Regulation of Transcription from Two *ssrS* Promoters in 6S RNA Biogenesis

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***ssrS*-encoded 6S RNA is an abundant noncoding RNA that binds σ^{70} -RNA polymerase and regulates expression at a subset of promoters in *Escherichia coli*. It is transcribed from two tandem promoters, *ssrS* P1 and *ssrS* P2. Regulation of transcription from two *ssrS* promoters in 6S RNA biogenesis was examined. Both P1 and P2 were growth phase-dependently regulated. Depletion of 6S RNA had no effect on growth-phase-dependent transcription from either promoter, whereas overexpression of 6S RNA increased P1 transcription and decreased P2 transcription, suggesting that transcription from P1 and P2 is subject to feedback activation and feedback inhibition, respectively. This feedback regulation disappeared in Δ *fis* strains, supporting involvement of Fis in this process. The differential feedback regulation may provide a means for maintaining appropriate cellular concentrations of 6S RNA.**

INTRODUCTION

Escherichia coli 6S RNA is a small RNA (184 nucleotides) that has an extended double-stranded structure with a large single-stranded bulge, like DNA structure in an open promoter complex (Barrick et al., 2005; Trotochaud and Wassarman, 2005). 6S RNA was first described in 1971 (Brownlee, 1971), but it was not until 2000 that its function as a regulator of σ^{70} -dependent gene transcription was identified (Wassarman and Storz, 2000). 6S RNA inhibits transcription from a subset of σ^{70} -dependent promoters by interacting with σ^{70} -RNA polymerase ($E\sigma^{70}$) during the stationary growth phase, an action that may result in activation of transcription from σ^S -dependent promoters (Trotochaud and Wassarman, 2004; Wassarman and Storz, 2000). Therefore, 6S RNA appears to act as a regulator of transcription during the change in growth from exponential to stationary phase. Rescue of 6S RNA-mediated inhibition of σ^{70} -dependent transcription, which is essential for recovery from the stationary phase, can be achieved by synthesis of short product RNA (pRNA) from 6S RNA (Cavanagh et al., 2012; Gildehaus et al., 2007; Shephard et al., 2010; Wurm et al., 2004). pRNA is directly synthesized by $E\sigma^{70}$ from the 6S RNA template, leading to the release of 6S RNA from the RNA po-

lymerase (Cavanagh et al., 2012; Gildehaus et al., 2007; Shephard et al., 2010; Wurm et al., 2004).

Because 6S RNA participates in the modification of general transcription upon changes in environmental growth conditions, such as nutrient exhaustion, in the transition from exponential to stationary phase growth, its cellular levels should be regulated in response to environmental signals. 6S RNA is transcribed from two tandem promoters, *ssrS* P1, which generates a short P1 transcript with an extra 9 nucleotides at the 5'-end of the mature 6S RNA, and *ssrS* P2, which generates a long P2 transcript with an extra 224 at 5'-nucleotides (Kim and Lee, 2004). P1 is a canonical σ^{70} -dependent promoter, whereas P2 is dependent on both σ^{70} and σ^S . Transcription from the two promoters is terminated by Rho factor approximately 90 nucleotides beyond the mature 3'-end (Chae et al., 2011). The sequential removal of the extra 3' and 5' sequences by exonucleases and RNase E/G, respectively, leads to formation of the mature 6S RNA (Chae et al., 2011; Kim and Lee, 2004). Upon changes in growth conditions, it is thought that 6S RNA levels are modulated by switching utilization of sigma factors to form distinct RNA polymerase holoenzymes and by regulating the efficiency of 6S RNA processing. However, how 6S RNA biogenesis is regulated in response to environmental changes is not yet understood in detail.

