6S RNA regulation of *relA* alters ppGpp levels in early stationary phase

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6S RNA is a small, non-coding RNA that interacts directly with σ^{70}-RNA polymerase and regulates transcription at many σ^{70}-dependent promoters. Here, we demonstrate that 6S RNA regulates transcription of *relA*, which encodes a ppGpp synthase. The 6S RNA-dependent regulation of *relA* expression results in increased ppGpp levels during early stationary phase in cells lacking 6S RNA. These changes in ppGpp levels, although modest, are sufficient to result in altered regulation of transcription from σ^{70}-dependent promoters sensitive to ppGpp, including those promoting expression of genes involved in amino acid biosynthesis and rRNA. These data place 6S RNA as another player in maintaining appropriate gene expression as cells transition into stationary phase. Independent of this ppGpp-mediated 6S RNA-dependent regulation, we also demonstrate that in later stationary phase, 6S RNA continues to downregulate transcription in general, and specifically at a subset of the amino acid promoters, but through a mechanism that is independent of ppGpp and which we hypothesize is through direct regulation. In addition, 6S RNA-dependent regulation of σ^{S} activity is not mediated through observed changes in ppGpp levels. We suggest a role for 6S RNA in modulating transcription of several global regulators directly, including *relA*, to downregulate expression of key pathways in response to changing environmental conditions.

INTRODUCTION

6S RNA is an untranslated, small RNA that was first discovered in *Escherichia coli* as a highly abundant RNA (Hindley, 1967). The cellular function of 6S RNA remained elusive for many years, but it is now known that it regulates gene expression through direct interaction with σ^{70}-RNA polymerase (σ^{70}-RNAP) (for reviews, see Willkomm & Hartmann, 2005; Wassarman, 2007). Although 6S RNA is present throughout growth, it accumulates to its highest levels during late stationary phase, when most Eσ^{70} is bound by 6S RNA (Wassarman & Storz, 2000).

Intriguingly, even during late stationary phase, 6S RNA-dependent downregulation of σ^{70}-dependent transcription is promoter-specific (Trotochaud & Wassarman, 2004). σ^{70}-Dependent promoters are primarily recognized through two core sequences: the -10 element (consensus TATAAT) and the -35 element (consensus TTGATA). In addition, a subset of promoters contains what is referred to as an extended -10 element (consensus TGnTATAAT), which is defined by an additional conserved TG 1 bp upstream of the -10 element (Voskuil *et al.*, 1995; Bown *et al.*, 1997). Previously, we determined that an extended -10 element and a -35 element with a weak match to the

consensus are two features that independently contribute to the 6S RNA sensitivity of promoter expression during late stationary phase (Cavanagh *et al.*, 2008).

To date, studies on 6S RNA regulation of transcription have focused primarily on late stationary phase (>18 h of growth in rich medium) when 6S RNA levels are maximal, although it also has been shown that the presence of 6S RNA leads to altered transcription during late exponential and early stationary phase (Trotochaud & Wassarman, 2004, 2006; Neusser *et al.*, 2010). Identified phenotypes associated with the loss of 6S RNA are most prominent in late and extended stationary phase, and include altered cell survival, particularly during competitive growth and growth at high pH (Trotochaud & Wassarman, 2004, 2006).

During the transition from exponential to stationary phase, a large number of regulators are involved in altering gene expression to facilitate cell adaptation and survival in a changing environment lacking optimal nutrients. One molecule important during this transition is ppGpp (Paul *et al.*, 2004; Gralla, 2005; Magnusson *et al.*, 2005; Potrykus & Cashel, 2008; Srivatsan & Wang, 2008). Decreasing availability of amino acids is largely sensed by RelA, a ppGpp synthase I that is associated with ribosomes. As uncharged tRNAs accumulate as a result of decreasing amino acid pools, RelA is activated and synthesizes ppGpp.

In turn, ppGpp binds to RNA polymerase and regulates transcription at ppGpp-sensitive promoters. Many of the promoters that are upregulated by ppGpp direct the expression of genes involved in amino acid biosynthesis and uptake (Barker *et al.*, 2001). Conversely, the rRNA genes are downregulated by ppGpp. In this manner, ppGpp is a key player in the response to declining nutrients and in the control of growth rate through regulation of ribosome synthesis.

Here, we demonstrate that 6S RNA regulates the expression of *relA*, which leads to changes in ppGpp levels during early stationary phase, and thus adds another layer of regulation to the complex network of responses that occur as cells transition to less favourable growth conditions. We show that 6S RNA-dependent changes in ppGpp levels during early stationary phase (i.e. 6 h of growth) are sufficient to lead to changes in the transcription of target genes such as those involved in amino acid biosynthesis and rRNA. Therefore, 6S RNA regulates transcription during this time frame in spite of being at subsaturating levels for binding to σ^{70}-RNAP. We also demonstrate that 6S RNA-dependent changes in transcription in late stationary phase (>24 h) are independent of ppGpp, thus revealing the diversity of mechanisms for 6S RNA-dependent regulation of gene expression during different times of cell growth and survival.

METHODS

Strains. *E. coli* strains (Table 1) were grown in EZ Rich Defined Medium [RDM; a MOPS-based medium (Neidhardt *et al.*, 1974) supplemented with 0.2 % glucose, 20 amino acids, vitamins and nucleobases] (Teknova) at 37 °C unless indicated otherwise. Promoter–*lacZ* fusions were all chromosomal as λ-phage lysogens, and were generated as described by Rao *et al.* (1994). Promoter regions for *relA*P1$_{(-100+50)}$ and *relA*P2$_{(-100+50)}$ were generated by PCR amplification from genomic DNA with oligonucleotides, and *relA*P1$_{(-42+2)}$ and *relA*P2$_{(-41+2)}$ by annealing oligonucleotides containing the entire promoter region with appropriate overhangs. Promoter regions were cloned into the *Eco*RI and *Hin*dIII sites in pMSB1 (Rao *et al.*, 1994). The *relA* promoter sequences are shown in Fig. 1(a); full oligonucleotide sequences are available upon request. The nomenclature for all promoter–*lacZ* fusions indicates the extent of promoter regions included in the reporter as the number of nucleotides upstream and downstream of the +1 transcription start site (see Table 1). All plasmid intermediates and the chromosomal regions containing the lysogen promoters were confirmed by sequencing. Promoter P1 transductions were performed as described by Silhavy *et al.* (1984) to move *ssrS1* (Lee *et al.*, 1985) and Δ*relA* (*relA251*::*kan*; Xiao *et al.*, 1991) into various strain backgrounds as needed.

