Review in Advance first posted online on April 10, 2014. (Changes may still occur before final publication online and in print.)

6S RNA, A Global Regulator of Transcription in *Escherichia coli*, *Bacillus subtilis*, and Beyond

Amy T. Cavanagh and Karen M. Wassarman

Department of Bacteriology, University of Wisconsin–Madison, Madison, Wisconsin 53706; email: wassarman@bact.wisc.edu

Annu. Rev. Microbiol. 2014. 68:45–60

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

This article's doi: 10.1146/annurev-micro-092611-150135

Keywords

sRNA, pRNA, stationary phase, global regulation, stress response, *ssrS*

Abstract

6S RNA is a small, noncoding RNA that interacts with the primary holoenzyme form of RNA polymerase. *Escherichia coli* 6S RNA is a global regulator that downregulates transcription and is important for modulating stress and optimizing survival during nutrient limitation. Studies in diverse organisms suggest a higher complexity in function than previously appreciated. Some bacteria have multiple 6S RNAs that appear to have independent functions. 6S RNA accumulation profiles also are quite divergent and suggest they integrate into cellular networks in a species-specific manner. Nevertheless, in all tested systems the common theme is a role for 6S RNA in survival. Finally, there has been much excitement about the ability of 6S RNA to be used as a template to synthesize product RNAs (pRNAs). This review highlights the details of 6S RNA in *E. coli* and compares and contrasts 6S RNAs in multiple species.

Contents

INTRODUCTION

More than a decade of intensive study of small, noncoding RNAs (sRNAs) in bacteria has identified hundreds of sRNAs. Functional studies revealed physiological roles and mechanisms of action for many of these regulatory RNAs, and although the details vary for how and when each sRNA functions, one unifying role for sRNA-dependent regulation is to modify gene expression in response to stress, changing environments, or other specialized growth conditions (63). The majority of studied sRNAs act by altering the stability and/or translatability of target mRNAs through base pairing (7). Other sRNAs have intrinsic function; for example, tmRNA acts as both tRNA and mRNA (29). A growing and divergent group of sRNAs interact with and modify proteins. Examples include sRNAs that interact with and sequester CsrA/Rsm (55), and 6S RNA, which interacts with RNA polymerase (RNAP) (60, 71, 76). 6S RNAs, how they function, and the physiological consequences of their action are the focus of this review. 6S RNA studies are progressing in many species, but *Escherichia coli* remains the best-studied example. Therefore, we discuss *E. coli* 6S RNA first and then compare the known properties and roles of 6S RNA in *Bacillus subtilis* and other organisms.

E. *COLI* 6S RNA

6S RNA–RNA Polymerase Interactions

E. coli 6S RNA was first identified in the 1960s because of its abundance (25), but functional studies lagged, in part hampered by the lack of insight from cells deleted for or overexpressing 6S RNA (26, 38). Additional characterization revealed that 6S RNA was part of an RNA-protein complex (40), and functional studies regained momentum when the *E. coli* genome sequence (9) and sensitive mass spectrometry techniques (36) allowed identification of RNAP subunits as potential binding partners (73). RNAP is a multisubunit enzyme with a transcriptionally competent core ($E = \alpha_2, \beta, \beta', \text{and } \omega$). Addition of a specificity subunit (σ) to form the holoenzyme ($E\sigma$) is required for transcription initiation. Most bacteria contain one primary σ factor (σ^{70} in *E. coli*) and a varying number of alternative σ factors important during different growth conditions (22). Copurification experiments revealed that 6S RNA was in a complex with $E\sigma^{70}$ (73), and in

vitro binding experiments using purified components confirmed the specificity of the interaction between 6S RNA and $E\sigma^{70}$ (21, 66). In particular, researchers have not detected 6S RNA binding to core RNAP, free σ factors, or alternative holoenzymes above background levels.

A highly conserved secondary structure, not the primary sequence, is essential for 6S RNA binding to RNAP (57, 66). Largely double-stranded with a single-stranded central region (**Figure 1**), this structure is tantalizingly similar to the conformation of DNA during transcription initiation when DNA surrounding the start site of transcription is melted (open complex), which led to models in which 6S RNA interacts with $E\sigma^{70}$ similarly to promoter DNA (3, 66). Binding of 6S RNA to RNAP blocks DNA from binding, and conversely binding of DNA to RNAP blocks RNA from binding (21, 72). The central region of 6S RNA is positioned near the active site of RNAP, and cross-linking studies revealed a close proximity between 6S RNA and σ^{70}, again reminiscent of DNA binding (21, 72, 73). Recent work mapped several regions of 6S RNA in close proximity to RNAP, leading to a model of 6S RNA docked within the available crystal structure of *E. coli* RNAP (44) that strongly supports the notion that 6S RNA makes many contacts to RNAP similar to contacts made by promoter DNA (61). In fact, 6S RNA binds at the active site similarly enough to promoter DNA for RNAP to use it as a template for RNA synthesis in a process called pRNA (product RNA) synthesis (see below) (21, 72).

However, there is at least one region where RNA and DNA bind RNAP differently. Region 4.2 of σ^{70} is critical for both 6S RNA and promoter DNA binding (13, 34). It interacts with the upstream region of 6S RNA, which is one of the few locations in the RNA where sequence at specific residues is critical for binding RNAP (57, 61). However, this upstream region does not resemble double-stranded promoter DNA, and the binding site for 6S RNA in region 4.2 was found to be overlapping with but distinct from the DNA binding site (34). Intriguingly, proposed upstream structures diverge considerably in many candidate 6S RNAs (3), raising questions about whether this contact is conserved.