Here, as an initial step to elucidate the regulatory circuit of 6S RNA biogenesis, we investigated regulation of transcription from each promoter during growth and then the potential of 6S RNA to regulate its transcription through effects on its own promoters. We showed that both P1 and P2 promoters were growth phase-dependently regulated. This growth-phase-dependent regulation of either promoter was not affected by 6S RNA depletion, whereas overexpression of 6S RNA increased P1 transcription and decreased P2 transcription. These effects of 6S RNA overexpression were related to the extent to which 6S RNA was elevated above the normal level. Thus, it is likely that P1 is feedback-activated and P2 is subject to feedback inhibition. This differential feedback regulation disappeared in strains lacking Fis, which is known to repress P2 transcription and slightly increase P1 transcription (Neusser et al., 2008), suggesting that Fis is involved in this feedback regulation. Because the sigma factor-dependence of these two promoters is

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different, this differential feedback regulation may serve to integrate sigma factors, providing a mechanism for maintaining required cellular levels of 6S RNA in response to environmental changes.

MATERIALS AND METHODS

Strains, plasmids, and bacterial growth

E. coli cells were cultivated in Luria-Bertani (LB) medium or M63 minimal medium (Wassarman et al., 2001) with or without ampicillin (100 µg/ml) or kanamycin (50 µg/ml) supplementation. An *ssrS::kan* cassette was transferred from strain MG1655 Δ *ssrS* to DJ480 to generate strain DJ480 Δ *ssrS* by P1 transduction (Chae et al., 2011); the *ssrS* deletion was confirmed by polymerase chain reaction (PCR). Plasmids pJY1 and pJY2 containing *ssrS* P1-*lacZ* and *ssrS* P2-*lacZ* transcriptional fusions, respectively, were prepared by amplifying the *ssrS* promoter regions (-334 to -203 for pJY1 and -92 to +10 for pJY2, where numbers denote positions relative to the 5'-end of 6S RNA) by PCR and cloning into the *EcoRI/BamHI* sites of pRS1553 (Simons et al., 1987). Single-copy chromosomal fusions were generated in DJ480 or DJ480 Δ *ssrS* from pJY1 and pJY2 through λ RS468, as described (Pepe et al., 1997; Powell et al., 1994; Simons et al., 1987), leading to the strains JY001 [DJ480 λ *ssrS*(P1)-*lacZ*], JY002 [DJ480 λ *ssrS*(P1)-*lacZ* Δ *ssrS*], JY003 [DJ480 λ *ssrS*(P2)-*lacZ*], and JY004 [DJ480 λ *ssrS*(P2)-*lacZ* Δ *ssrS*]. Single-copy chromosomal fusions were confirmed by PCR, as described (Powell et al., 1994). A *fis::kan* cassette was also transferred from the Keio collection to JY001 [DJ480 λ *ssrS*(P1)-*lacZ*] and JY003 [DJ480 λ *ssrS*(P2)-*lacZ*] to generate JY005 [DJ480 λ *ssrS*(P1)-*lacZ* Δ *fis*] and JY006 [DJ480 λ *ssrS*(P2)-*lacZ* Δ *fis*] strains. The *fis* deletion was confirmed by polymerase chain reaction (PCR). The plasmid pHM4T, an isopropylthio- β -galactoside (IPTG)-inducible RNA expression vector derived from the plasmid pHM1 (Han et al., 2010), was used to construct plasmids p6S-S and p6S-L, which generate ectopic 6S RNA upon IPTG induction. The *ssrS* regions for p6S-S (-9 to +258) and p6S-L (-224 to +258) were PCR-amplified. In the case of the -224 to +258 *ssrS* fragment, the -10 element of P1 (positions -21 to -16) was changed from 'TAGAGT' to 'CTCGAG' using the SOEing method (Ho et al., 1989). This mutation inactivates the internal P1 promoter; thus, the -224 to +258 fragment can generate transcripts only through IPTG induction. PCR-amplified DNA fragments were cloned into the *EcoRI/XbaI* sites of pHM4T. The oligonucleotides employed are listed in Table 1.