The *ssrS1* allele contains a *bla* insertion into the 6S RNA gene (*ssrS*) (Lee *et al.*, 1985). Alleles with a precise replacement of the 6S RNA coding sequence with a tetracycline-resistance cassette (*ssrS2*) or a 13 bp scar after Flp-mediated removal of the tetracycline-resistance cassette (*ssrS3*) have been described previously (Trotochaud & Wassarman, 2006). For all three alleles (*ssrS1*, *ssrS2* and *ssrS3*), no detectable 6S RNA is expressed, and expression of the downstream gene (*ygfA*) is the same as in the wild-type, indicating that they are non-polar on *ygfA*, and the β-galactosidase activity of reporter genes

and growth phenotypes are indistinguishable (Trotochaud & Wassarman, 2006; Cavanagh *et al.*, 2008).

For the microarray experiment, RNA was isolated from two independent cultures each of wild-type (MG1655; KW489) or 6S RNA null (*ssrS3*; KW490) cells grown at 37 °C for 16 h after dilution to OD$_{600}$ 0.05 into RDM. RNA samples were sent to Nimblegen for analysis using the standard protocol for measuring mRNA levels on *E. coli* expression microarrays (Design T183333 60-mer, *E. coli* K-12). The full dataset will be published elsewhere (A. T. Cavanagh & K. M. Wassarman, unpublished data).

ppGpp analysis. Levels of ppGpp were measured after formic acid extraction from cells as described by Schneider *et al.* (2003). In short, overnight cultures grown in RDM at 37 °C were diluted to OD$_{600}$ 0.1 into 2 ml RDM containing 1 mM K$_2$HPO$_4$ and 40 μCi (1.48 MBq) [^{32}P]orthophosphoric acid (PerkinElmer) and further incubated at 37 °C for the times indicated. For nucleotide extraction, 200 μl cell culture was added to 40 μl cold 2 M formic acid and incubated on ice for 20 min. Following centrifugation, 20 μl extract was spotted onto a polyethylenimine (PEI)-cellulose F TLC plate (EMD Chemicals) and separated in 0.85 M KH$_2$PO$_4$ (pH 3.4). ppGpp was identified by migration profile as well as by its absence in Δ*relA*Δ*spoT* strain backgrounds, and the level of ppGpp was quantified on a Typhoon phosphorimager. Note that previous discrepancies concerning nucleotide isolation and quantification primarily resulted from formaldehyde-based isolation methodologies (Schneider & Gourse, 2004). Therefore, formic acid extraction was used here, which allows comparison of ppGpp levels between cell types over time.

β-Galactosidase assays. β-Galactosidase activity was measured at 30 °C as previously described (Trotochaud & Wassarman, 2004), and activity is expressed in Miller units (ΔOD$_{420}$ min^{-1} per OD$_{600}$ unit) (Miller, 1972). Briefly, cultures inoculated from a single colony were grown for 18 h in RDM, diluted approximately 1 : 100 in fresh medium (to OD$_{600}$ 0.04) and grown for 24 h, at which time they were analysed (24 h time point) or rediluted into fresh medium (1 : 100) and grown for an additional 6 h prior to analysis (6 h time point). Cell growth was at 37 °C for all strain backgrounds except those containing the *hisG*–*lacZ* reporter, which was at 30 °C as it is a 'system I' lysogen that is temperature-sensitive (Rao *et al.*, 1994). For β-galactosidase assays, cells were lysed with SDS and chloroform. At least three independent cultures per strain were used per experiment, and experiments were repeated at least three times.

RESULTS

Microarray expression analysis comparing mRNA levels between wild-type and 6S RNA null cells (*ssrS3*) demonstrated that expression of many genes was altered in a 6S RNA-dependent manner (A. T. Cavanagh & K. M. Wassarman, unpublished data). One mRNA that increased in the absence of 6S RNA was *relA*, which encodes ppGpp synthase I. Specifically, in cells lacking 6S RNA, *relA* mRNA was increased 2.3-fold relative to wild-type cells in late stationary phase (16 h of growth in RDM). Given the importance of ppGpp in regulating gene expression in response to nutrient limitation, a time when 6S RNA function is also important, we examined the relationship between 6S RNA function, *relA* expression and ppGpp levels. Expression of *spoT*, which encodes the bifunctional SpoT enzyme that can synthesize and degrade ppGpp (Magnusson *et al.*, 2005; Potrykus & Cashel, 2008), was

