functions as an RNA directly, not as part of a message. One can imagine an RNA acting both as a coding RNA and as a functional RNA. One such example exists, in *Staphylococcus aureus* (reviewed in Reference 42). It is also clear that some RNA sequences that are parts of messages [5′ or 3′ untranslated regions (UTRs)] carry out important sensing and regulatory roles, changing their folding and behavior as a function of temperature or binding to small molecule effectors (43, 88). However, although these are clearly RNA regulators, they act solely in *cis* and their mode of action is distinct enough that they will not be covered here.
2. The transcript is expressed. For the discussion here, that means that prediction by computational means or even detection by microarray is not yet considered adequate. Confirmation by Northern blot has been most generally used. Because the majority of these regulatory RNAs are small, we expect the transcript to be less than 300 nucleotides.

The entries in Table 1 were identified by a variety of approaches, discussed in more detail below. About 12 were found in what we can call the classic phase of sRNA discovery, either by direct labeling of RNAs or by chance during other work. More recently, in the modern phase of sRNA hunting, a variety of global approaches have expanded the list to over 50; others have been predicted computationally or detected by microarray but not confirmed by Northern blot.

Metabolic Labeling and Serendipity: The Classic Phase of sRNA Discovery

As the RNA species expressed by *E. coli* were first being cataloged, a number of abundant and frequently stable RNAs were identified by metabolic labeling and direct analysis by various fractionation procedures. These included RNAs such as the 4.5S RNA, part of the secretion machinery, and the 10S RNA, later found to be the catalytic part of the RNase P ribozyme (10Sb, encoded by *rnpB*). TmRNA,

TABLE 1 Small RNAs in *E. coli*

Category	Number	Examples	Size (nt)	Mechanism/role	Regulators/comments	Reference(s)
Structural, enzymatic	2	4.5S	114	Secretion	Abundant, essential	(37)
		RNaseP	377	Ribozyme, processing	Abundant, essential	(29)
Quality control	1	TmRNA	363	Translation quality control	Abundant, uses SmpB	(111)
Protein inhibitors	3	6S	184	Inhibits RNA polymerase	Up in stationary	(106)
		CsrB	360	CsrA inhibition	BarA/UvrY	(78)
		CsrC	270	CsrA inhibition	BarA/UvrY	(107)
cis-acting antisense	9	SokA	55	HokA antitoxin	Plasmid-like?	(71)
		RdlD	66	LdrD antitoxin	Unknown	(47)
Hfq-binding, antisense	22	DsrA	85	Stimulates *rpoS* Inhibits *hns*	Low temp., LeuO	(58, 75) (53)
		RprA	105	Stimulates *rpoS*	RcsC/B phosphorelay	(59)
		OxyS	109	Anti-*rpoS*, *fhlA*	OxyR	(2, 3)
		RyhB/SraI	90	Anti-*sdh*, *sodB*	Fur	(61)
		Spot 42	109	Anti-*galK*	CRP/cAMP	(68)
		MicF	93	Anti-*ompF*	SoxR/S	(22)
		MicC	108	Anti-*ompC*	Inverse to MicF	(19, 19a)
		DicF	56	Anti-*ftsZ*	Phage promoter	(10)
Antisense	3	RyeA/SraC	275	Anti-RyeB	Unknown	(100)
Unknown	26		40–300	Multiple searches[a]		

[a]See text.

Nt, nucleotide.

which serves an important role in translational quality control (see below), was initially identified as an RNA of a size similar to RNase P RNA (10Sa RNA, encoded by *ssrA*) (54). 6S RNA, which modulates RNA polymerase activity, and Spot 42, a sRNA that acts as an antisense regulator (see below), were also detected by metabolic labeling (39). Clearly, this approach favors relatively abundant and/or stable RNAs.

The small regulatory RNAs first studied in *E. coli* were found by accident, by observation of phenotypes conferred by multicopy plasmids or by detection of RNAs during the study of particular operons. Observation that a multicopy plasmid encoding the porin OmpC also downregulated expression of a second porin, OmpF, led to discovery of *micF*, transcribed divergently from *ompC* (4, 64). The regulatory RNAs OxyS and GcvB were detected as transcripts made divergently from the genes for the LysR family regulatory proteins OxyR and GcvA in transcription studies (2, 97). The synthesis of these sRNAs is regulated by the divergent regulator protein in a manner analogous to other LysR family proteins that regulate divergent protein-encoding genes (82). DsrA was identified during studies of capsule regulation as a gene capable of increasing capsule synthesis when present on a multicopy plasmid being studied for other reasons (84). Another sRNA, RprA, was identified in a screen of a multicopy plasmid library for plasmids that suppressed a phenotype of a *dsrA* mutant (57). CsrB was found as a prominent and stable RNA species that binds to the translational regulatory protein CsrA (56). DicF, which inhibits *ftsZ* translation, is encoded within a cryptic prophage. It was found by analysis of plasmids encoding another prophage function, *dicB*, when growth inhibition was present, even when *dicB* was absent (10).

Computation and Global Detection: The Modern Phase of RNA Hunting

RNA PREDICTIONS The more we learn about the sRNAs, the closer we come to defining characteristics that will allow us to predict them from the genome sequence of an organism. The set of sRNAs found by metabolic labeling and by serendipity had some characteristics in common. These properties have served as a guide in further searches. With the exception of 6S RNA and *dicF*, sRNAs are transcribed from single gene operons. These operons (and therefore the RNAs themselves) frequently end with a rho-independent terminator (seen at the DNA level as an inverted repeat followed by a run of Ts); other stem-loops are frequently seen in the predicted structure of these RNAs. They are encoded in intergenic regions (that is, between protein coding genes). Finally, these sRNAs are highly conserved in organisms such as *Salmonella* and *Klebsiella*. These properties were used in four different global hunts for novel sRNAs. The classes of RNAs found in these searches are summarized in Table 1.