Preparation of total cellular RNA

Cells were grown overnight in LB broth containing ampicillin (100 µg/ml) at 37°C with vigorous shaking. The overnight cultures were diluted 100-fold in fresh LB medium and grown for 2 h to dilute out the remaining 6S RNA and β -galactosidase (to the extent possible) that had been expressed during the stationary phase. After further diluting 1:20 in fresh LB, cells were grown at 37°C for a specific length of time with or without IPTG. In the case of cells grown without IPTG, IPTG was added and cells were cultured for an additional incubation period. Total cellular RNA was extracted from the culture as previously described (Kim et al., 1996).

Northern blot analysis

Total cellular RNA (10 µg) was fractionated on 6% polyacrylamide gels containing 7 M urea and electro-transferred onto a Hybond-XL membrane (GE Healthcare). Oligonucleotide probes

were labeled with [γ -³²P]ATP using T4 polynucleotide kinase (Enzymonics). The oligonucleotides *ssrS*+185_R and a5S were used for probing 6S RNA and 5S RNA, respectively (Table 1). The membranes were hybridized in RapidHyb buffer (GE Healthcare) according to the manufacturer's instructions, and were visualized and quantified using Image Analyzer FLA7000 (Fuji) and TINA v2.0 software.

β -Galactosidase assay

Cells were grown as for preparation of total cellular RNA except that ampicillin was not included in cultures of cells containing no plasmids. Relative β -galactosidase activities were determined, as described previously (Miller, 1992). At least triplicate measurements were performed for each strain.

RESULTS

Growth-dependence of *ssrS* promoters

6S RNA accumulates considerably during growth (Trotochaud and Wassarman, 2004), and its growth-dependent accumulation depends on transcription from two promoters, *ssrS* P1 and *ssrS* P2 (Kim and Lee, 2004). Previous studies examining the levels of primary transcripts from P1 and P2 have shown that both transcripts increase with growth in the exponential phase, whereas during the transition from the exponential to the stationary phase, the level of P1 transcripts substantially declines and the level of P2 transcripts remains relatively constant. However, the levels of primary transcripts do not fully represent the relative activities of the corresponding promoters because the fates of each transcript may differ. To determine how *ssrS* promoter activities are changed during growth, we introduced *ssrS* P1- or P2-*lacZ* fusion constructs into the *E. coli* chromosome using lambda phages to generate JY001 and JY003 strains, respectively, and measured their β -galactosidase activities during growth (Fig. 1A). The P1 promoter region used consisted of the sequence from -92 to +10 relative to the 5'-end of 6S RNA (+1), leading to transcription initiation at -9; the corresponding P2 promoter region covered -334 to -203, with transcription-initiation occurring at -224. We found that P1 was 2-3 times more active than P2 in the exponential phase and the activities of both promoters gradually increased until the cells reached the late-exponential phase. Beyond that point, P1 activity fell sharply; P2 activity also decreased, but to a much lesser extent (to ~80%). Then, both promoter activities approached nearly the same level.

Effects of 6S RNA on the activities of *ssrS* promoters

Many transcription factors regulate the expression of their own genes by modulating promoter activity (Stekel and Jenkins, 2008). Accordingly, we asked whether *ssrS* promoters could be regulated by their own product, 6S RNA. To test whether 6S RNA affects transcription from *ssrS* promoters, we first investigated the expression profiles of *ssrS* promoter-*lacZ* fusions during growth by measuring β -galactosidase activity in *ssrS*⁺ and Δ *ssrS* background cells grown in rich LB medium (Fig. 1B). The Δ *ssrS* strains (JY002 and JY004) showed the same expression profiles as those of *ssrS*⁺ cells (JY001 and JY003), suggesting that the absence of 6S RNA did not affect the activity of either promoter throughout growth. We also examined the effects of the absence of 6S RNA on the *ssrS* promoter activities in a minimal medium where the promoter activities may be more tightly regulated. Transcription from both *ssrS* promoters was not significantly affected by the depletion of 6S RNA in the minimal medium either (Fig. 1C).