Table 1. Bacterial strains used in this study

Strain	Genotype	Reference or source
KW489	*E. coli* K-12 MG1655	
KW490	KW489 *ssrS3*	Cavanagh *et al.* (2008)
RLG3499	MG1655 *pyrE+ lacI lacZ* [VH1000]	Gaal *et al.* (2001)
KW348	RLG3499 *ssrS1*	Trotochaud & Wassarman (2006)
RLG10110	RLG3499 λ*relA*P1(−100 + 50)–*lacZ*	This work
KW702	RLG10110 *ssrS1*	This work
RLG10111	RLG3499 λ*relA*P2(−100 + 50)–*lacZ*	This work
KW703	RLG10111 *ssrS1*	This work
KW704	RLG3499 λ*relA*P1(−41 + 2)–*lacZ*	This work
KW705	KW704 *ssrS1*	This work
KW706	KW704 *relA251*::kan^r	This work
KW707	KW704 *ssrS1 relA251*::kan^r	This work
KW708	RLG3499 λ*relA*P2(−42 + 2)–*lacZ*	This work
KW709	KW708 *ssrS1*	This work
KW710	KW708 *relA251*::kan^r	This work
KW711	KW708 *ssrS1 relA251*::kan^r	This work
RLG4418	RLG3499 λ*hisG*(−60 + 15)–*lacZ*	Barker *et al.* (2001)
KW359	RLG4418 *ssrS1*	Cavanagh *et al.* (2008)
KW712	RLG4418 *relA251*::kan^r	This work
KW713	RLG4418 *ssrS1 relA251*::kan^r	This work
RLG5080	RLG3499 λ*thrABC*(−72 + 1)–*lacZ*	Barker *et al.* (2001)
KW459	RLG5080 *ssrS1*	Cavanagh *et al.* (2008)
KW714	RLG5080 *relA251*::kan^r	This work
KW715	RLG5080 *ssrS1 relA251*::kan^r	This work
RLG4422	RLG3499 λ*livJ*(−60 + 13)–*lacZ*	Barker *et al.* (2001)
KW464	RLG4422 *ssrS1*	Cavanagh *et al.* (2008)
KW716	RLG4422 *relA251*::kan^r	This work
KW717	RLG4422 *ssrS1 relA251*::kan^r	This work
RLG4818	RLG3499 λ*pheA*(−73 + 10)–*lacZ*	Barker *et al.* (2001)
KW351	RLG4818 *ssrS1*	Cavanagh *et al.* (2008)
KW718	RLG4818 *relA251*::kan^r	This work
KW719	RLG4818 *ssrS1 relA251*::kan^r	This work
RLG6358	RLG3499 λ*rrnB*P1(−41 + 1)–*lacZ*	Hirvonen *et al.* (2001)
KW238	RLG6358 *ssrS1*	Trotochaud & Wassarman (2004)
KW720	RLG6358 *relA251*::kan^r	This work
KW721	RLG6358 *ssrS1 relA251*::kan^r	This work
KW460	RLG3499 λ*lacUV5*(−48 + 4)–*lacZ*	Cavanagh *et al.* (2008)
KW461	KW460 *ssrS1*	Cavanagh *et al.* (2008)
KW722	KW460 *relA251*::kan^r	This work
KW723	KW460 *ssrS1 relA251*::kan^r	This work
RLG3760	RLG3499 λ*bolA1*(−54 + 16)–*lacZ*	Gaal *et al.* (2001)
KW378	RLG3760 *ssrS1*	Trotochaud & Wassarman (2004)
KW724	RLG3760 *relA251*::kan^r	This work
KW725	RLG3760 *ssrS1 relA251*::kan^r	This work

relatively unchanged (0.85 in cells lacking 6S RNA compared with wild-type cells).

6S RNA regulates transcription of *relA*

relA expression is driven by two identified promoters, P1 and P2 (see Fig. 1a) (Metzger *et al.*, 1988; Nakagawa *et al.*, 2006). *relA*P1, located 178 bp upstream of the translational start site for *relA*, is constitutively active; *relA*P2, located 626 bp upstream of *relA*, is induced during the transition into stationary phase (Metzger *et al.*, 1988; Nakagawa *et al.*,

2006). Interestingly, *relA*P1 has a fairly weak −35 element when compared with the consensus (TgGAac) and *relA*P2 contains an extended −10 element; both these features are independent indicators of promoters sensitive to 6S RNA (Cavanagh *et al.*, 2008), and therefore we predicted that both *relA*P1 and *relA*P2 would be directly downregulated by 6S RNA. To test whether these promoters respond to 6S RNA regulation, promoter–*lacZ* fusions were generated to allow expression to be monitored by β-galactosidase activity in cells with 6S RNA or lacking 6S RNA (*ssrS1*). In agreement with our predictions, both *relA*P1$_{(−100 + 50)}$
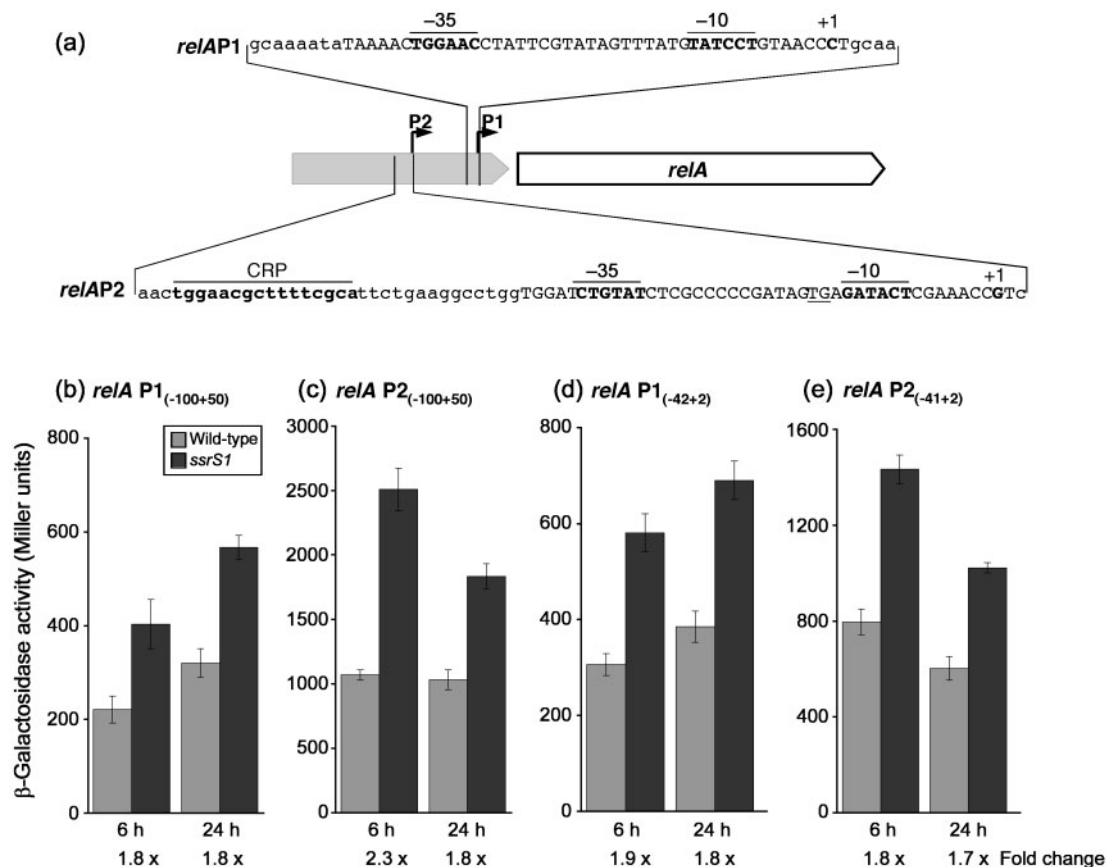
Fig. 1. *relA* P1 and P2 promoters are both sensitive to 6S RNA. (a) Schematic of *relA* (white box) and its genomic location. Promoters P1 and P2 are indicated by arrows, and the sequence of each promoter region is shown above (P1) or below (P2) the schematic. Note that both P1 and P2 are quite distant upstream from the translational start of *relA* and are located within the upstream gene (*rlmD*) represented by a grey box. The start site for transcription (+1), the −10 and −35 elements, and the CRP binding sequence are shown in bold and labelled above the sequences. The TG of the extended −10 element for P2 is underlined. Upper-case type indicates the extent of sequence included in the minimal promoters *relA*P1$_{(-42+2)}$ and *relA*P2$_{(-41+2)}$. Note that the full sequence included in *relA*P1$_{(-100+50)}$ and *relA*P2$_{(-100+50)}$ extends beyond the sequences shown. (b−e) β-Galactosidase activity from *relA* promoter–*lacZ* reporters. Wild-type and 6S RNA null (*ssrS1*) strains containing chromosomal copies of the promoters indicated were grown to late stationary phase (24 h) in RDM at 37 °C. Fold change is the β-galactosidase activity in *ssrS1* divided by the β-galactosidase activity in the wild-type background. Data shown are the mean of at least three independent experiments with three duplicate samples per experiment. Error bars, SD.