Regulation of Transcription

Transcription (σ^{70} dependent). 6S RNA interacts with RNAP, which suggested it would influence transcription. 6S RNA accumulates to high levels during late stationary phase (~10,000 copies per cell), a time when the vast majority of $E\sigma^{70}$ is bound by 6S RNA (73). Thus, early studies focused on 6S RNA–dependent changes in transcription during late stationary phase, although regulation of transcription had also been observed earlier, even during exponential phase, when 6S RNA levels are reduced but still substantial (>1,000 copies per cell) (31, 65, 67, 73). Initial studies suggested 6S RNA might generally inhibit σ^{70}-dependent transcription (73), but further analysis revealed a more complex story, as several σ^{70}-dependent promoters are downregulated by 6S RNA whereas other promoters remain unchanged (13, 65, 73).

How could binding to the primary transcription machinery have promoter-specific effects? Simple competition between 6S RNA and promoter DNA for free $E\sigma^{70}$ seemed possible, but this model would predict that promoter affinity and kinetics of RNAP binding would direct 6S RNA sensitivity, which was not supported experimentally (13). σ^{70}-Dependent promoters are recognized by two sequences upstream of the transcription start site (-10 and -35 elements) and some promoters have additional conserved nucleotides forming an extended -10 element. Mutation of specific promoter features was able to interconvert 6S RNA–sensitive and 6S RNA–insensitive promoters and revealed that the presence of a weak -35 or an extended -10 element independently determines sensitivity to 6S RNA (13). Strength of the core -10 element did not alter 6S RNA sensitivity, further suggesting a more complex mechanism than simple competition. Most tested reporters contained minimal promoter sequences, thereby increasing the likelihood that observed

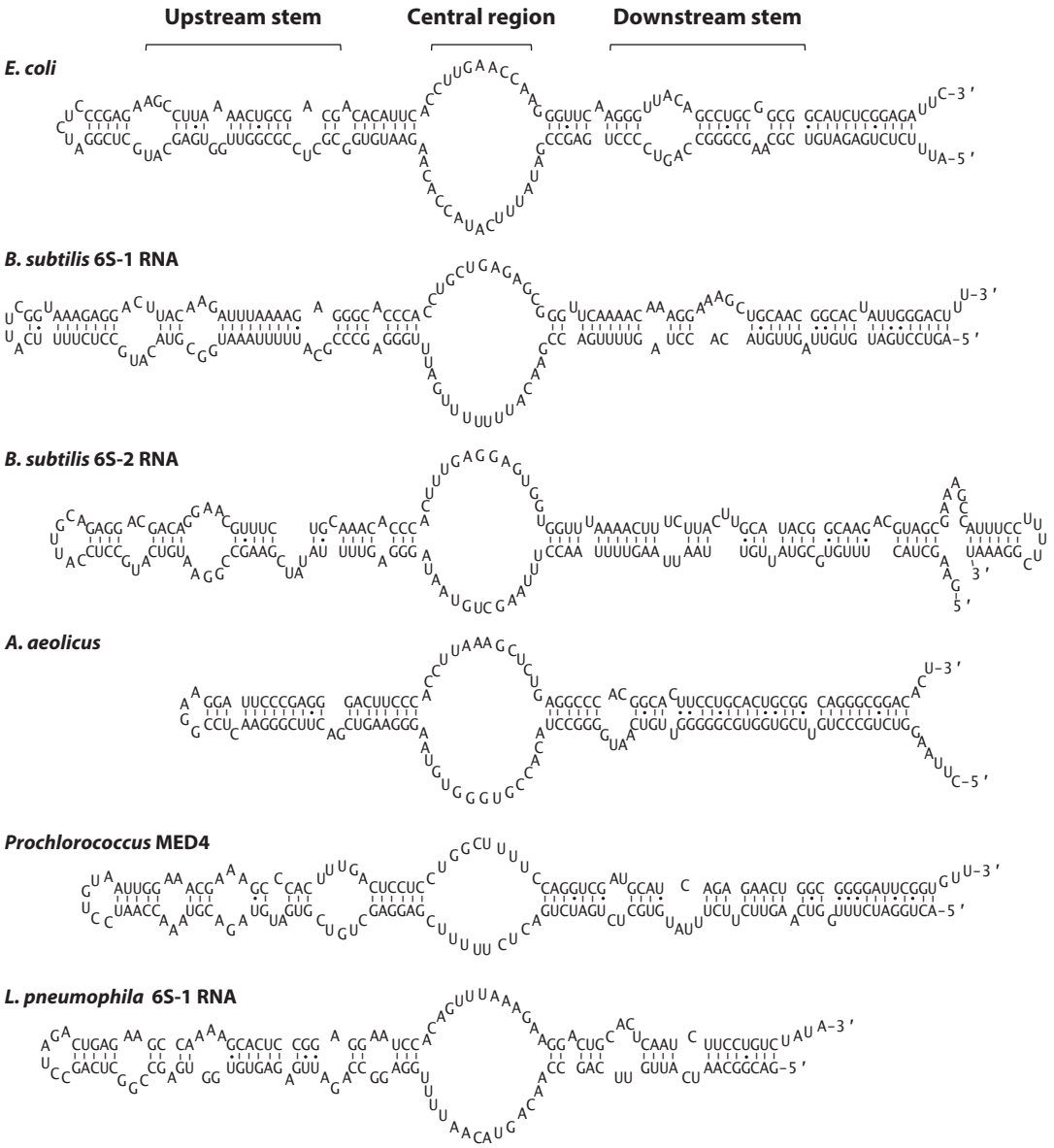

Figure 1

6S RNAs from *Escherichia coli*, *Bacillus subtilis*, *Aquifex aeolicus*, *Prochlorococcus* MED4, and *Legionella pneumophila* are shown in predicted secondary structures (2, 3, 19, 66, 77). Divergent 6S RNAs share a common secondary structure but have little primary sequence similarity.

changes were due to direct 6S RNA effects rather than secondary effects mediated through *trans*-acting factors.