Table 1. Oligonucleotides used in this study

Primers	Sequence (5' to 3')	Use
P2_F	AGT GAA TTC GTT CAC TGC GTG TGA	pJY1 preparation
P2_R	TCC GGA TCC CAA TGA AGA CCA ACT GTT CAG	
P1_F	AGT GAA TTC TTA CTT GAA CAA GGT CGC A	pJY2 preparation
P1_R	TCC GGA TCC TCA GAG AAA TTT TGT CTT CAC GG	
<i>ssrS</i> -224_F	CGG AAT TCA CTG AAC AGT TGG TCT TCA TTG	p6S-S and p6S-L preparation
<i>ssrS</i> -9_F	CGG AAT TCG AAG ACA AAA TTT CTC TGA G	
<i>ssrS</i> +258_R	GCT CTA GAT TTG CGG ATT TCT TGT CGG	
<i>ssrS</i> +185_R	CGC AAG CTT GGG AAT CTC CGA GAT GCC GCC	Anti-6S RNA probe
a5S	CGG CAT GGG GTC AGG TGG	Anti-5S rRNA probe

Fig. 1. Growth phase-dependence of *ssrS* promoters. (A) Schematic diagrams of *ssrS-lacZ* transcriptional constructs. Strains JY001 and JY002 carry the *ssrS*(P1)-*lacZ* fusion construct, whereas strains JY003 and JY004 carry the *ssrS*(P2)-*lacZ* fusion construct. The *ssrS* promoter regions used for the construction of the fusions are -92 to +10 for P1 and -334 to -203 for P2. Each transcription start site is indicated by arrows. Primers a, b, and c used for confirmation of the corresponding lysogens are indicated. *ssrS* promoter activities and growth curves in rich LB medium (B) and in M63 minimal medium (C). Levels of β -galactosidase expressed from P1 or P2 are presented in Miller units. Filled and open symbols represent *ssrS*⁺ and Δ *ssrS* cells, respectively. Filled circle and open circle, *ssrS*(P1)-*lacZ*; filled triangle and open triangle, *ssrS*(P2)-*lacZ*. The data are representative results from one of three independent experiments.

Fig. 2. Ectopic expression of 6S RNA. Ectopic expression of 6S RNA from p6S-S or p6S-L by IPTG. *ssrS*-deleted cells containing RNA expression plasmids were grown to an OD₆₀₀ of ~0.4, after which 10⁻¹ mM IPTG was added to the culture. Total cellular RNA was prepared at the indicated time points after IPTG addition. Each RNA sample (10 μg) was fractionated on a 6% polyacrylamide gel containing 7 M urea. 6S RNA transcripts were analyzed by northern blotting. pHM, control RNA expression vector pHM4T.

Fig. 3. Effect of 6S RNA overexpression on *ssrS* promoter activities. *ssrS* promoter activities and growth curves, measured as β-galactosidase levels, for (A) JY001 [P1-*lacZ ssrS**], (B) JY002 [P1-*lacZ ΔssrS*], (C) JY003 [P2-*lacZ ssrS**], and (D) JY004 [P2-*lacZ ΔssrS*]. Cells were grown in the presence of 10⁻¹ mM IPTG in LB medium. Plasmids p6S-S and p6S-L generate P1 transcripts and P2 transcripts upon IPTG induction. pHM4T, control RNA expression vector.

We next examined whether higher cellular levels of 6S RNA affected expression from the *ssrS* promoters. Overexpression of 6S RNA was accomplished using the IPTG-inducible RNA expression vector, pHM4T. DNA fragments spanning nucleotides -9 (initiation site) to +258 for P1 transcripts and -224 (ini-

tiation site) to +258 for P2 transcripts were cloned into pHM4T to generate the plasmids p6S-S and p6S-L, respectively. Transcription of natural 6S RNA is terminated by Rho factor near +280 and the 3'-end region of the terminated transcripts contains the N-terminal coding region of *ygfA* (Chae et al., 2011).