and *relA*P2$_{(-100+50)}$ had increased expression in cells lacking 6S RNA relative to the wild-type (1.8- to 2.3-fold) in both early and late stationary phase (6 and 24 h of growth in RDM) (Fig. 1b, c, Table 2). For comparison, the microarray data were from cells grown for 16 h in the same medium. Together, these data suggest that *relA* mRNA levels are altered in a 6S RNA-dependent manner throughout stationary phase.

The *relA*P2 promoter contains a cAMP receptor protein (CRP) binding site that is activated by CRP (Nakagawa *et al.*, 2006) and the *relA*P2$_{(-100+50)}$ reporter included this CRP site. The microarray data also indicated that *crp* mRNA levels were increased (1.9-fold) in cells lacking 6S RNA compared with wild-type cells (A. T.

Cavanagh & K. M. Wassarman, unpublished data). To test whether changes in transcription at *relA*P2$_{(-100+50)}$ and *relA*P1$_{(-100+50)}$ were likely to result from direct regulation by 6S RNA or might be due to indirect regulation via changes in *trans*-acting factors such as CRP, we also tested reporters that contained only minimal core promoter sequences lacking known binding sites [*relA*P1$_{(-42+2)}$ and *relA*P2$_{(-41+2)}$; see Fig. 1a]. The minimal *relA*P1$_{(-42+2)}$ reporter remained sensitive to 6S RNA, similar to the longer *relA*P1$_{(-100+50)}$ (compare Fig. 1d and Fig. 1b). The minimal *relA*P2$_{(-41+2)}$ reporter also remained sensitive to 6S RNA, although to a lesser extent than the reporter that also contained the CRP binding site (*relA*P2$_{(-100+50)}$) (compare Fig. 1e and Fig. 1c). In agreement with CRP activation at this promoter, the overall level of expression

Table 2. Comparison of promoter activities in cells with and without 6S RNA

Results are given as fold change (β-galactosidase activity in *ssrS1* divided by β-galactosidase activity in wild-type cells). Measurements were made with wild-type *relA* (*relA*$^+$) cells or cells lacking *relA* ($\Delta relA$) grown in RDM for 6 or 24 h as indicated. For promoter activities and the SD associated with analysis, see Fig. 2.

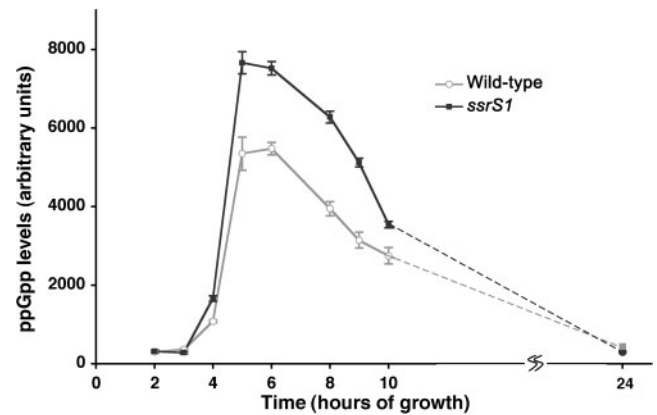| Promoter	6 h		24 h	
	relA$^+$	$\Delta relA$	*relA*$^+$	$\Delta relA$
hisG	1.7	1.1	1.4	1.6
thrA	1.5	1.1	1.1	1.0
livJ	1.3	1.1	1.9	1.9
pheA	1.4	1.0	1.0	1.0
*rrnB*P1	0.7	1.0	1.1	1.0
lacUV5	0.9	0.9	1.0	1.0
*relA*P1$_{(-42+2)}$	1.9	1.8	1.8	1.8
*relA*P2$_{(-41+2)}$	1.8	1.8	1.7	1.7
bolA	0.5	0.4	0.5	0.5

Fig. 2. Maximal ppGpp levels are higher in cells lacking 6S RNA. Nucleotides extracted by formic acid from cells grown in RDM containing [^{32}P]orthophosphate were separated by TLC. Relative [^{32}P]ppGpp levels are given in arbitrary intensity units as quantified on a Typhoon phosphorimager. A typical dataset with means of replicates of each cell type is shown: wild-type cells (RLG3499; lower line) and cells lacking 6S RNA (*ssrS1*; KW348; upper line). Error bars, SD.

of the minimal *relA*P2$_{(-41+2)}$ was decreased relative to *relA*P2$_{(-100+50)}$. *hns* has also been reported to activate transcription from *relA*P2 (Nakagawa *et al.*, 2006). However, we observed a decrease in *hns* mRNA levels in cells lacking 6S RNA compared with wild-type cells (A. T. Cavanagh & K. M. Wassarman, unpublished data), suggesting that a 6S RNA-dependent decrease in *hns* cannot account for the observed 6S RNA-dependent increase in *relA* expression.