Two global expression studies found that hundreds of mRNAs change in levels in a 6S RNA–dependent manner (13, 47), which will include those directly regulated by 6S RNA as well as any changed through secondary effects. The first study focused on late stationary phase and

312 genes expressed in stationary phase that have mapped transcription start sites to facilitate comparison of promoter elements (13). Even with the composite direct and secondary effects there was good agreement (70–80%) between observations and predictions for 6S RNA sensitivity, lending additional support to the experimentally derived model that the strength of the −35 element or the presence of an extended −10 element determines 6S RNA sensitivity.

The second global study examined midlog and early stationary phase and also found an abundance of genes were differentially expressed, confirming a role for 6S RNA at earlier times of growth (47), in agreement with results from individually tested promoters (65, 67). However, this study concluded that 6S RNA sensitivity did not correlate with promoter features.

The discrepant conclusions regarding the role of promoter elements in 6S RNA sensitivity are likely due to the difference in growth phase examined (late stationary versus log and early stationary) and/or the number and type of genes examined (312 with mapped promoters versus all genes). Although it is possible that promoter features determining 6S RNA sensitivity are different throughout growth, this seems unlikely, as all tested reporter genes respond similarly to 6S RNA in early and late stationary phase except for a subset known to change via secondary effects (12, 65, 67). Another possibility is a difference in relative contribution of direct and secondary effects to each study, and only promoters directly regulated by 6S RNA are relevant for this mechanistic question. Many genes have more than one promoter and are organized in operons, and, thus, analysis of all individual genes may include more secondary effects than analysis of a subset of genes chosen based on the presence of a primary mapped promoter. Furthermore, secondary effects might be more abundant during early stationary phase, especially given that the level of ppGpp, a global regulator, is elevated in the absence of 6S RNA during early but not late stationary phase (12, 47).

Promoter-specific regulation is not observed in vitro, which has hampered detailed mechanistic understanding of direct 6S RNA regulation. 6S RNA does downregulate transcription in vitro (21, 66), but all tested σ^{70}-dependent promoters are strongly downregulated by 6S RNA in vitro (>10-fold compared with 2- to 5-fold in vivo), including promoters insensitive to 6S RNA in vivo. In vitro conditions may lack a critical component, although cell lysates also do not reflect in vivo observations. Higher-order chromosome structure may be necessary, or another aspect of in vitro assays may not properly reflect in vivo conditions. For instance, dynamic exchange between DNA and RNA on RNAP would be expected in vivo at some level to account for observed regulation, but the 6S RNA–RNAP interaction is very tight and minimal dissociation is observed under typical in vitro conditions (21, 34, 66, 72). 6S RNA and DNA have overlapping binding sites on RNAP, suggesting they will compete at some level. However, it seems unlikely that DNA and RNA compete for free holoenzyme given that overall promoter affinity does not determine sensitivity to 6S RNA. Region 4.2 of σ^{70} may be an important site for competition (13, 34), although when and how are unclear. Whether one nucleic acid can dissociate the other in vivo, and whether there might be transient complexes containing RNAP, DNA, and 6S RNA not detected in vitro remain to be seen.

Transcription (σ^S dependent). The presence of 6S RNA also leads to upregulation of σ^S-dependent transcription in vivo, although not all σ^S-dependent promoters are sensitive, suggesting promoter specificity for 6S RNA–regulation of σ^S activity as well (13, 31, 47, 65, 73). 6S RNA does not form stable, specific complexes with σ^S-RNAP, and σ^S protein levels do not change in response to 6S RNA (65, 66, 73). It has been proposed that 6S RNA binding to σ^{70}-RNAP might alter competition between σ factors, resulting in enhanced σ^S activity. Alternatively, *trans*-acting factors regulated by 6S RNA in a σ^{70}-dependent fashion might affect σ^S activity, although none of the factors known to alter σ^S activity (4, 23, 33) respond to 6S RNA in a manner consistent with

observations (A.T. Cavanagh & K.M. Wassarman, unpublished observations). 6S RNA–dependent changes in ppGpp levels (see below) do not account for observed changes in $E\sigma^S$ activity (12). Once again, in vitro assays have not been helpful; σ^S-dependent promoters are inhibited by 6S RNA in vitro, in contrast to upregulation of $E\sigma^S$ activity in vivo (21, 65). Further experiments are necessary to sort out the 6S RNA effects on σ^S activity.

Physiological Outcomes of 6S RNA Function

Cells lacking 6S RNA grow indistinguishably from wild type through exponential phase and into stationary phase (38) despite changes in expression of hundreds (~250) of genes in exponential phase and an even greater number (~800) in stationary phase (13, 47). However, cells lacking 6S RNA are at a disadvantage in competitive growth and are decreased for survival during long-term nutrient deprivation (65), and cells overexpressing 6S RNA in the absence of σ^S have reduced viability (73). 6S RNA–dependent changes in transcription at individual genes are modest (2- to 5-fold), but the overall transcription change when amplified by hundreds of genes is great, and it has been proposed that this large-scale misregulation may make cells lacking 6S RNA inefficient in utilizing diminishing resources in late stationary phase. Alternatively, competition and/or long-term growth defects may result from changes in transcription of one or a few specific gene(s) critical for long-term survival. The precise details linking specific gene expression changes to these mutant phenotypes are not yet understood.