Two groups have searched within intergenic regions for conservation with near relatives (*Klebsiella* and *Salmonella*, primarily). One of these groups (105) used location of the conservation within the intergenic region, expression detected on

microarrays, and elements of structure to choose among conserved candidates for Northern blot confirmation. Another group (77) developed computational approaches to detect conservation of secondary structure (inverted repeats encoding stem-loops) within intergenic regions. Two other groups (5, 19) used computational methods to predict a promoter and a terminator within a given intergenic region with an orientation and spacing consistent with a sRNA (orphan promoters, terminators); Argaman et al. also demanded conservation for the predicted sRNA. Another group (17) carried out a purely computational search in *E. coli* using the characteristics of known ncRNAs in a learning program but did not extend the study to experimental confirmation of the large number of predicted ncRNAs.

GLOBAL ANALYSIS OF EXPRESSION Microarray analysis of transcripts from a given growth condition should reveal the existence of freestanding transcripts in places where no gene has been annotated. The first requirement is that the RNA be sufficiently expressed under the chosen growth condition to be detectable. This approach requires the use of arrays containing probes in the intergenic regions, currently available in Affymetrix oligonucleotide-based microarrays of *E. coli*. Signals detected on Affymetrix microarrays were used as secondary evidence in one conservation-based screen (105). In another study (94), a number of possible sRNAs were predicted on the basis of expression detected with the Affymetrix arrays in intergenic regions for 13 different growth conditions. Nineteen of 34 sRNAs known at the time were found in this analysis; 9 additional sRNAs were predicted, some of which have been identified as candidates in other searches. In a more recent search, RNAs bound to Hfq, an RNA chaperone previously implicated in the action of many regulatory RNAs (see below), were isolated by coimmunoprecipitation with Hfq and annealed to the microarray for identification; of 20 predicted Hfq-binding new sRNAs, 5 were confirmed by Northern blot (115). Notably, the new sRNAs that bind Hfq tightly were generally not well conserved, a probable explanation for the failure to detect them in the conservation-dependent searches and a warning that significant numbers of nonconserved but functional regulatory RNAs may exist.

Direct cloning of RNAs of a given size range is an approach that has been widely used to identify microRNAs in eukaryotic cells and archaea (16), and has also recently been applied to *E. coli* (100). This is the modern equivalent to the metabolic labeling studies done earlier; it requires nothing of the sRNA other than that it be expressed. Some of the cloned RNAs isolated by this approach are unique; others overlap with those detected by other methods. This approach may be useful for isolating sRNAs expressed at a high level under a given growth condition or for an organism for which microarrays are not available. In addition, only direct cloning is likely to find sRNAs encoded on the opposite strand to ORFs, unless a microarray with the other strand is specifically used. Even with this approach, sRNAs encoded on the sense strand of a gene are going to be difficult to distinguish from breakdown products of the relevant messenger RNAs without much further analysis. For now, such putative sRNAs may remain uncataloged.

In summary, global approaches show that a significant number of sRNAs exist in the bacterial chromosome and that both computational and expression-based methods are productive in finding them; each has its limitations. The primary limitation of most methods of detecting sRNAs is a significant bias toward the intergenic region; sRNAs within genes, on either strand, are likely to be harder to detect.

There are approximately 4000 known ORFs in *E. coli* and 50–100 small regulatory RNAs (66 listed in Table 1). Thus, they represent about 2% of the number of protein-coding genes. Until searches for sRNAs have been carried out as thoroughly in other organisms, we will not know how typical this is. Even for *E. coli*, because each sRNA may regulate multiple target genes, it suggests a great many regulatory signals exist that we do not yet understand. For the majority of the RNAs in Table 1, little or nothing is known about function.

FUNCTIONAL CLASSES OF RNAs: AN EMPHASIS ON TRANSLATIONAL REGULATION

Structural and Enzymatic RNAs

Two highly conserved RNAs in Table 1 encode RNase P, an enzyme involved in processing tRNAs and rRNAs (29) and 4.5S RNA, used in secretion of cotranslationally secreted membrane and exported proteins (37). These two RNAs are also the only essential sRNAs currently known in *E. coli*. The involvement of these ncRNAs with the translation apparatus is echoed by the roles of many other sRNAs in translation quality control and translational regulation. Whether this reflects an evolutionary root in the translation machinery or the effectiveness of RNA:RNA interactions is not clear. However, in the absence of other information, it may be safe to assume that a given sRNA will act on message stability or translation.

Quality Control

One sRNA, tmRNA, is listed under Quality Control in Table 1. As with the structural RNAs, related RNAs are found in a wide range of other bacteria, as well as in the mitochondrial genomes of some eukaryotes (109, 110). The name tmRNA reflects its properties as both a tRNA and an mRNA; it is also called SsrA, a name that reflects its first detection (small stable RNA) (54). It was recognized to have portions of its structure that folded like tRNAs, and, like a tRNA, it could be charged with an amino acid (alanine) (51). Surprisingly, a group studying truncated versions of a cloned interferon protein found C-terminal extensions of 11 amino acids not encoded by the interferon gene and corresponding to a very short (10-amino-acid) coding region within the *ssrA* gene (96). This work led to our current model of tmRNA action as part of the process of quality control of translation (48). Normally, protein translation stops when the ribosome encounters a nonsense

codon. Not only does this cause a stop in translation, it also invokes the action of termination factors, leading to release of the finished polypeptide. If translation stalls or stops without a termination codon, for instance because the message stops prematurely or rare codons are present, tmRNA can enter the empty A site of the ribosome, the charged alanine on the tRNA portion of tmRNA is transferred to the stalled polypeptide chain, and translation resumes, but from the 10-amino-acid ORF internal to the tmRNA. Translation ends at the nonsense codon within the tmRNA reading frame, releasing a polypeptide with an 11-amino-acid C-terminal tag (the SsrA tag). This sequence is sufficient to direct the polypeptide to one of a number of energy-dependent proteases, usually ClpXP, rapidly clearing the cell of this presumably abnormal polypeptide (31, 36). This also clears the otherwise stalled ribosome, and a variety of evidence suggests that this function is at least as important as destroying the polypeptide. tmRNA requires the SmpB protein, which binds to the ribosome and helps deliver the tmRNA (46, 98). (For recent reviews and a more detailed discussion, see References 28, 45, 111.)