6S RNA-dependent regulation of *relA* transcription leads to altered ppGpp levels

The observed 6S RNA-dependent change in *relA* mRNA levels was rather modest; therefore, we next tested whether these changes in *relA* mRNA were sufficient to lead to a change in ppGpp levels. Wild-type and 6S RNA null (*ssrS1*) cells were grown in RDM containing [^{32}P]orthophosphate. At the times indicated, nucleotides were extracted from cells by formic acid and separated by TLC (see Methods). As expected, in wild-type cells, ppGpp accumulated during the transition into stationary phase (4–5 h of growth), remained elevated for several hours in stationary phase, and then decreased to undetectable levels by 24 h of growth (Fig. 2) (see also Murray *et al.*, 2003). Interestingly, in cells lacking 6S RNA, initiation of ppGpp accumulation occurred with similar timing, but the maximal level of ppGpp in early stationary phase was 1.5- to 1.6-fold higher in cells lacking 6S RNA than in wild-type cells, as also recently reported by Neusser *et al.* (2010). By 24 h of growth, ppGpp levels were undetectable in both cell types, indicating that the longer-term shut-off of ppGpp synthesis is 6S RNA-independent. ppGpp levels were undetectable in this assay throughout this time frame in $\Delta relA$ and $\Delta relA\Delta spoT$ strain backgrounds (data not shown). It is

clear that the observed 6S RNA-dependent downregulation of *relA* expression in wild-type cells is sufficient to lead to a detectable reduction in ppGpp levels in early stationary phase.

6S RNA-dependent changes in ppGpp lead to regulation of transcription in early stationary phase

We next tested whether the 6S RNA-dependent change in ppGpp levels might be sufficient to lead to altered transcription of promoters sensitive to ppGpp. For these experiments we chose to examine promoter–*lacZ* reporters previously described in studies examining ppGpp and 6S RNA-dependent regulation (Barker *et al.*, 2001; Trotochaud & Wassarman, 2004; Cavanagh *et al.*, 2008). Note that although many of the genes examined also are regulated by attenuation or other mechanisms after transcription initiation, the reporters used here contain only minimal promoter sequences lacking elements mediating attenuation, and therefore should be representative of regulation at transcription initiation.

For promoters that are upregulated by ppGpp (i.e. *livJ*, *hisG*, *thrA* and *pheA*), expression was increased in *ssrS1* compared with wild-type cells at 6 h of growth, a time when ppGpp levels were significantly different between cells with or without 6S RNA (Fig. 3a, Table 2). A promoter that is negatively regulated by ppGpp, *rrnB*P, had decreased expression in *ssrS1* compared with wild-type cells, and a ppGpp-insensitive promoter (i.e. *lacUV5*) was unchanged.

To further test whether these 6S RNA-dependent changes were mediated through changes in ppGpp levels, we
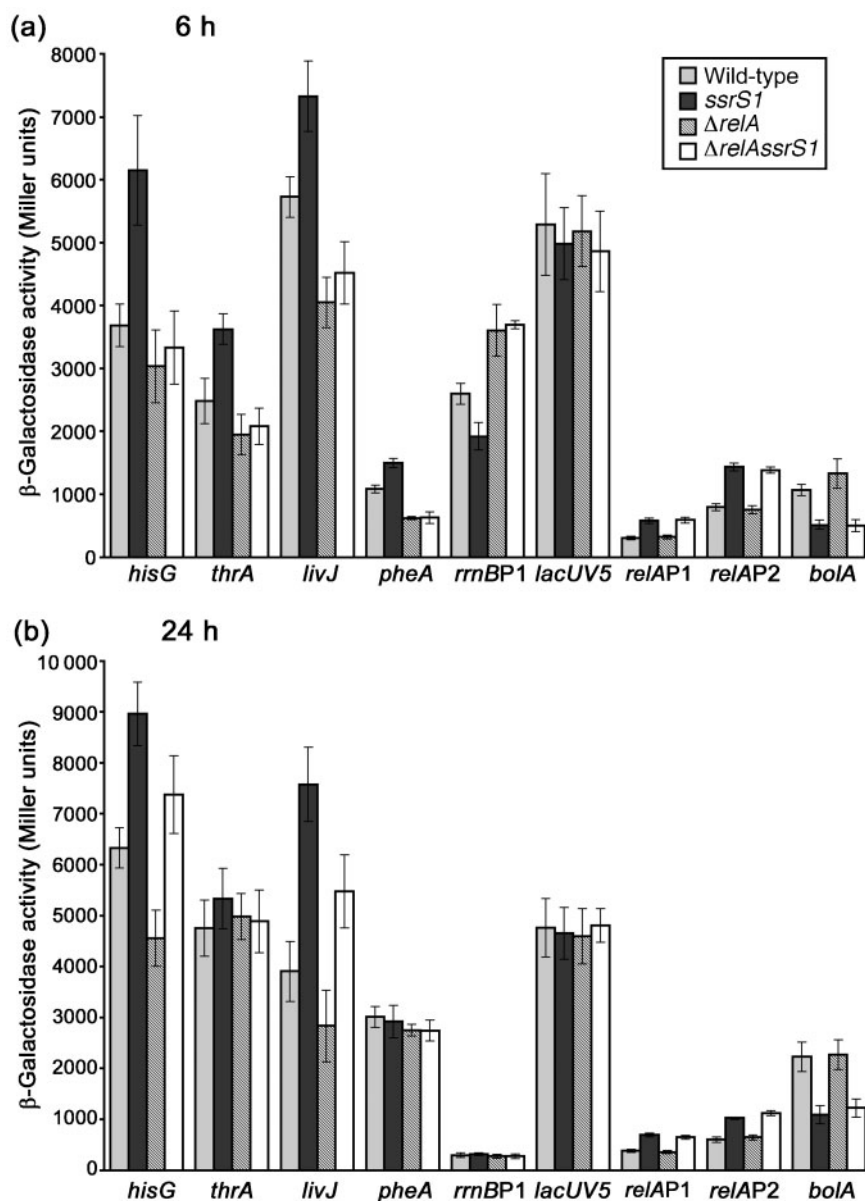
Fig. 3. β-Galactosidase activities of various promoter–*lacZ* reporter genes in early stationary phase (6 h) and late stationary phase (24 h). Wild-type, 6S RNA null (*ssrS1*), *relA* null ($\Delta relA$) and double mutant ($\Delta relAssrS1$) strain backgrounds containing chromosomal copies of the promoters indicated were grown to (a) early stationary phase (6 h) or (b) late stationary phase (24 h) in RDM. The *relA* promoters are *relA*P1$_{(-42+1)}$ and *relA*P2$_{(-41+2)}$. Data shown are mean of at least three independent experiments with three duplicate samples per experiment. Error bars, SD.