Cells lacking 6S RNA also survive better than wild type at elevated pH, and it has been demonstrated that this phenotype is due to direct 6S RNA downregulation of transcription of *pspF* (67). *pspF* is transcribed from a single σ^{70}-dependent promoter containing an extended -10 element, a feature known to be important for 6S RNA sensitivity. PspF, in turn, is a transcriptional activator of two σ^{54}-dependent promoters (*pspABCDE* operon and *pspG*) (30), and it is the secondary effects on these genes that mediate changes in survival at high pH. The *psp* genes illustrate the importance of separating direct and secondary effects to address mechanistic versus physiological questions. *pspF*, *pspA*, *pspB*, *pspC*, *pspD*, *pspE*, and *pspG* are all increased in cells lacking 6S RNA. However, the combination of genetics and analysis of minimal promoter reporters was required to sort out the details that *pspF* is the direct 6S RNA–regulated gene and that the other changes are secondary (67). It would not have been possible to predict promoter features responsible for direct 6S RNA regulation at *pspF* without first knowing that the other six genes are regulated secondarily, although the secondary effects are critical from a physiological perspective. In addition, 6S RNA–dependent changes in *pspF* mRNA are only ~2.5 fold, demonstrating that even modest changes in one direct gene can be sufficient for measurable changes in growth/survival.

The gene expression studies hint that 6S RNA–dependent regulation is integrated into global pathways (13, 47, 60). *relA*, which is directly downregulated by 6S RNA (12), encodes a ppGpp synthase responsible for ppGpp accumulation in early stationary phase (59). A global regulator itself, ppGpp binds RNAP and alters transcription of many sensitive genes (43, 53, 59), although it is not required for 6S RNA function (12). In cells lacking 6S RNA, ppGpp levels are increased and predicted changes in ppGpp-sensitive transcription are observed (12, 47). *spoT*, encoding an enzyme with both ppGpp hydrolase and synthetase activities (59), also has been reported to play a role in 6S RNA–dependent increases in ppGpp (47), although the mechanism is not yet clear. In addition, mRNAs encoding several primary regulators (e.g., Crp, Fnr) and general translation machinery are changed in a 6S RNA–dependent manner, further suggesting that 6S RNA coordinates global regulation (13, 47).

It is intriguing that 6S RNA upregulates σ^S and downregulates *relA*, albeit through different mechanisms, as both are global regulators important during stationary phase. Additionally, PspF,

also downregulated by 6S RNA, is important in specific stress responses. 6S RNA appears to have a role in modulating, rather than turning on or off, stress response. Of note, 6S RNA does not prevent stress response, as both the ppGpp and Psp responses remain activated by stress in the presence of 6S RNA. The response is merely dampened, perhaps conserving valuable resources under nutrient-limiting conditions and facilitating long-term survival.

BEYOND *E. COLI*: 6S RNAS IN DIVERSE SPECIES

Of great interest is how 6S RNAs function in other organisms and whether divergent 6S RNAs act similarly to *E. coli* 6S RNA. Both computational and experimental approaches have found candidate 6S RNAs or 6S RNA–encoding genes (3, 66). With the discovery of diverse RNAs, it becomes important to contemplate what defines a 6S RNA and what assumptions can be made about function. We propose that 6S RNAs are RNAs that bind the primary form of their cognate RNAP holoenzyme in a manner resembling promoter DNA binding. This definition is specific enough to promote useful generalizations and comparisons between diverse species, is likely to define a class of mechanistically similar RNAs, and is relatively straightforward to test experimentally, but it also leaves potential for other classes of RNAs that might interact with or modify RNAP behavior by other mechanisms. Here we discuss current understanding of 6S RNAs in organisms beyond *E. coli*. We begin with *B. subtilis*, where mechanistic and physiological studies allow direct comparison with *E. coli* to address the ubiquity of 6S RNA traits and functions. We include species abbreviations in RNA names to help clarify the different RNAs (e.g., *E. coli* 6S RNA = Ec6S RNA).

6S-1 and 6S-2 RNAs in *B. subtilis*

Interestingly, the gram-positive bacterium *B. subtilis* has two 6S RNAs (3, 66). Here we use the original 6S RNA nomenclature and gene names: 6S-1 RNA encoded by *bsrA* (also called Bs190 RNA and 6SB RNA, and *ssrSB*) and 6S-2 RNA encoded by *bsrB* (also called Bs203 RNA and 6SA RNA, and *ssrSA*). Both RNAs were identified as abundant RNAs of unknown function (1, 64) and later identified as 6S RNAs based on coimmunoprecipitation with the housekeeping form of RNAP ($E\sigma^A$ in *B. subtilis*) (66). Bs6S-1 and Bs6S-2 RNAs share little primary sequence homology with each other or with Ec6S RNA, but they have similar secondary structures (**Figure 1**), bind very tightly to RNAP, and can be used for pRNA synthesis in vitro, although Bs6S-2 RNA is not as efficient a template as Bs6S-1 or Ec6S RNAs (see below) (3, 5, 10, 11, 14, 66). Together with observations that the central single-stranded region is required for binding (14), these data suggest Bs6S-1 and Bs6S-2 RNAs bind RNAP in a manner similar to Ec6S RNA binding to RNAP. A third *B. subtilis* RNA was reported to bind another form of RNAP (66), but further investigation revealed it was an artifact of the antibody used (C.S. Chin & K.M. Wassarman, unpublished observations).