RNA Regulators

The majority of the ncRNAs in Table 1 probably function as regulatory molecules. For a protein or an RNA to act as a regulator, it must have the following characteristics, discussed in more detail below:

1. Synthesis and/or activation only under specific conditions. For the sRNAs studied thus far, synthesis in response to a stress allows the regulatory switch to be turned on. Another class of RNA regulators, 5′ UTRs that act as riboSensors, bind small molecule effectors; binding results in changes in the folding of the RNA and therefore changes transcription termination or translational activity (reviewed in References 88, 108). It seems quite possible that some sRNAs will also be found to be regulated and/or to act by binding to small molecules. Changes in folding as a result of intracellular conditions (temperature, osmotic strength) may also act as regulatory signals.
2. Specificity of action. For the sRNAs, sequence and structure may both contribute to specificity, with many sRNAs acting by complementary pairing with a target RNA.
3. Limited action. Every regulator needs to be shut off at an appropriate time. Synthesis can be shut down, but in addition activity should be lost as well when the activation signal is gone. At least some of the sRNAs appear to be inactivated by degradation associated with their activity; they may work stoichiometrically rather than catalytically (60). It remains to be seen if this is a universal property of these sRNAs and, if not, what other mechanisms exist to limit action.

RNAs THAT REGULATE PROTEIN ACTIVITY A major category of regulatory RNAs are those that act by binding to a protein and modifying its activity. There are

currently two known target proteins in *E. coli* regulated directly by sRNA binding: RNA polymerase, regulated by binding to the 6S RNA (106), and CsrA, the carbon storage regulatory protein, regulated by binding to at least two RNAs, CsrB and CsrC (56, 107). These RNAs are discussed only briefly here, but both clearly represent well-conserved families of regulatory RNAs.

6S RNA is made in increased amounts as cells enter stationary phase and, almost unique among the well-studied sRNAs, is processed from the mRNA for an ORF of unknown function (38). 6S RNA is unique in another way as well, as it is the only known sRNA that acts on the transcription process rather than posttranscriptionally. Although mutations that eliminate 6S have no strong phenotype, they do show a change in RNA polymerase promoter selectivity (106). Because 6S RNA binds to and inhibits RNA polymerase containing the vegetative sigma factor (sigma 70) but not that containing the stationary phase sigma factor (sigma 38 or RpoS), it seems to help alter promoter recognition during stationary phase. The structure of 6S RNA, which is well conserved, has been suggested to resemble a sigma 70 promoter, providing a basis for 6S binding to polymerase and suggesting that binding should be within the promoter-binding regions of RNA polymerase (106).

CsrA is a protein that acts as a translational regulator. It binds sequences in the 5′ UTR of its target mRNAs to inhibit translation, redirecting carbon utilization. CsrB and CsrC are RNAs; each contains repeating sequence motifs, each capable of binding CsrA and resembling the sequence that CsrA binds in its target mRNAs (reviewed in Reference 78). Both the protein target, CsrA, and the sRNAs are widely found in gram-negative species and affect pathogenesis, biofilm formation, and swarming (34, 41, 42, 55). Regulatory signals for synthesis and degradation of this class of sRNAs are just beginning to be defined (18, 32, 72, 92).

RNA:RNA PAIRING FOR SPECIFICITY OF ACTION A significant number of the sRNAs in Table 1 are believed to act as antisense regulators, meaning that they work by pairing to messenger RNAs, affecting stability or translation of the message.

A few antisense RNA regulators are encoded on the opposite strand of the DNA from the regulated mRNA (*cis*-acting), resulting in the potential for complete pairing. This is akin to plasmid- and phage-encoded antisense molecules (11, 102). We assume that for these, the functional regulatory RNA has as its only target this *cis*-encoded transcript, although this need not be the case.

Some bacterial *cis*-acting RNAs, such as SokA and RdlD (Table 1), are also similar in function to one class of antisense RNAs found in plasmids. They negatively regulate the expression of a toxin and therefore are components of what have been called toxin/antitoxin systems (reviewed in Reference 26). Although the function of these chromosomally encoded toxins is still a matter of debate, in plasmids they contribute to plasmid stability by killing plasmidless cells. Loss of the plasmid DNA means no new antitoxin synthesis; the unstable antitoxin decays, triggering killing of the plasmidless host. For some of these systems, the antitoxin is an RNA that inhibits translation of the toxin, directly or indirectly; the antitoxin RNA is more unstable than the toxin messenger.

The majority of the known bacterially encoded antisense RNAs are encoded far from their targets; the vast majority also bind to and require the RNA chaperone Hfq. Furthermore, a recent genomewide search for Hfq-binding RNAs may have come close to saturating the search for this class of RNAs (115). The remainder of this review focuses on the Hfq-binding class of sRNAs, all of which are believed to act as antisense RNAs. For a few RNAs in this large class, we understand how their expression is regulated and have identified at least some of their targets; other sRNAs of unknown function are being actively studied. However, even when all these are understood, this class of sRNAs represents only about one-third of the identified sRNAs in *E. coli* (115), so there are many other sRNAs about which we know even less.

DEFINING A ROLE FOR Hfq IN sRNA FUNCTION

Hfq was first identified as a biochemical activity, a host factor, along with ribosomal protein S1, required for the replication of the RNA phage Qβ (9). It was found to bind strongly to RNA, particularly AU-rich single-stranded RNA. It is part of a complex operon that also includes the *amiB* and *miaA* genes involved in RNA modification as well as the *hflKC* genes, regulators of an ATP-dependent protease, FtsH. In studies of this operon, Winkler and colleagues created insertion mutations in *hfq* (95). The properties of the *hfq* mutants led to recognition that Hfq was necessary for translation of RpoS, the stress sigma factor of *E. coli* (69). Hfq was shown to be important for overcoming an inhibitory hairpin upstream of *rpos* (13) (see below and Figure 1*A*, color insert). The hairpin occludes ribosome binding to the RpoS translation start site; the bypass mutants abolish the hairpin so that translation is constitutive (see Figure 1*A* and below).

Independently, two sRNAs, DsrA and OxyS, were found to regulate *rpoS* translation (2, 85). Both sRNAs were found to bind Hfq and require it for their activity (86, 113). These sRNAs act by pairing with complementary sequences in their mRNA targets, suggesting that Hfq is important for this pairing. This was demonstrated in vitro for OxyS (114) and for another pairing sRNA, Spot 42 (67).