Fig. 4. Effect of 6S RNA overexpression on *ssrS* promoter activities in M63 minimal medium. *ssrS* promoter activities measured as β-galactosidase levels at 12 h, for (A) JY001 [P1-*lacZ ssrS*⁺], (B) JY002 [P1-*lacZ ΔssrS*], (C) JY003 [P2-*lacZ ssrS*⁺], and (D) JY004 [P2-*lacZ ΔssrS*]. Cells were grown in the presence of 10⁻¹ mM IPTG. Plasmids p6S-S and p6S-L generate P1 transcripts and P2 transcripts upon IPTG induction. pHM4T, control RNA expression vector.

Fig. 5. Relationship between cellular levels of 6S RNA and *ssrS* promoter activities. 6S RNA expression from plasmids p6S-S or p6S-L was modulated by varying IPTG concentration (0, 10⁻⁵, 10⁻³, and 10⁻¹ mM). (A, B) Promoter activities were monitored in strains JY002 [P1-*lacZ ΔssrS*] and JY004 [P2-*lacZ ΔssrS*] carrying p6S-S or p6S-L grown to stationary phase (5.5 h growth after dilution of overnight cultures into fresh medium). Strains JY001 [P1-*lacZ ssrS*⁺] and JY003 [P2-*lacZ ssrS*⁺] carrying the parental RNA expression vector pHM4T were used as controls. (C, D) Northern blot comparing cellular levels of 6S RNA among the strains used to assay *ssrS* promoter activities. The relative levels of 6S RNA, normalized to 5S RNA, are presented below the northern data. pHM, control RNA expression vector pHM4T.

Since this region may interfere with cellular metabolism, the sequences before the start codon (+259) of *ygfA* were cloned into the RNA expression vector. The IPTG-induced transcription was terminated at the *rmB* terminator in the vector. 6S RNA transcripts terminated at heterologous terminators are

quickly processed at the 3'-end as much as the transcripts terminated by Rho factor (Chae et al., 2011). The 5'-leader sequences of pre-6S RNA transcripts produced by p6S-S and p6S-L plasmids upon IPTG induction were the same as those generated from natural P1 and P2 promoters, respectively,

Fig. 6. Effects of 6S RNA overexpression in Δfis strains. Promoter activities were monitored in strains JY005 [P1-*lacZ* Δfis] and JY006 [P2-*lacZ* Δfis] carrying pHM4T, p6S-S or p6S-L grown to stationary phase (5.5 h growth after dilution of overnight cultures into fresh LB medium). Strains JY001 [P1-*lacZ* *ssrS*⁺] and JY003 [P2-*lacZ* *ssrS*⁺] carrying the parental RNA expression vector pHM4T were used as controls. 6S RNA was expressed from plas-mids p6S-S or p6S-L by 10⁻¹ mM IPTG.