measured expression of all reporters in $\Delta relA$ and $\Delta relAssrS1$ cells (Fig. 3a). Expression from the amino acid and rRNA promoters was sensitive to *relA* under these conditions, as expected (Barker *et al.*, 2001); the amino acid promoters were increased in wild-type compared with $\Delta relA$ cells, and *rrnB*P1 was decreased. However, to address whether 6S RNA-dependent changes are likely to be mediated through changes in ppGpp, the expression of reporters was compared between $\Delta relA$ and $\Delta relAssrS1$ strain backgrounds. For *hisG*, *thrA*, *livJ*, *pheA* and *rrnB*P1,

expression was similar in $\Delta relA$ and $\Delta relAssrS1$ strain backgrounds, demonstrating that the 6S RNA-dependent effects in early stationary phase require *relA*, and suggesting that they result from changes in ppGpp levels (Fig. 3a, Table 2). In contrast, expression from other σ^{70}-dependent promoters not sensitive to ppGpp [e.g. *lacUV5*, *relA*P1$_{(-42+2)}$ and *relA*P2$_{(-41+2)}$] and a σ^{S}-dependent promoter (*bolA*) had the same fold changes for *ssrS1* compared with the wild-type and for $\Delta relAssrS1$ compared with $\Delta relA$ strain backgrounds (Fig. 3a, Table 2), indicating

that not all 6S RNA-dependent changes in expression in early stationary phase require *relA*. Further deletion of *spoT* had no additional effect, as expression of the reporters tested (*pheA*, *livJ*, *lacUV5* and *bolA*) in a ΔrelAΔspoT strain background was indistinguishable from that of the ΔrelA background in the presence or absence of 6S RNA in early stationary phase (data not shown). Steady-state levels of 6S RNA were unaffected by ppGpp, as the accumulation of 6S RNA from exponential to late stationary phase in ΔrelAΔspoT cells was indistinguishable from that in wild-type cells (data not shown).

6S RNA-dependent regulation of transcription in late stationary phase is not mediated by ppGpp

Previously, we reported that some of the promoters examined here (i.e. *livJ* and *hisG*) were sensitive to 6S RNA regulation during late stationary phase (16–24 h of growth), and at that time it was presumed to be via direct regulation (Cavanagh *et al.*, 2008). In light of the above observations that the effects of 6S RNA on these promoters are likely to be indirect via changes in *relA* expression (and ppGpp levels) during early stationary phase (6 h of growth), we next revisited 6S RNA-dependent changes in expression of these genes at later time frames. Of note, previous work had shown that *thrA*, *pheA* and *rrnB*P1 are not sensitive to 6S RNA in late stationary phase, whereas *livJ* and *hisG* are sensitive, lending support to the suggestion that there are 6S RNA effects on the transcription of the first set of genes at later times independent of those mediated through ppGpp in early stationary phase. In addition, ppGpp levels are reduced to background by 24 h of growth, decreasing the likelihood that it is a major regulator at these later time frames in stationary phase.

We monitored expression of our reporter genes in various strain backgrounds as indicated (e.g. with or without 6S RNA, with or without *relA*) after 24 h of growth (Fig. 3b, Table 2). For promoters previously shown to be regulated by 6S RNA at 24 h (*livJ*, *hisG*), we observed that the increase in expression in the absence of 6S RNA was independent of *relA* at 24 h, as indicated by a similar fold change between wild-type and *ssrS1* cells and between ΔrelA and ΔrelAssrS1 cells for both *livJ* and *hisG*. Similarly, the σ^S-dependent promoter *bolA* remained decreased in cells lacking 6S RNA in the presence or absence of *relA*. 6S RNA regulation in late stationary phase was also not influenced by SpoT, as expression of the reporters tested (*pheA*, *livJ*, *lacUV5* and *bolA*) in a ΔrelAΔspoT strain background was indistinguishable from that of the ΔrelA background in the presence or absence of 6S RNA at 24 h of growth (data not shown). Therefore, we conclude that 6S RNA-dependent regulation of these promoters in late stationary phase is not mediated by ppGpp. In contrast, *thrA*, *pheA*, *rrnB*P1 and *lacUV5* were insensitive to 6S RNA at 24 h of growth, irrespective of the presence or absence of *relA*.

DISCUSSION

We have demonstrated that *relA* transcription is a target for 6S RNA regulation, as first suggested by an expression microarray experiment and confirmed by analysis of promoter–*lacZ* fusions. Although the changes in *relA* mRNA levels are modest, the changes are sufficient to direct a corresponding change in ppGpp levels. In cells lacking 6S RNA, maximum ppGpp levels are higher than in wild-type cells during early stationary phase. However, in both wild-type and 6S RNA null cells, the timing of initial ppGpp accumulation appears to be similar, and ppGpp levels are undetectable in long-term stationary phase (>24 h). In wild-type cells, ppGpp accumulates when RelA protein is activated in response to the presence of uncharged tRNAs in the ribosome, as a signal that amino acids are limiting. Later, in stationary phase, ppGpp levels decrease again, in part driven by continued degradation of ppGpp and a decrease in activation of RelA as the translational capacity of the cell is reduced in response to nutrient limitation (see Potrykus & Cashel, 2008). We propose that 6S RNA does not alter the mechanism of activation of RelA, nor the signals and molecules involved in reducing ppGpp levels in later stationary phase. Instead, 6S RNA regulates the transcription of *relA*, thereby altering the levels of RelA available to respond to these signals, and leading to changes in the levels, but not the timing, of ppGpp accumulation.