The class of Hfq-binding RNAs is large and contains some of the best-understood small regulatory RNAs. In a test of 46 known sRNAs found in various searches, 15 were found to bind Hfq tightly; at least 5 other sRNAs were defined by their binding to Hfq, bringing the total to at least 22, and possibly as many as 36 (115). Most likely, these all act by pairing to target messages. How Hfq acts to promote their action is discussed below in light of the best-studied cases.

PHYSIOLOGICAL FUNCTION OF Hfq-BINDING sRNAs

Four small Hfq-binding RNAs, DsrA, OxyS, Spot 42, and RyhB, have been studied in some detail. Experiments with each of them provide different insights into how the Hfq-binding RNAs can act.

All four of these RNAs are approximately 100 nucleotides in length. Each is encoded by a freestanding gene with a single promoter, and each ends with a rho-independent terminator. The promoters resemble standard promoters but are all tightly regulated; only when synthesis is induced will a given sRNA have a significant biological effect. Computer predictions and probing of the structures of the RNAs in vitro suggests all are well structured; the longest single-stranded region is also the region in which Hfq binds (Figure 1).

What do the Hfq-dependent sRNAs do for cellular physiology? Many (but not all) are well conserved in related bacteria, suggesting an important function (and suggesting conservation of targets as well). There are very few cases in which the regulation of the RNA, the targets, and the physiological outcome are all well understood. I first review the regulatory circuits for the four RNAs listed above, followed by a discussion of the information currently available on their mechanism of action.

Regulation of RpoS Translation

The *rpoS* gene encodes an alternative sigma factor, used by *E. coli* in times of stress (starvation, pH or osmotic shock, stationary phase) to transcribe large numbers of stress-response genes (reviewed in Reference 35). The RpoS response differs from some of the specific stress-response pathways in the range of signals that lead to its induction. RpoS levels are regulated primarily at the level of translation and at the level of protein turnover. The default state for *rpoS* translation is "off;" only low levels of RpoS or of an *rpoS-lac* translational fusion are expressed under optimum growth conditions. This inhibition of translation depends on the structure of the mRNA upstream of the *rpoS* start codon. A long 5′ region can fold into a hairpin that inhibits ribosome binding. RpoS translation increases rapidly after stress treatments; this increase requires Hfq and, we now know, sRNAs. The inhibitory structure was defined by Brown & Elliott (13) by searching for mutations that bypassed the Hfq requirement and made translation constitutive. As shown in Figure 1*A* for DsrA, two sRNAs, DsrA and RprA, are complementary to the upstream stem of the hairpin and both activate translation of *rpoS* by pairing. Mutations in DsrA or RprA that disrupt pairing can be restored to function by compensating mutations in the *rpoS* RNA pairing target (58, 59). The *dsrA* promoter is active only at low temperatures ($<30^{\circ}$C) (74, 75). It is also negatively regulated by LeuO, although the physiological role of LeuO is not known (50, 74). Low temperature expression of DsrA leads to expression of RpoS during exponential growth at lower temperatures (85). Why is RpoS needed at low temperatures? Recent results show that products of the *otsA* and *otsB* genes, which regulate levels of the osmoprotectant trehalose, protect the cell from cold temperatures (44). These genes are RpoS-dependent. Therefore, low temperature leads to higher levels of RpoS, resulting in increased expression of these gene products and protection from very low (4°C) temperatures. In addition, overexpressed DsrA negatively regulates *hns*, encoding a pleiotropic transcriptional regulator (52, 84). HNS negatively regulates a number

of genes that are osmotically inducible, and therefore DsrA negative regulation of *hns* may help the cell survive cold temperature and/or osmotic stress. There are other proposed targets of DsrA as well (52); it is unclear how they contribute to this physiological response.

RprA was found as a multicopy suppressor of *dsrA* mutants (57). It is regulated via the phosphorelay cascade RcsC/YojN/RcsB (59). This cascade is responsible for regulation of capsule synthesis as well as regulation of some osmotically inducible genes, one of the many promoters of *ftsZ*, and a number of other genes as well (14, 20, 27, 33). The phosphorelay is activated by solid surfaces and regulates up to 150 genes, most associated with the cell membrane or cell surface (24), possibly all contributing to biofilm formation. Some genes regulated by activation of RcsC in recent global analyses could be indirect targets, regulated by RprA or by RpoS when it is positively regulated by RprA. Additional direct targets of RprA are likely to exist but have not yet been identified. Regulation of RpoS by DsrA and RprA is noteworthy as the only current example of positive regulation of a gene by a small antisense RNA. At least two other sRNAs have been identified that positively regulate RpoS translational fusions (105); the signals that lead to their induction have not been defined.

Changes in ionic strength affect RpoS translation by sRNAs without necessarily increasing the synthesis of the sRNAs (57); how this works is not yet clear (112).

Finally, negative regulation of RpoS induction by sRNAs also occurs. OxyS, regulated by OxyR and induced in response to oxidative stress, negatively regulates RpoS as well as a number of other targets, including *fhlA* (2). Because the same signals that lead to OxyS induction (activation of OxyR) also lead to induction of a set of genes that deal with oxidative stress, it has been suggested that this negative regulation shuts down less specific repair pathways in favor of the specific ones (113). FhlA activates synthesis of the formate hydrogenlyase complex in the presence of formate; metal cofactors for this might lead to H_2O_2-induced damage (2). The regulation of *fhlA* by OxyS is direct, the result of antisense pairing both with the ribosome binding region and a second region within the *fhlA* gene (3) (Figure 1*B*). The mechanism of negative regulation of RpoS by OxyS is not understood.