Of particular interest was whether Bs6S-1 and Bs6S-2 RNAs had redundant or independent functions. Cells lacking Bs6S-1 RNA sporulate earlier than wild type or cells lacking Bs6S-2 RNA (15). *B. subtilis* responds to nutrient limitation quite distinctly from *E. coli*, often resulting in sporulation (24). Key players in early sporulation events are upregulated earlier in cells lacking Bs6S-1 RNA than in wild type and have been proposed to be secondary effects resulting from Bs6S-1 RNA–dependent changes in nutrient utilization. A more detailed understanding of direct effects of Bs6S-1 RNA is required for a full appreciation of the role of Bs6S-1 RNA in determining the timing of sporulation.

Both Bs6S-1 and Bs6S-2 RNAs bind in the active site of $E\sigma^A$, yet only Bs6S-1 RNA influences the timing of sporulation, suggesting that these two RNAs regulate different genes. Both RNAs

have been shown to inhibit transcription in vitro (10), although it is unclear whether the promoters tested are regulated in vivo. Preliminary in vivo studies indicate that Bs6S-1 and Bs6S-2 RNAs regulate different promoters in vivo, even at times when both are present (A.T. Cavanagh & K.M. Wassarman, unpublished observations). The details underlying differences in promoter specificity and whether they can be reproduced in vitro await further study; however, these observations predict a greater complexity in mechanism(s) of 6S RNA regulation of transcription than previously anticipated. In addition, they support independent functions for Bs6S-1 and Bs6S-2 RNAs in the cell, consistent with different phenotypes associated with loss of Bs6S-1 or Bs6S-2 RNA. Further questions include how multiple 6S RNAs are regulated in the same cell and when having multiple 6S RNAs might be advantageous.

6S RNAs in Other Species

Although understanding of mechanistic and physiological aspects of 6S RNAs from diverse organisms is still evolving, here we highlight enticing similarities and differences from *E. coli* and *B. subtilis* 6S RNAs. Like *B. subtilis*, *Legionella pneumophila* is reported to have two 6S RNAs: 6S RNA (referred to here as 6S1 RNA for clarity) and 6S2 RNA (19, 20, 75). Lp6S1 RNA, predicted by bioinformatics, has a secondary structure similar to that of *E. coli* and *B. subtilis* 6S RNAs (**Figure 1**), accumulates to high levels postexponentially, and forms a complex with RNAP (19). However, only a fraction of Lp6S1 RNA coimmunoprecipitates with RNAP, suggesting that the complex may be less abundant or less stable than in *E. coli* or *B. subtilis*, where 50–90% of each 6S RNA coimmunoprecipitates with RNAP (66, 73). Lp6S1 RNA–dependent changes in gene expression were surprising in that the majority of genes were upregulated in the presence of Lp6S1 RNA and only a handful downregulated (19). For comparison, in *E. coli*, where the direct effect of Ec6S RNA is to downregulate σ^{70}-dependent transcription, hundreds of genes are downregulated, although many genes are also upregulated, presumably because of secondary effects (13, 47). This difference may represent a diverse mechanism for Lp6S1 RNA function. Alternatively, the presence of Lp6S2 RNA may affect observed changes in transcription. Nevertheless, many of the Lp6S1-regulated genes have intracellular multiplication functions, and phenotypic analysis revealed cells lacking Lp6S1 RNA had reduced intracellular survival in host cells (19), expanding the list of 6S RNAs that affect cellular survival.

Much less is known about Lp6S2 RNA. It was identified by RNAseq and suggested to be a 6S RNA based on predicted secondary structure (75). The presence of a second candidate 6S RNA in this gram-negative organism unrelated to the gram-positive *B. subtilis* raises interesting questions regarding the potential for multiple 6S RNAs in divergent species. Somewhat similarly to Bs6S-1 and Bs6S-2 RNAs, the Lp6S1 and Lp6S2 RNAs have different expression profiles (19, 75). It remains to be seen if Lp6S2 RNA does indeed function as a 6S RNA and when it functions.

Many 6S RNAs accumulate under conditions that hint at interesting cellular roles. Cyanobacteria 6S RNAs provide several examples. 6S RNA in *Synechococcus* sp PCC6301 is decreased in stationary phase compared with exponential phase (74), in direct opposition to Ec6S and Bs6S-1 RNAs and more similar to Bs6S-2 RNA. *Prochlorococcus* MED4 6S RNA levels are cell-cycle dependent rather than growth-rate dependent and also change with light, consistent with the high-light adaptation of this strain (2). Reduced fitness has been observed in *Synechocystis* PCC6803 cells lacking 6S RNA when grown on plates but not when grown in liquid culture, which has been proposed to be due to a difference in light stress (54).

Many α-proteobacterial 6S RNA candidates have been identified (3). *Wolbachia*, an obligate intracellular α-proteobacterium, has a 6S RNA candidate that shows differential expression in

somatic versus germ-line cells, perhaps regulating or regulated by differences in timing of replication (18). The *Wolbachia* 6S RNA accumulates during fast replication, in contrast to *E. coli* 6S RNA that accumulates during slow growth in stationary phase. For the plant symbiont *Bradyrhizobium japonicum*, cells in root nodules have increased 6S RNA levels compared with free-living cells (42, 70).