We also demonstrated that the 6S RNA-dependent changes in ppGpp during early stationary phase are sufficient to result in higher expression of genes positively regulated by ppGpp (i.e. amino acid promoters) and lower expression of a negatively regulated promoter (i.e. *rrnB*P1). However, altered ppGpp levels cannot account for all 6S RNA-dependent changes in transcription; 6S RNA regulates many promoters in late stationary phase when ppGpp levels are undetectable, and promoters that are not inherently sensitive to ppGpp also remain 6S RNA-regulated in the absence of ppGpp (e.g. ΔrelA strains) even during early stationary phase (see Fig. 3). In addition, altered ppGpp levels cannot explain the 6S RNA-dependent changes in σ^S activity, as demonstrated by continued 6S RNA regulation of a σ^S-dependent promoter (*bolA*) throughout growth in the absence of ppGpp (e.g. in ΔrelA and ΔrelAΔspoT strain backgrounds). Together, these results demonstrate that 6S RNA is an additional regulator that contributes to changes in the expression of genes required to facilitate the transition from exponential to stationary phase.

6S RNA is required for appropriate ppGpp accumulation, but ppGpp does not influence 6S RNA activity

There are many regulators important during the transition from exponential to stationary phase, such as ppGpp, σ^S and now 6S RNA. Here, we demonstrate that 6S RNA function is necessary for appropriate regulation of ppGpp

levels during early stationary phase, raising interesting questions about whether there are additional links between these regulators. For example, given that ppGpp binds directly to σ^{70}-RNAP, one could speculate that ppGpp influences 6S RNA activity directly. However, we demonstrate here that ppGpp does not influence the ability of 6S RNA to function. Specifically, in late stationary phase, when ppGpp levels are below detection, many promoters are downregulated by 6S RNA (see Fig. 3b, *hisG*, *livJ*, *rel*AP1 and *rel*AP2, and additional promoters in Trotochaud & Wassarman, 2004; Cavanagh *et al.*, 2008), and these promoters remain similarly 6S RNA-sensitive in $\Delta relA$ and $\Delta relA\Delta spoT$ strain backgrounds compared with the wild-type. In early stationary phase, promoters that are not inherently sensitive to ppGpp remain sensitive to 6S RNA, even in the absence of ppGpp, indicating that 6S RNA function does not require ppGpp (e.g. *rel*AP1 and *rel*AP2; Fig. 3). In contrast, ppGpp is required for 6S RNA-dependent changes in transcription from other promoters during early stationary phase (e.g. *hisG*, *thrA*, *livJ*, *pheA* and *rrn*BP1; see Fig. 3a). However, we suggest that these promoters are responding to changes in ppGpp levels in early stationary phase, and therefore are only indirectly regulated by 6S RNA. We conclude that 6S RNA activity is required for appropriate ppGpp accumulation during early stationary phase, but that ppGpp does not contribute to the ability of 6S RNA to function.

6S RNA-dependent changes in ppGpp levels are not responsible for 6S RNA regulation of σ^S activity

We have shown previously that several σ^S-dependent promoters are upregulated in the presence of 6S RNA, although other σ^S-dependent promoters are insensitive to 6S RNA (Trotochaud & Wassarman, 2004), and microarray studies have suggested that expression of additional σ^S-dependent genes is altered in cells lacking 6S RNA (Cavanagh *et al.*, 2008; Neusser *et al.*, 2010). We do not detect stable interactions between 6S RNA and σ^S-RNAP *in vivo* or *in vitro* above background binding of non-specific RNAs, and steady-state levels of σ^S protein are unchanged in cells with and without 6S RNA (Wassarman & Storz, 2000; Trotochaud & Wassarman, 2005). Therefore, we proposed that 6S RNA-dependent regulation of σ^S activity is indirect. Given that ppGpp can increase alternative σ factor activity by a number of proposed mechanisms (Jishage *et al.*, 2002; Bernardo *et al.*, 2006; Costanzo *et al.*, 2008), we wondered whether the 6S RNA upregulation of σ^S-dependent promoters is mediated through changes in ppGpp. However, we found that a σ^S-dependent promoter previously shown to be sensitive to 6S RNA (*bolA*) (Trotochaud & Wassarman, 2004) remained similarly sensitive to 6S RNA in both early and late stationary phase in $\Delta relA$ and $\Delta relA\Delta spoT$ strain backgrounds compared with the wild-type (Fig. 3, Table 2; data not shown). In addition, previous studies on σ^S-dependent promoters were carried out in late stationary phase, when ppGpp

levels are undetectable (Trotochaud & Wassarman, 2004). Although it is possible that some σ^S-promoters will respond to the 6S RNA-dependent changes in ppGpp levels, these data demonstrate that 6S RNA upregulation of σ^S-dependent transcription cannot simply be explained by the changes in ppGpp levels. Further studies will be needed to explain how 6S RNA function leads to altered σ^S-dependent transcription.

6S RNA regulation of transcription: direct versus indirect mechanisms

As we aim to elucidate the mechanisms by which 6S RNA regulates transcription and the cellular consequences of this regulation, it becomes increasingly important to try to separate direct and indirect effects. Here, we have clearly shown that in early stationary phase, 6S RNA-dependent changes in expression of amino acid promoters and rRNA promoters are indirect, and result from altered *relA* expression. However, we propose that the 6S RNA-dependent change in *relA* expression is due, at least in part, to direct regulation (see below). Typically, direct transcriptional regulation would be verified using purified *in vitro* transcription assays. Unfortunately, this approach has not proven useful in studies of 6S RNA to date; the *in vitro* transcription conditions tested have not recapitulated promoter-specific regulation by 6S RNA, but instead all tested σ^{70}-dependent promoters have been similarly downregulated (Trotochaud & Wassarman, 2005; Wassarman & Saecker, 2006). It is possible that other factors involved in 6S RNA function *in vivo* are lacking in the *in vitro* assay, or that the *in vitro* conditions are not dynamic enough to represent *in vivo* conditions. We favour the second possibility, based on observations that 6S RNA and promoter DNA bound to σ^{70}-RNAP have very slow off-rates *in vitro*, suggesting that the tested *in vitro* conditions are not able to effectively reproduce the presumed competition occurring *in vivo* (Wassarman & Saecker, 2006; Klocko & Wassarman, 2009). However, we note that 6S RNA regulation of σ^{70}-dependent transcription does not result from competition with σ^S, as tested σ^{70}-dependent promoters (e.g. *rsd*P2; see Trotochaud & Wassarman, 2004) remain 6S RNA-sensitive in an *rpoS*::Tn*10* strain background (data not shown).