RyhB and Iron Metabolism

RyhB RNA provides an answer to a puzzle in iron regulation, the positive regulation of some genes by the negative regulator Fur. Iron levels need to be carefully regulated because abundant iron can cause damage, but it is also an essential nutrient. In *E. coli* and many other bacteria, much of this regulation depends on the Fur protein, a repressor. When iron is plentiful, the Fur repressor binds Fe^{2+} and is active. When Fe^{2+} is limiting, Fur no longer represses, and the large number of genes in the Fur regulon are induced; these genes encode proteins involved in iron assimilation. Some genes, however, are positively regulated by Fur repressor and Fe^{2+}. The positively regulated genes encode nonessential Fe-S proteins and ferritins, proteins that store Fe; it makes sense to stop making these proteins when

iron is limiting. But how does Fur act to positively regulate these genes? Studies on regulation of *sodB*, encoding superoxide dismutase, one of these positively regulated genes, demonstrated that the regulation was posttranscriptional (23). When RyhB was found in a global search (105), examination of the literature and computer analysis suggested that it would be regulated by Fur and might pair with and regulate the *sdh* operon, encoding another Fe-S protein, succinate dehydrogenase. This proved to be the case; Fur negatively regulates *ryhB*, and RyhB negatively regulates *sdh*, *sodB* (see Figure 1*C*), and other Fe-S protein operons, leading to rapid degradation of the message for these operons (61). There is positive regulation of many operons encoding Fe-S proteins by Fe not only in *E. coli* (62) but also in other organisms, including mammals. Certainly some of this is due to RyhB-like molecules; in other cases, another mechanism of posttranscriptional regulation is used, the regulation of translation by the state of aconitase binding to Fe (reviewed in Reference 49).

Spot 42 and Sugar Metabolism

Another regulatory mystery, this time in the regulation of the *gal* operon, is also explained by a sRNA, Spot 42. The ratio of UDP-galactose epimerase (product of *galE*, the first gene in the operon) to galactokinase (product of *galK*, the third gene in the operon) varies. When cyclic AMP is low, the ratio of GalE:GalK is high compared with growth under conditions that lead to high cyclic AMP; this effect is independent of the *gal* operon promoters. This makes physiological sense because although galactokinase is only needed when galactose is available to metabolize, UDP-galactose epimerase has a second role in synthesis of UDP-galactose, a building block for the cell wall and capsule (1). When cells are growing on glucose, cyclic AMP is low; *galE* but not *galK* translation is needed; on galactose, cyclic AMP levels are higher, but both *galE* and *galK* need to be translated. Spot 42, one of the first sRNAs to be identified (40), provides the link between CRP and cAMP and polarity in the *gal* operon. Spot 42 pairs with and negatively regulates translation of *galK* without perturbing *galE* translation (68) (Figure 1*D*). Spot 42 is made under the negative regulation of CRP and cAMP, leading to higher levels of sRNA synthesis when cells are growing on glucose and lower levels when cells are growing on less favorable carbon sources (80). Spot 42 also downregulates components of the *suc* operon, again playing a role in allowing different genes within an operon to be independently regulated; other targets may be found (1). Spot 42 is currently unique in its role in regulating polarity within an operon.

Other Antisense RNA Roles

A number of other sRNAs that act by antisense pairing have been studied and are described very briefly here. MicF RNA is made when the SoxR/S regulators are activated; it downregulates translation of one of the major porins, OmpF (reviewed in Reference 22). Recent work demonstrates that MicF binds Hfq strongly (115);

presumably Hfq is necessary for its activity. A newly identified sRNA, MicC, regulates the other major porin, OmpC, under complementary conditions (19a). These sRNAs presumably help the cell respond to environmental conditions beyond those that are sensed by the phosphorelay system that regulates both the *ompF* and *ompC* genes (73). Changing the ratio of OmpF to OmpC in the cell envelope modulates entry of small molecules into the cell.

DicF, encoded by a cryptic prophage, negatively regulates translation of the *ftsZ* cell division gene. This sRNA, which is processed from a longer message, would not normally be expressed in a lysogen (10, 93). Possibly under conditions of prophage induction, inhibition of *ftsZ* translation is useful for the inducing phage because encoded downstream from DicF is another cell division inhibitor, the DicB protein (8). DNA damage, used by many prophage as an inducing signal, also induces yet another inhibitor of FtsZ activity, SulA (104), suggesting a possible common theme in inhibiting cell division after DNA damage.

THE MECHANISM OF ANTISENSE RNA ACTIVITY

The group of RNAs discussed above all pair with their targets, and the specificity of action depends on that pairing (Figure 1). The extent of pairing that is needed and the nature of the initial interactions have not yet been studied in detail for any of these sRNAs. What is known suggests that one or two regions, each with 8–9 base pairs, are sufficient to allow specific regulation. The best-studied examples are the *cis*-acting sRNAs; in these, short regions of pairing can initiate interactions; these regions are frequently in the loops of stem-loops and may then extend to longer regions of interaction (26, 103). It seems likely the nature of the pairing for the *trans*-acting sRNAs will be similar.

Pairing and a Role for Hfq

In vivo, all four of the sRNAs discussed above require Hfq for activity. How does Hfq act? Hfq is a well-conserved hexameric protein; electron microscopy and crystallography show that it forms a ring and has structural homology to eukaryotic Sm and Sm-like proteins that function in RNA splicing (81, 83, 91). Hfq binds AU-rich single-stranded RNA, with a preference for binding next to a structured (stem-loop) region (12, 67, 114) (see Figure 1). The single-stranded RNA binds to the inside of the ring along the top surface (83). Recent research on Hfq is reviewed in more detail in Reference 97a.

One effect of Hfq is clearly to increase the stability of the sRNAs. In vivo, when new RNA synthesis is stopped with rifampicin and stability is followed by Northern blots, these RNAs are frequently quite stable, with half-lives ranging from a few minutes to greater than 30 min; most are considerably less stable in *hfq* mutants (60, 67, 100). Consistent with Hfq stabilization of these RNAs, there is a lower accumulation of many sRNAs in *hfq* mutants (115). Thus, one role for Hfq could be just to stabilize the sRNAs. As expected from this model, overproduction of DsrA can partially bypass the requirement for Hfq (86).

A variety of in vitro experiments suggests that Hfq has a more active role in stimulating sRNA activity than simply to stabilize the RNA substrates. Hfq is an RNA chaperone with the ability to stimulate splicing of a bacteriophage intron (66). Hfq also binds to the target mRNAs of the sRNAs. This has been determined both in vivo, where some messenger RNAs can be precipitated with Hfq (115), and in vitro, where a number of mRNA targets bind Hfq specifically. In some cases, this binding site is near the region involved in pairing (25, 67, 99, 114). Only modest effects of Hfq binding on stability of these messages have been detected (in the absence of the sRNA) (99). For *ompA* mRNA, stability of the message is greater in the absence of Hfq, which has been interpreted as interference with *ompA* translation by Hfq binding, coupled with protection of the *ompA* message from degradation by the act of translation (101). An alternative explanation would be that Hfq allows a yet unidentified sRNA to target the *ompA* message for translation inhibition and degradation, as would be seen, for instance, for the *sodB* message, also more stable in an *hfq* mutant.