except that the sequence from position -21 to -16 in the transcripts from p6S-L was changed as a result of mutating the -10 element of the internal P1 site to inactivate transcription from it. We found that the two transcripts were induced by IPTG and properly processed to mature 6S RNA (Fig. 2). The amounts of mature 6S RNA generated from IPTG-induced -9 and -224 primary transcripts from p6S-S and p6S-L, respectively, were almost same, suggesting that P1 and P2 transcripts contribute equally to the biogenesis of 6S RNA in the cell. This 6S RNA expression system was used to generate higher levels of 6S RNA in the cells. Because the 5'-leader sequences, in addition to the mature 6S RNA sequence, could also be involved in the putative regulatory circuit controlling the biogenesis of 6S RNA, we used both plasmids p6S-S and p6S-L to express 6S RNA. The plasmids were introduced into bacterial strains JY001 and JY003, and ectopic expression of 6S RNA was induced with IPTG. Measurements of β -galactosidase activity in plasmid-carrying cells grown in the presence of 10⁻¹ mM IPTG in LB medium showed that overexpression of 6S RNA, whether derived from p6S-S or p6S-L, increased P1 activity and decreased P2 activity at points beyond the mid-exponential growth phase (Fig. 3). These effects became manifest in the stationary phase. The fact that both p6S-S and p6S-L produced the same effects suggested that mature 6S RNA, not primary transcripts, was involved in regulating its own expression. Because there was no difference in the profiles of *ssrS* promoter activities between *ssrS*-deleted cells and *ssrS*⁺ cells during growth as shown in Fig. 1, it is likely that 6S RNA modulates its own promoters when present in excess of its normal cellular level in wild-type cells. We also examined effects of overexpression of 6S RNA on the *ssrS* promoter activities in the stationary growth phase in the minimal medium (Fig. 4). Overexpression of 6S RNA increased P1 transcription marginally and decreased P2 transcription. It remains to be demonstrated why P1 transcription was less increased in the minimal medium than in LB.

To more directly examine the relationship between the cellular levels of 6S RNA and changes in promoter activities, we used the *ssrS*-deletion strains, JY002 and JY004. As noted above, P1 activity increased and P2 activity decreased during the stationary growth phase in the presence of 10⁻¹ mM IPTG. To determine how the cellular levels of 6S RNA correlate with this divergent effect of overexpressed 6S RNA on the two promoters, we modulated 6S RNA expression by varying IPTG concentration and monitored β -galactosidase expression from P1 and P2 in stationary-phase cells (Fig. 5). Both P1 and P2 responded to 6S RNA only when 6S RNA was present at levels above the normal level in wild-type cells, and the effects of 6S RNA on the promoters were related to the extra amounts of 6S RNA in the cells.

Since it is known that Fis represses *ssrS* P2 transcription, while slightly inducing the promoter activity of *ssrS* P1 transcription (Neusser et al., 2008), we examined whether the effects of 6S RNA overexpression would depend on Fis. Ectopic expression of 6S RNA was induced in *fis*⁺ and Δfis backgrounds and β -galactosidase activities from the *ssrS* promoter-*lacZ* fusions were measured (Fig. 6). The increased P1 activity and decreased P2 activity observed in *fis*⁺ strains (JY001, JY003) cells disappeared in Δfis strains (JY005, JY006), suggesting that overexpressed 6S RNA affects the *ssrS* promoter activities in a Fis-dependent manner.

DISCUSSION

6S RNA accumulates in *E. coli* as cells progress their growth. As the biogenesis of 6S RNA requires not only transcription from the proximal P1 and distal P2 promoters but also processing of the resulting primary transcripts, growth-phase-dependent regulation at both transcriptional and post-transcriptional levels could be involved in regulating 6S RNA accumulation. After transcription from the σ^{70} -dependent P1 promoter and the σ^{70} - σ^S -dependent P2 promoter, the mature 5'-end of 6S RNA is generated from P1 transcripts by RNase E and G, and from P2 transcripts exclusively by RNase E. Therefore, the accumulation of 6S RNA during growth could be achieved through differential utilization of both sigma factors and endoribonucleases. In this study, we found that almost the same levels of mature 6S RNA were generated from ectopically overexpressed P1 transcripts and P2 transcripts, suggesting that the level of contribution of each *ssrS* promoter to 6S RNA biogenesis mostly depends on its promoter activity rather than the processing rate of its transcripts.

The activities of both P1 and P2 were maximal at the late-exponential growth phase and decreased on entering the stationary growth phase. P1 activity was 2-3 times stronger than that of P2 during exponential growth, but the activities of the two promoters became similar in the stationary growth phase. This growth-phase-dependent regulation of P1 and P2 could explain the profile of 6S RNA accumulation during growth (Wassarman and Storz, 2000).