Other candidate 6S RNAs have been identified in pathogens. *Clostridium acetobutylicum* 6S RNA increases during general stress (17, 58, 68). *Burkholderia cenocepacia* 6S RNA increases during oxidative stress, which may indicate a role in responding to host immune systems (52). *Yersinia pestis* 6S RNA levels are changed during lung infection compared with levels in in vitro culture, although *Y. pestis* cells lacking 6S RNA were reported to have normal virulence (80).

The functional significance of all these intriguing expression profiles awaits further investigation, but they certainly suggest a global role for 6S RNA in response to environmental shifts and/or survival during stress, but with variations appropriate to the niche and needs of different bacteria. Other 6S RNA candidates have been shown to be expressed under at least one condition [e.g., *Aquifex aeolicus* (77), *Bordetella pertussis* (66), *Caulobacter crescentus* (37), *Clostridium difficile* (58), *Helicobacter pylori* (56), *Pseudomonas aeruginosa* (69), *Rhodobacter sphaeroides* (8), *Rhodospeudomonas palustris* (42), *Streptomyces coelicolor* (50, 51)], and there are many, many additional predicted 6S RNA genes.

Of primary importance is whether these 6S RNA candidates bind RNAP, but direct interaction has been tested in only a handful of cases. In vivo complexes have been demonstrated in *E. coli*, *B. subtilis*, and *L. pneumonia* (Lp6S1), and complexes with cognate RNAPs have been studied in vitro for *E. coli* and *B. subtilis*. A somewhat indirect, yet compelling, argument for RNAP binding is the presence of 6S RNA–templated pRNAs in *H. pylori* and *B. japonicum* (42, 56). Several cyanobacterial 6S RNAs have been shown to bind RNAP, regulate transcription, and direct pRNA synthesis in vitro using *E. coli* $E\sigma^{70}$ (54). However, one caveat regarding heterologous studies is that surprising differences in behavior of *B. subtilis* and *E. coli* RNAPs in 6S RNA function have been described (11) (see below).

Secondary structure similarity to known 6S RNAs has been the principal feature defining 6S RNA candidates. However, some candidate 6S RNAs have notable changes in structure. *L. pneumophila* 6S1 RNA has a short downstream stem, and cyanobacterial 6S RNAs have reduced central regions, although all these RNAs can bind RNAP, suggesting a flexibility in structure in these regions (19, 54, 57). *A. aeolicus* and many α-proteobacteria have a shorter upstream stem (3, 77), which is a region of the RNA critical for binding RNAP in *E. coli*, presumably because of interaction with σ^{70}-region 4.2 (13, 34, 57, 61). The *A. aeolicus* RNA and several tested α-proteobacterial RNAs do not bind *E. coli* RNAP (K.M. Wassarman, unpublished observations), although these RNAs may very well interact with their cognate RNAPs. Definitive conclusions await experimental testing, but if these divergent RNAs are indeed 6S RNAs (i.e., bind to primary RNAP), they suggest a complexity in RNA-RNAP interactions in different species not previously appreciated; if not, they establish another class of sRNAs.

New evidence is accumulating that additional sRNAs may interact with RNAP in a manner different enough from *E. coli* and *B. subtilis* 6S RNAs to define independent classes of RNAs. For example, in *Mycobacteria smegmatus*, Ms1 RNA interacts with core RNAP rather than the primary holoenzyme, in spite of having originally been identified based on secondary structure similarity to 6S RNA [(51); J. Hnilicová & L. Krásný, personal communication]. In addition, RNAP has been reported to add nontemplated nucleotides on the end of several sRNAs (78), which describes yet another type of RNA:RNAP interaction, although the physiological significance remains unclear.

REGULATION OF 6S RNA

Generation of 6S RNA

Understanding 6S RNA biogenesis and its regulation may provide insight into the cellular role of 6S RNA. We focus on Ec6S RNA biogenesis, as little is known about this process in other organisms. Two promoters direct transcription of *ssrS*, the gene encoding 6S RNA: P1 is σ^{70}-dependent, and P2 is utilized by both Eσ^{70} and Eσ^{S}, which hinted that 6S RNA accumulation in stationary phase might be driven by σ^{S} (26, 32, 39). However, 6S RNA accumulates normally in cells lacking σ^{S} (73). Both promoters are upregulated during transition into stationary phase, and neither is regulated by the stringent response or 6S RNA at endogenous levels (27, 39, 46). Several additional factors have been implicated in regulation of *ssrS* transcription (e.g., HNS, LRP, StpA, and Fis) (46). This study revealed that these regulators specifically downregulate *ssrS* transcription during stationary phase, and thus they cannot be directly responsible for the observed accumulation of 6S RNA during stationary phase. However, their action suggests that a complex interplay of cellular components contribute to 6S RNA synthesis.

Although the mechanism(s) driving 6S RNA accumulation remains unclear, transcriptional regulation is unlikely to be solely responsible. Posttranscriptional regulation is supported by observations that 6S RNA accumulation profiles remain similar when expressed from an unregulated, exogenous promoter, although absolute levels can vary (73). Mature Ec6S RNA is processed from larger precursor RNAs (16, 26, 32, 41), and differential processing efficiency could contribute to accumulation of 6S RNA, although there is no evidence that processing events are regulated in *E. coli*. However, 6S RNAs of different sizes have been observed in high-light adapted cyanobacteria, which might suggest regulated processing (2). Differential stability of 6S RNA in exponential and stationary phase also could contribute to stationary phase accumulation. Free 6S RNA is expected to be more accessible to cellular RNases, and thus less stable, than 6S RNA bound and protected by RNAP. Mechanisms that alter the fraction of 6S RNA bound to RNAP, such as pRNA synthesis (see below), could significantly affect 6S RNA stability and levels. Mature 6S RNA has been reported to be very stable (40), but this study used rifampicin, which is now known to prevent pRNA synthesis and is likely to alter 6S RNA stability.