If Hfq is binding to both mRNA and sRNA, does it help to bring them together? In vitro, Hfq stimulates the pairing of both Spot 42 and OxyS to their target message (67, 114); recent experiments with RyhB show similar stimulation of pairing (25). Two possible roles for Hfq in this stimulation of pairing have been suggested; both may be true. In one model, interactions between the RNAs and Hfq increase local concentrations, aiding RNA:RNA interaction. For instance, if one ring of Hfq binds to the regulatory RNA and another to the target message, interactions between the two rings could promote the interaction of the two RNAs. Because it is not clear how the right rings of Hfq would find each other, it seems likely that this Hfq:Hfq interaction, if it occurs, might stabilize interactions already taking place by direct RNA:RNA pairing. The second model suggests more of a chaperone role. Hfq binding to a sRNA or to a target may change and/or stabilize RNA structure in such a way that complementary sequences are more available for pairing. This has been studied in vitro in a few instances. Although Hfq binding did not change the structure of RyhB or DsrA, it did change the structure of the RyhB target *sodB*, improving its ability to pair with RyhB (12, 25). Subtle changes in OxyS structure were detected on Hfq binding (114). Possibly Hfq also recruits other activities that have more direct roles in changing RNA structure. A recent paper suggests that Hfq itself has ATPase activity and that it associates with ribosomal protein S1 and, through that association, with RNA polymerase (90); this would suggest significantly more complex roles for Hfq.

Outcomes of Pairing: Changing Translation and mRNA Stability

Although all the sRNAs shown in Figure 1 act by pairing and require Hfq, they have different final effects on their targets. It is not known if this reflects essential differences in the way these sRNAs act. The effects of pairing discussed below are not mutually exclusive; changes in structure can lead to changes in translation and vice versa.

Pairing can change folding of the target or sRNA. For the plasmid-encoded *cis*-acting sRNAs, this can affect transcription termination as well as translation and message stability (reviewed in References 11, 26, 30, 102).

Pairing can change ribosome accessibility. This may affect message degradation. In some cases, the pairing can improve ribosome accessibility, as it does for RpoS when positively regulated by DsrA (58). In one recent in vitro study, DsrA was found to bind directly to the 30S ribosome (112); although such a binding activity might increase the local concentration of DsrA near a message about to be translated, it is not yet clear if this is necessary for DsrA activity in vivo. In the case of RyhB pairing with *sodB*, in vitro tests demonstrated inhibition of translation, dependent on pairing (99). When the relevant ribosome access site is internal to an operon, the RNA can cause polarity, as with Spot 42 regulation in the *gal* operon (68).

Pairing can lead to rapid mRNA degradation, as observed with RyhB (60). We do not yet know if this degradation is indirect, a consequence of blocking ribosome access, but it clearly makes the process irreversible.

TURNOVER OF sRNAs AS A CONSEQUENCE OF PAIRING In addition to these effects on the target mRNA, pairing can lead to rapid degradation of sRNAs. This conclusion is currently indirect; RyhB, DsrA, and OxyS were rapidly degraded in the presence of ongoing transcription, although they are very stable in rifampicin-treated cells (60). We interpret this to mean that these RNAs are degraded upon pairing with their mRNA targets. When new transcription is inhibited with rifampicin, the mRNA targets are degraded and no new ones are made; under these conditions, the sRNAs become stable. This rapid turnover as a consequence of pairing means that RyhB and the other small, pairing RNAs act stoichiometrically rather than catalytically and that they will only continue to act as long as the signals for their synthesis are present. Thus, this degradation during use provides an intrinsic shutdown mechanism.

Beyond Pairing: Role of RNase E in sRNA Action

Because a major outcome of pairing can be degradation of the sRNA and the target mRNA, how is this taking place? Mutations in *rnc*, encoding RNase III, a double-stranded endonuclease and the protein most like the ribonuclease in eukaryotes that processes RNAi and microRNAs (Dicer), had no effect on degradation of RyhB or its target mRNA (60). The other major endoribonuclease, RNase E, is an essential enzyme involved in processing many RNAs, most significantly tRNA, as well as RNase P and tmRNA. It is also involved in mRNA degradation; temperature-sensitive mutants in the active site affect the rate of turnover of mRNAs (reviewed in Reference 15). Finally, RNase E is the scaffold for assembly of a protein complex called the degradosome, made up of polynucleotide phosphorylase, enolase, and an RNA helicase, in addition to RNase E. Deletions of the degradosome-binding domain of RNase E are not lethal.

Recent work on a number of systems suggests that RNase E plays a critical role in the functioning of Hfq-binding sRNAs. Intriguingly, the recognition motif for RNase E cleavage is a single-stranded AU-rich region, reminiscent of the sequences recognized by Hfq. It now has been shown that Hfq and RNase E at least sometimes see the same targets. RyhB becomes very unstable in an *hfq* mutant (as do a number of other sRNAs) and is stabilized in an RNase E mutant (60, 65). In vitro, cleavage of RyhB by RNase E is inhibited by Hfq (65). Similar observations were made for DsrA (65), and the cleavage site for RNase E defined in vitro could give rise to a truncated DsrA RNA observed in vivo in other experiments (74). In a separate series of experiments, we found that certain processing intermediates of the transcript from the *argX-proM* tRNA operon bind Hfq near RNase E cleavage sites; processing by RNase E appears to be slowed when Hfq is present (115). In general, it seems safe to say that many, if not all, Hfq-binding sites have the potential to be RNase E cleavage sites and vice versa; the equilibrium between amounts and specific affinity may determine how much Hfq binding protects a given RNA from RNase E.