The inability to recapitulate the promoter specificity for 6S RNA regulation using *in vitro* assays has made definitive testing of direct versus indirect 6S RNA effects more difficult. However, we previously identified two promoter features that correlated with 6S RNA regulation (a weak -35 element and an extended -10 element) using reporters with minimal promoter sequences that lacked known binding sites for *trans*-acting factors to minimize the potential for indirect effects. More importantly, we were able to convert sensitive to insensitive promoters as well as insensitive to sensitive promoters with minor sequence changes only in core promoter elements of these reporters, even in several unrelated promoters that do not

share flanking sequences (e.g. *livJ*, *lacUV5*, *hupBP2*, *pspF* and *galP2*) (Cavanagh *et al.*, 2008). These results strongly support the model that these elements are important for direct 6S RNA regulation, presumably through competition between 6S RNA and promoter DNA for binding to σ^{70}-RNAP.

Based on our previous study, we predicted that 6S RNA-dependent regulation of *relA* is likely to be direct at both P1 and P2, which have a weak -35 element and an extended -10 element, respectively. Both minimal promoter fusions were 6S RNA-sensitive, consistent with our hypothesis (see Fig. 1c, d). However, we also note that *relA*P2$_{(-100+50)}$ is more sensitive to 6S RNA than *relA*P2$_{(-41+2)}$, suggesting that there are additional effects at the longer promoter, most likely mediated through the CRP binding site and 6S RNA-dependent changes in *crp* expression (see Fig. 1c, e). Results from a microarray experiment comparing global mRNA levels in cells with and without 6S RNA revealed that *crp* mRNA levels were increased 1.9-fold in cells lacking 6S RNA. Transcription of *crp* is complex, with multiple promoters, making it more difficult to predict whether it is directly regulated by 6S RNA without further experimentation; however, we note that one of the *crp* promoters contains an extended -10 element (Mitchell *et al.*, 2003), suggesting that it is a possibility.

Intriguingly, we have been able to distinguish two mechanisms by which 6S RNA regulates expression from some amino acid biosynthesis and uptake genes: (1) an indirect mechanism in early stationary phase (6 h), in which 6S RNA-dependent changes in ppGpp levels lead to changes in transcription at all tested ppGpp-sensitive promoters; and (2) a *relA*-independent mechanism in late stationary phase ($\geqslant$24 h), in which 6S RNA-dependent changes in transcription are not mediated through changes in levels of ppGpp. We maintain our previous predictions that the late stationary phase regulation by 6S RNA is direct, based on the presence of weak -35 elements in the sensitive promoters (*hisG* and *livJ*), the observation that *relA* is dispensable at this time (see Fig. 3b, Table 2), and the ability to convert the *livJ* reporter to a 6S RNA-insensitive promoter by changing the -35 element to consensus (Cavanagh *et al.*, 2008). However, it remains possible that the observed changes at these promoters are mediated through another gene with altered expression, analogous to the role of *relA* with respect to ppGpp-sensitive promoters in early stationary phase and of *crp* with respect to *relA*P2.

The role of 6S RNA in stationary phase

Regardless of whether we can fully distinguish direct from indirect 6S RNA regulation of transcription, we can gain insight into how the function of 6S RNA affects cellular physiology by considering the global changes in gene expression, as all changes (direct and indirect) will contribute. We previously reported that hundreds of genes are altered in expression in late stationary phase in a 6S

RNA-dependent manner (Cavanagh *et al.*, 2008), and recently others have reported large numbers of changes in earlier time frames as well (Neusser *et al.*, 2010). The two studies used microarray analysis to compare global gene expression changes in genes with and without 6S RNA, and therefore changes in mRNA levels represent both 6S RNA-direct and 6S RNA-indirect changes. As such, it would be surprising to find signals important for direct 6S RNA regulation to be present in all genes altered in expression, nor should it be unexpected that some genes have decreased expression in the absence of 6S RNA, even among those with σ^{70}-dependent promoters. Here, we set out to test whether there was a relationship between 6S RNA and ppGpp, as both are regulators of transcription during stationary phase; therefore, we focused on several well-studied promoters known to be sensitive to ppGpp (e.g. amino acid promoters and *rrnB*P1). Our findings are consistent with a recent report that also observed 6S RNA-dependent changes in ppGpp levels and focused primarily on genes encoding the translation machinery that are sensitive to ppGpp (Neusser *et al.*, 2010).

It is intriguing to speculate why one stationary phase regulator, 6S RNA, downregulates another regulator, *relA*, that is important during the transition into stationary phase. The timing of ppGpp accumulation is not dramatically altered in cells lacking 6S RNA compared with wild-type cells; instead, it appears to be the maximal level of ppGpp accumulation that is changed (see Fig. 2). One model suggests that the role of 6S RNA in stationary phase is to dampen the expression of many genes, perhaps to conserve energy as cells encounter suboptimal growth conditions (Trotochaud & Wassarman, 2006). As such, 6S RNA would facilitate the integration of multiple signal inputs by downregulating key regulators in multiple pathways and preventing overactivation of any one response. For example, one gene downregulated by 6S RNA, *pspF*, is a transcriptional activator that responds to several stresses, including elevated pH (Model *et al.*, 1997; Darwin, 2005). We have shown that the presence of 6S RNA decreases the set-point of *pspF* expression at neutral pH but does not alter the ability of the *psp* system to respond to high pH conditions (Trotochaud & Wassarman, 2006). Many key regulators appear to be differentially expressed in cells lacking 6S RNA compared with wild-type cells, such as CRP (see above) and OxyR (A. T. Cavanagh & K. M. Wassarman, unpublished data), suggesting that several diverse pathways are affected by 6S RNA function, and that understanding the full extent of how 6S RNA function alters cell physiology will require further studies to unravel these complexities.

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