Regulation of 6S RNA by pRNA Synthesis

One of the most exciting discoveries about 6S RNA in the last decade was that it can be used as a template by RNAP to produce de novo product RNA (pRNA) by a process called pRNA synthesis. Mechanistic in vitro assays have revealed many details about pRNA synthesis in *E. coli* and *B. subtilis* (5, 6, 11, 14, 21, 49, 57, 62, 72, 79) (**Figure 2**). pRNA synthesis initiation parallels transcription initiation and includes an abortive cycle, apparent scrunching of the template, and release of σ^{70} after initiation. However, in contrast to highly stable transcription elongation complexes, 6S RNA-pRNA-core complexes are unstable, in part because of an internal stem structure stimulated by pRNA synthesis, which leads to the release of 6S RNA:pRNA hybrids that are unable to rebind RNAP.

In vivo studies also have been informative (5, 14, 72, 79) and have revealed that abundant pRNA synthesis occurs very soon after stationary phase cells are shifted to fresh medium (i.e., during outgrowth), at least for Ec6S RNA in *E. coli* and Bs6S-1 RNA in *B. subtilis* (see below for Bs6S-2 RNA). Little pRNA synthesis is detected in late stationary phase. Nucleotide levels increase substantially during outgrowth (45), and in vitro pRNA synthesis is sensitive to nucleotide concentrations (11, 72), suggesting nucleotide levels may trigger the switch from 6S RNA inhibition (e.g., 6S RNA:RNAP) in stationary phase to 6S RNA–templated pRNA synthesis during

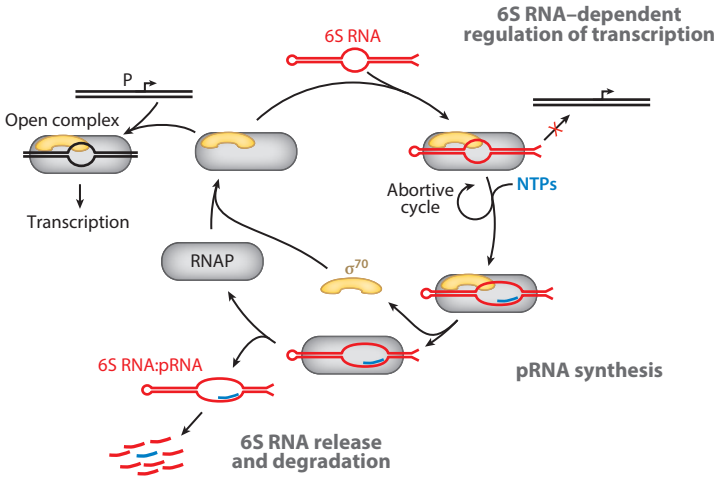

Figure 2.

Model for the regulation of 6S RNA function and RNA polymerase availability by pRNA synthesis. The model is shown with the *Escherichia coli* components, but it is expected to represent 6S RNA function more globally. 6S RNA, promoter DNA (P), core RNA polymerase (RNAP), σ^{70}, 6S RNA:pRNA duplexes, open complex, and nucleotide substrates (NTPs) are as labeled. The processes important for 6S RNA function discussed here are shown in bold (regulation of transcription, pRNA synthesis, and 6S RNA release and degradation).

outgrowth. How much pRNA synthesis occurs in exponential phase is less clear, although pRNA has been detected in exponential phase, suggesting pRNA synthesis is not limited to outgrowth (5, 56).

It has been predicted that pRNA synthesis has multiple outcomes with potential physiological relevance: (*a*) release of RNAP from 6S RNA, thereby relieving RNAP from 6S RNA inhibition and leading to increased transcription required for outgrowth and (*b*) destabilization of 6S RNA due to the inability of released 6S RNA:pRNA to rebind RNAP, making 6S RNA more accessible for degradation (**Figure 2**). Consistent with a role for pRNA synthesis in regulating 6S RNA levels, Ec6S RNA decreases substantially between stationary phase and early log phase, but the level of a mutant 6S RNA that does not support pRNA synthesis remains high (14, 73). In addition, *E. coli* cells expressing the mutant RNA are delayed in their ability to reenter exponential growth (14), demonstrating a critical role for pRNA synthesis at this time, presumably through regulation of 6S RNA and free RNAP levels. Mathematical modeling and simulation suggest these two parameters, release of RNAP and destabilization of 6S RNA, are sufficient to have a dramatic effect on transcription (48).

Somewhat surprisingly, pRNAs templated by Bs6S-2 RNA have not been detected in vivo, and the level of pRNA synthesis from Bs6S-2 RNA in vitro can be much lower than for Bs6S-1 RNA by *B. subtilis* Eσ^{A}, suggesting Bs6S-2 RNA is not an efficient template for pRNA synthesis (5, 14, 28). The Bs6S-2 RNA:pRNA hybrid would be considerably weaker than Bs6S-1 RNA:pRNA. This observation led to the alternate hypothesis that Bs6S-2 RNA does support pRNA synthesis but that pRNA readily dissociates, thereby allowing rebinding of released Bs6S-2 RNA and presumed degradation of the pRNA (10). Consistent with either a lack of pRNA-synthesis-mediated release or a rapid rebinding to RNAP, *B. subtilis* cells expressing Bs6S-2 RNA alone (i.e., lacking Bs6S-1 RNA) are delayed in outgrowth (14). It is unclear how the presence of Bs6S-1 RNA in wild-type cells relieves Bs6S-2 RNA inhibition of outgrowth, although it is not through changes in Bs6S-2

RNA levels or the fraction of Bs6S-2 RNA bound to RNAP, and Bs6S-1 RNA is not able to actively displace Bs6S-2 RNA on RNAP in vitro (10, 14).