RNase E is required for the degradation of both mRNA and the sRNA (RyhB) in vivo (60, 65). *sodB* is normally degraded in an RNase E–dependent fashion, so that it is difficult to determine if the RyhB-stimulated degradation is secondary to blocking translation or a direct consequence of pairing. In either case, however, the degradation of sRNAs is also stimulated upon pairing. Possibly pairing changes or displaces Hfq binding, allowing RNase E or other degradosome components entry to sites that otherwise are shielded.

It is not yet clear how important the degradosome is for sRNA action. RyhB and *sodB* degradation are both slowed in degradosome mutants, although the specific roles of degradosome components has not been explored (60). It is tempting to suggest that helicase unwinding, for instance, will be particularly important in the degradation of the highly structured sRNAs, possibly after the initial endonucleolytic cuts are made.

Our Current Picture of Antisense RNA Action: Summary and Unsolved Problems

We can now put together a picture of the mode of action of Hfq-binding sRNAs. Thus far, all these sRNAs act by pairing with target mRNAs (Figure 1). Pairing is stimulated in vivo by Hfq, in part by binding to and stabilizing the sRNAs, but probably by more actively stimulating pairing. In most cases, negative regulation of the translation activity and stability of the message is a result of pairing. RNase E gains access to both message and sRNA, cleaving both; exonucleases then complete the degradation. How Hfq is displaced from the paired complex to allow RNase E attack is not yet known. In cases in which regulation is positive (sRNA stimulation of RpoS translation) or in which the mRNA is not degraded (Spot 42 regulation within the *gal* operon), destruction of the mRNA must be blocked. Whether this reflects differences in the nature of pairing, in the relative location of the

Hfq-binding site and pairing regions, or other properties of either sRNA or message is not yet clear.

The model systems studied thus far provide only a glimpse at how these sRNAs work. In vivo studies identified Hfq's role in sRNA action. What other proteins are involved in this process? HU, a histone-like protein in *E. coli*, binds well to RNA, and mutants lacking HU have RpoS translation defects, suggesting a possible role for HU in sRNA function (6, 7). Hfq has been found associated with S1 both for Qβ growth and in binding to RNA polymerase (90); they may also act together for sRNAs. Sequences required for optimal pairing within the sRNA and target messages have not been defined in most cases. Studies of plasmid antisense RNAs suggest that the details of the interactions will be critical in fully defining how these RNAs act.

Finally, in vivo competition between multiple targets for a given sRNA may be critical under some conditions, particularly because the sRNA can be consumed by pairing with one abundant target. Thus far, this has not been assessed in vivo and is missing from most in vitro studies.

SIGNS OF sRNAs

Now that we know they exist, we can be more alert to the signs that a sRNA might be playing an important role in a given regulatory circuit. In cases where things do not quite fit, a sRNA may be lurking somewhere. Here are some examples:

1. Unexpected direction of regulation. RyhB provides an explanation for how Fur, a repressor, could positively regulate genes (see above). Fur negatively regulates RyhB; RyhB in turn negatively regulates a set of genes encoding iron-binding proteins. In general, for any known negative regulator, an observation of positive regulation could be due to a similar cascade; two negatives make a positive. Similarly, a positive regulator can act negatively on a target message by activating synthesis of a negatively regulating sRNA. Inverted regulation does not have to be via sRNAs, but because the sRNAs are generally missed in mutant hunts, it may take special attention to find them.
2. Orphan regulator binding sites. Genomewide definition of functional regulatory sites can be done by computational methods, by multicopy titration, or by genomewide mapping of regulatory protein binding sites by chromatin coimmunoprecipitation. If any of these approaches demonstrate a binding site for a regulator, and the nearby ORF does not show the appropriate regulation, this may be evidence of a regulatory site for a sRNA. In fact, sRNAs that are regulated by the Fur repressor in *Pseudomonas aeruginosa* were recently identified by locating a Fur binding site and a transcription terminator sequence in reasonable proximity in an intergenic region (116).
3. Regulation without a site. Some sRNAs lead to rapid degradation of their target messages; this should be detectable in experiments with arrays and

in those with transcriptional fusions that contain the relevant sRNA target site. Therefore, we need to be aware that not every change in transcription is due to changes in synthesis. Evidence of regulation of a given gene in array experiments, for instance, coupled with failure to find a regulatory protein binding site in the relevant promoter, may indicate that regulation is indirect, via a sRNA.

4. Regulated translation. In a genomewide search for conservation in intergenic regions, many highly conserved 5′ UTRs were observed (105). Hfq also binds to the message from many genes with 5′ UTRs (115). Although neither of these is evidence of a target for a sRNA, researchers should keep that possibility in mind.

CONCLUSIONS

sRNAs Provide some Unique Advantages

Why use a sRNA instead of a protein regulator? One advantage may be their size and therefore the speed of the response. Once synthesis starts, it takes very little time to complete the regulator. If it is degraded as it is used, no other mechanism for turnover is needed.

sRNAs that regulate translation also provide a simple way to impose an overarching level of regulation on a group of genes or operons that may be regulated in many different ways at the level of transcription. A sRNA acting at the level of translation or messenger stability will always be epistatic to transcriptional regulation of the same gene. This is most evident for RyhB, which can simultaneously downregulate many genes; if iron is limiting, this becomes necessary irrespective of the individual inducing signals for each of the genes. Multiple sRNAs, each made under different conditions, can regulate a single target, allowing integration of many environmental signals. Thus far, this is best exemplified by the sRNAs that regulate RpoS translation (76).

Other advantages are likely to appear as we learn more about these multitalented molecules. It seems likely that every major regulatory network will contain at least one sRNA. Recent reports on effects of *hfq* mutants on virulence in many organisms suggest that these sRNAs will play critical roles in pathogenesis as well (70, 79, 87).

Where Did They Come From?

The above arguments for the advantages of sRNAs imply that they are of value to current organisms and not simply a relic from the ancient "RNA world." In fact, we do not know whether any of these regulatory RNAs are relatively newly evolved or from where they have evolved. The Hfq-binding sRNAs are tRNA-like in size but apparently not in sequence or structure, and the widespread occurrence of Hfq suggests that the RNAs will be widespread as well. Sequence similarities

can only be detected thus far between the Hfq-binding sRNAs from *E. coli* and its relatively close neighbors—*Salmonella*, *Klebsiella*, *Yersinia*, and *Vibrio*. *Pseudomonas aeruginosa* contains at least some functionally similar sRNAs with no apparent sequence similarity. Does this reflect rapid evolution from a common source, or convergent evolution? As we find them in more organisms, the nature or lack of relatedness should become more apparent and help us define the critical properties that are conserved across species.