Because 6S RNA is a regulatory RNA that inhibits transcription by interacting specifically with $E\sigma^{70}$, it can be regarded as a transcription factor. Regulation of transcription factor genes by their own products is often an important component in the regulation of transcription factor gene expression, and must be understood to fully appreciate the global regulatory dynamics governing the expression of such genes. Since 6S RNA acts specifically on $E\sigma^{70}$, it is reasonable to assume that 6S RNA could affect transcription from its own σ^{70} -dependent promoters.

However, whether 6S RNA is actually involved in regulating its own expression is unknown. Our data demonstrate that transcription from both *ssrS* promoters was unaffected by the depletion of 6S RNA, whereas overexpression of 6S RNA increased P1 transcription and decreased P2 transcription in the stationary growth phase. The effects of 6S RNA overexpression were related to the extent to which 6S RNA levels exceeded the normal levels in wild-type cells. Thus, 6S RNA appears to affect the activity of its own promoters only after it reaches a certain level, suggesting that P1 and P2 are subject to feedback activation and feedback inhibition, respectively. However, whether these effects are direct or indirect is not yet clear. All studies on promoter specificity of 6S RNA in intact cells have used cells lacking 6S RNA, and have shown that transcription from a subset of σ^{70} -dependent promoters increased in cells lacking 6S RNA, claiming that 6S RNA down-regulate those promoters (Neusser et al., 2010; Trotochaud and Wassarman, 2006; Wassarman, 2007; Wassarman and Storz, 2000). 6S RNA prefers σ^{70} -dependent promoters containing an extended -10 element and a weak -35 element for its inhibitory action (Cavanagh et al., 2008). Since *ssrS* P1 (σ^{70} -dependent) and P2 (σ^{70} - σ^S -dependent) promoters were not affected by the absence of 6S RNA, both the *ssrS* promoters do not belong to σ^{70} -dependent promoters down-regulated by 6S RNA. Thus, the mechanism employed for feedback regulation of transcription from the *ssrS* promoters might be different from the activation mechanism of promoters in the absence of 6S RNA.

Many transcriptional factors have been suggested as effectors for 6S RNA transcription (Neusser et al., 2008). Among them, the DNA-binding protein Fis, which affects the topology and supercoiling of the *E. coli* chromosome to regulate the expression of many genes, represses *ssrS* P2 transcription *in vitro*, while slightly inducing the promoter activity of *ssrS* P1. Because these data correlate with the effects of ectopic overexpression of 6S RNA on the activities of the respective promoters and their Fis-dependency, one might argue that the effects observed here could reflect the regulation of *fis* expression by overexpressed 6S RNA. Although how *fis* expression is regulated by overexpressed 6S RNA remains to be demonstrated, effects of overexpressed 6S RNA on its own expression seem to be indirect.

Since P1 was up-regulated and P2 was down-regulated in the tested growth conditions, this divergent regulation resulted in only a small net change in 6S RNA transcription. However, the net changes could be larger in cells that reciprocally modulate P1 and P2 activities for responding to diverse environmental transitions. Because regulation of *ssrS* transcription was observed when 6S RNA was overexpressed, it would be also intriguing to know under what conditions a higher level of 6S RNA is expressed. It might be possible that the higher level expression could occur through enhancement of 6S RNA transcription by various transcription factors in response to environmental changes.

In summary, transcription from *ssrS* P1 and *ssrS* P2 promoters was growth phase-dependently regulated. Depletion of 6S RNA had no effect on growth-phase-dependent transcription from either promoter. Transcription from P1 and P2 was feedback-activated and feedback-inhibited, respectively. This feedback regulation depends on Fis. The differential feedback regulation could provide cells another level of regulating 6S RNA biogenesis, serving as a means for maintaining appropriate amounts of 6S RNA in response to environmental changes. For example, feedback-inhibition of the σ^S -dependent P2 promoter

may be essential for the inhibition of continued synthesis of 6S RNA necessary for efficient recovery and subsequent regrowth of stationary phase cells in fresh medium.

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