What determines the different pRNA synthesis efficiencies of Bs6S-1 and Bs6S-2 RNAs? pRNA from Bs6S-1 RNA initiates with GTP, whereas pRNA from 6S-2 RNA initiates with ATP, suggesting a possible explanation. Further study of pRNA synthesis on heterologous RNAs differing at only one position, thereby directing initiation of pRNA synthesis with either GTP or ATP, revealed *B. subtilis* $E\sigma^A$ has a strong preference to initiate with GTP (11). Furthermore, an outgrowth delay was observed in *B. subtilis* cells expressing the heterologous RNA initiating with ATP but not GTP. These results are consistent with a lack of efficient pRNA synthesis in vivo rather than rapid rebinding, as there would be minimal differences in stability of these RNA:pRNA duplexes. Interestingly, Bs6S-2 RNA levels are not decreased in exponential phase (1, 3, 5, 66), which may be a result of inefficient pRNA synthesis.

In contrast to *B. subtilis* $E\sigma^A$, *E. coli* $E\sigma^{70}$ will initiate pRNA synthesis with any nucleotide, and the wild-type Ec6S RNA directs initiation with ATP, revealing a major difference in behavior of these RNAPs for pRNA synthesis (11). Mechanistic studies are needed to elucidate what directs these changes in behavior. However, *B. subtilis* has evolved intricate regulatory networks, not present in *E. coli*, designed to respond specifically to GTP levels (35). So perhaps pRNA synthesis in *B. subtilis* also is designed to respond to GTP levels, though this does not explain mechanistically why *B. subtilis* $E\sigma^A$ does not initiate efficiently with ATP.

The global role of pRNA synthesis, such as in regulating 6S RNA activity and/or its accumulation profiles, will require further study of diverse 6S RNAs in many organisms. It will be particularly interesting to learn if and how pRNA synthesis functions in bacteria that have 6S RNAs with very different accumulation profiles, and what signals determine pRNA synthesis timing and efficiency.

CONCLUSIONS

6S RNAs are a growing class of sRNAs defined by the ability to bind the primary form of RNAP in a manner similar to promoter DNA binding. Many questions remain, even for the best-studied *E. coli* and *B. subtilis* 6S RNAs, and the identification of each new 6S RNA has led to further intriguing questions about how and when 6S RNAs function. Mechanistic details underlying 6S RNA regulation of transcription remain unclear, even for *E. coli*, where features of promoters sensitive to Ec6S RNA have been determined. *B. subtilis* 6S-1 and 6S-2 RNAs are suggested to regulate with different promoter specificity in spite of both binding $E\sigma^A$, and *L. pneumophila* 6S1 RNA may directly activate, rather than inhibit, transcription. Any models describing 6S RNA–dependent regulation must be able to account for these different behaviors in diverse organisms, but first more details in all species are required.

Expression studies have revealed that 6S RNA is a global regulator, directing changes in expression of an abundance of genes, including many that encode high-level regulators. Of note, 6S RNA regulation of transcription is best described as modulatory given the modest level of change at individual genes and is probably not a mechanism to turn genes off. Based on *E. coli*, it is suggested that 6S RNA dampens aspects of stress response, presumably to conserve available resources and optimize survival when nutrients are limiting. The role of 6S RNA in survival appears to be a common theme, although the precise details can differ between organisms. 6S RNA accumulation profiles also vary, suggesting diversity in when and where these RNAs might be most important to the cell. However, we suggest 6S RNA function may contribute significantly to transcriptional regulation even when levels are not maximal, such as in exponential phase in *E. coli*, when 6S RNA is only one-tenth as abundant as in stationary phase.

Of great interest is the use of 6S RNA as a template by RNAP during pRNA synthesis. We suggest pRNA synthesis is one important mechanism to regulate 6S RNA activity by removing 6S RNA from RNAP in a manner that prevents rebinding of 6S RNA (as 6S RNA:pRNA) and contributes to the destabilization of 6S RNA (**Figure 2**). Although it is tempting to speculate an independent activity for pRNA, there is no evidence to suggest a function beyond the role its synthesis plays in regulating 6S RNA. Furthermore, it remains unclear whether pRNA synthesis is a ubiquitous 6S RNA trait or whether some 6S RNAs are not regulated in this manner, as has been suggested for Bs6S-2 RNA. The potential regulation of 6S RNA by other factors, such as RNAP-interacting factors or transcription factors, is another area to be investigated. Such studies may also highlight how 6S RNA is able to convert the normal cellular RNAP into an RNA-dependent RNA polymerase.

A clearer understanding of how 6S RNAs behave in diverse organisms is expected to provide a better picture of how these RNAs function generally but is also likely to provide more information about how individual species are able to take advantage of this specialized strategy to manage stress and survival. All in all, there is still much to be learned about 6S RNA, from detailed mechanistic to broader physiological questions.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Apologies to those whose original contributions were not cited because of space constraints. Work in our lab is supported by the National Institutes of Health (GM67955) and the Greater Milwaukee Foundation through a Shaw Scientist Award (K.M.W.).

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