As more sRNAs are identified, the challenge of finding out what they do will become even more pressing than it is now. The next few years are likely to see the development of new ways to study them and identify their targets, much as the past few years has led to new and better ways to find them. In the end, the network of connections between the components of an organism such as *E. coli* will need to include the RNA regulators in addition to the protein regulators if we will ever truly be able to predict how an organism will react under a given condition.

ACKNOWLEDGMENTS

I thank the many colleagues who shared unpublished results and provided invaluable discussions on the topics covered in this review. I thank Gisela Storz, Cari Vanderpool, David Fitzgerald, Karl Thompson, and Eric Massé for comments on the manuscript.

The *Annual Review of Microbiology* is online at http://micro.annualreviews.org

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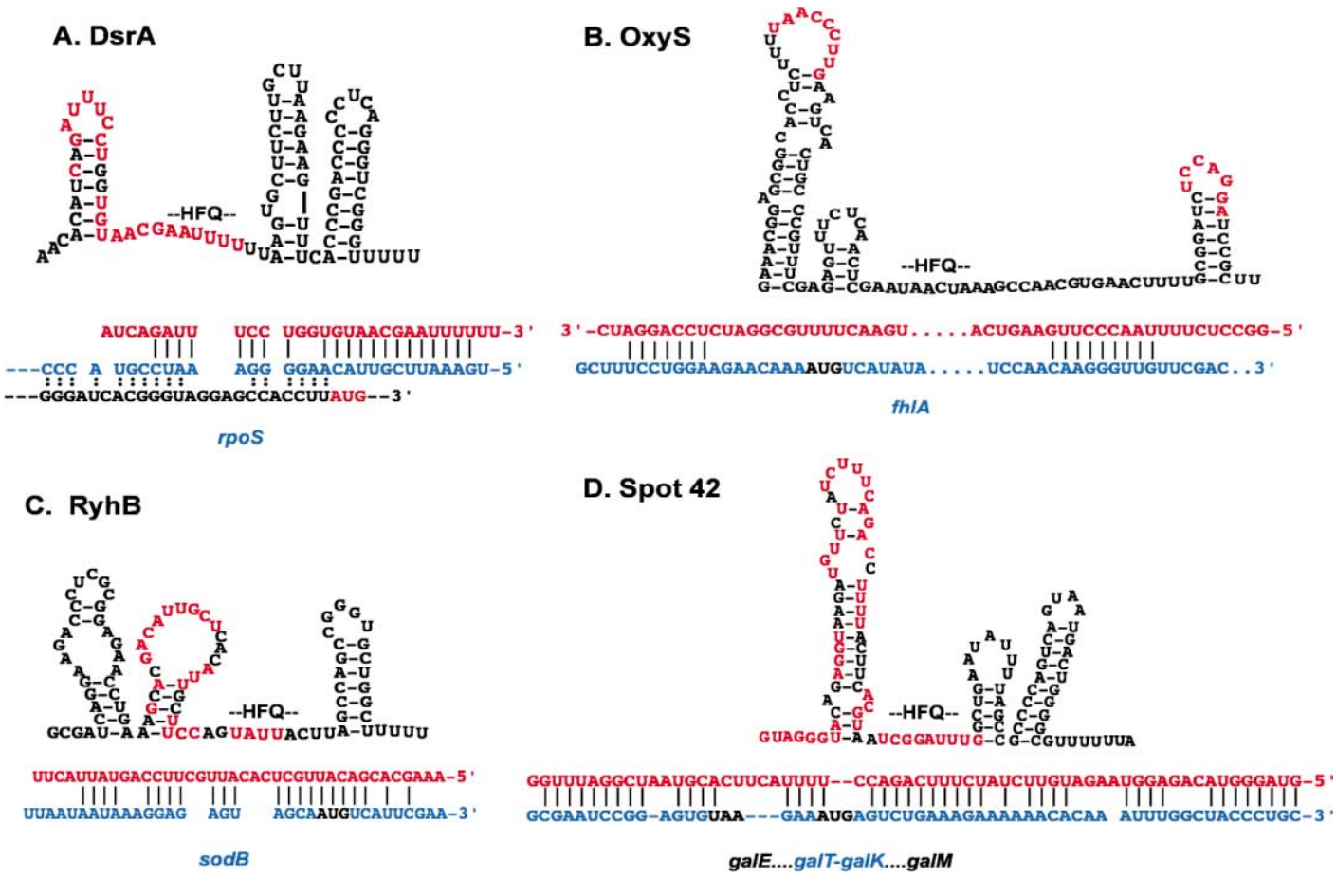

Figure 1 sRNAs that act by pairing. Each panel illustrates the structure of the sRNA, as determined by in vitro probing experiments. The bases that pair with the target shown are in red. The single-stranded regions that bind Hfq and/or are protected by Hfq are indicated by a --Hfq--. Below each sRNA is a linear representation of the pairing of the sRNA (*red*) with a target (*blue*). The AUG in the target sequences are shown in *black*. In all these cases, some part of the pairing has been experimentally tested; in none has the full pairing been tested. (*A*) DsrA. The structure is as determined by (12). The target, *rpoS*, is positively regulated by DsrA, by pairing to the upstream RNA (*blue*); alternatively, the blue strand pairs with the black strand to inhibit ribosome binding and translation (indicated by a colon between the paired nucleotides) (76). The blue and black strands are connected by an additional 63 nucleotides. (*B*) OxyS. The structure and pairing is as determined by (2, 3); Hfq binding is from (114). (*C*) RyhB. The structure and Hfq binding is from (25); the pairing is an extension of that determined by Geissmann & Touati on the basis of visual examination of the sequence. (*D*) Spot 42. The structure and Hfq binding is as determined by (67, 68). The UAA (*black*) is the terminator codon for *galT*; the AUG is the start codon for *galK*.

Annual Review of Microbiology
Volume 58, 2004

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