Promoter specificity for 6S RNA regulation of transcription is determined by core promoter sequences and competition for region 4.2 of σ^{70}

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Summary

6S RNA binds σ^{70}-RNA polymerase and downregulates transcription at many σ^{70}-dependent promoters, but others escape regulation even during stationary phase when the majority of the transcription machinery is bound by the RNA. We report that core promoter elements determine this promoter specificity; a weak −35 element allows a promoter to be 6S RNA sensitive, and an extended −10 element similarly determines 6S RNA inhibition except when a consensus −35 element is present. These two features together predicted that hundreds of mapped *Escherichia coli* promoters might be subject to 6S RNA dampening in stationary phase. Microarray analysis confirmed 6S RNA-dependent downregulation of expression from 68% of the predicted genes, which corresponds to 49% of the expressed genes containing mapped *E. coli* promoters and establishes 6S RNA as a global regulator in stationary phase. We also demonstrate a critical role for region 4.2 of σ^{70} in RNA polymerase interactions with 6S RNA. Region 4.2 binds the −35 element during transcription initiation; therefore we propose one mechanism for 6S RNA regulation of transcription is through competition for binding region 4.2 of σ^{70}.

Introduction

The 6S RNA is an untranslated, small RNA that was first discovered in *Escherichia coli* as a highly abundant RNA (Hindley, 1967). Although its cellular function remained elusive for many years, it is now known that 6S RNA regulates transcription through direct interaction with RNA polymerase (RNAP) [see Wassarman (2007) and

Willkomm and Hartmann (2005) for review]. Bacterial RNAP contains a multisubunit core enzyme (β-, β′-, ω- and two α-subunits) and a specificity subunit (σ) that together form the holoenzyme (Eσ). Although core RNAP can carry out transcription elongation, the holoenzyme form is required for DNA promoter recognition and transcription initiation. *E. coli* contains seven σ-subunits: the housekeeping σ^{70} in addition to six alternative σ factors important during growth in suboptimal environments (Gruber and Gross, 2003). 6S RNA binds specifically and tightly to Eσ^{70}, resulting in decreased transcription at many σ^{70}-dependent promoters (Trotochaud and Wassarman, 2004; 2005). Although 6S RNA is present throughout growth, it accumulates substantially during late stationary phase when the vast majority of Eσ^{70} is bound by 6S RNA (Wassarman and Storz, 2000). Cells lacking 6S RNA are altered in cell survival, particularly during competitive growth in stationary phase and growth at high pH (Trotochaud and Wassarman, 2004; 2006).

A highly conserved secondary structure is required for 6S RNA binding to Eσ^{70} (Trotochaud and Wassarman, 2005) (Fig. 1). The RNA is primarily double-stranded with a large central bulge (Barrick *et al.*, 2005; Trotochaud and Wassarman, 2005); a structure reminiscent of the conformation of DNA within the 'open complex' formed during transcription initiation when the DNA surrounding the start site of transcription is unwound. Similar to a DNA promoter, 6S RNA resides within the active site of RNAP and can be used as a template by Eσ^{70} to synthesize product RNAs (pRNA) (Wassarman and Saecker, 2006; Gildehaus *et al.*, 2007). pRNA synthesis results in the release of 6S RNA from RNAP, which appears to be the mechanism to liberate Eσ^{70} from 6S RNA regulation upon outgrowth from stationary phase (Wassarman and Saecker, 2006). However, in stationary phase the 6S RNA:Eσ^{70} complex is quite stable, presumably due to the inability of pRNA synthesis to occur under low nucleotide concentrations present at this time (Wassarman and Storz, 2000; Murray *et al.*, 2003; Wassarman and Saecker, 2006), although other factors also may contribute to 6S RNA:Eσ^{70} stability in stationary phase. The presence of 6S RNA within the active site of Eσ^{70} blocks promoter DNA from binding to RNAP (Wassarman and Saecker,
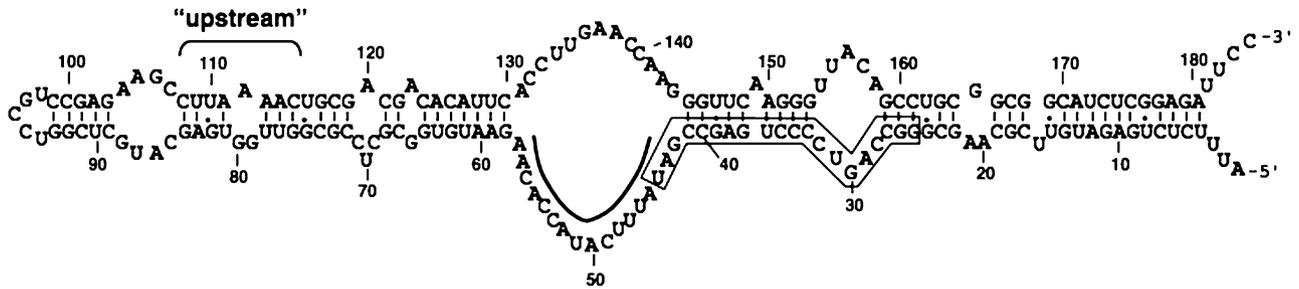
Fig. 1. *E. coli* 6S RNA. Illustration of 6S RNA in a secondary structure supported by phylogenetic and experimental analyses (see Barrick *et al.*, 2005; Trotochaud and Wassarman, 2005). The region from G42-A57 (indicated by bar) has been replaced with CAC in the inactive 6S(M5) RNA. The sequence in 6S RNA complementary to the longest pRNA is boxed. The 'upstream region' indicates a potential region for interaction with σ⁷⁰ region 4.2 based on estimated distances from the single-stranded region and analogy to DNA binding.

2006; Gildehaus *et al.*, 2007); thus the mechanism of 6S RNA inhibition of transcription during stationary phase appears to be by sequestration of Eσ^{70} from DNA.

σ^{70}-dependent promoters are primarily recognized through two core sequences called the −35 element (consensus TTGACA) and −10 element (consensus TATAAT) based on their approximate distances from the start site of transcription at position +1. Both core promoter elements are recognized through direct interactions with σ^{70}; region 4 of σ^{70} contacts the −35 element and region 2 of σ^{70} contacts the −10 element (Dombroski *et al.*, 1992; Campbell *et al.*, 2002; Murakami *et al.*, 2002). Some promoters contain an 'extended −10 element' that is defined by a conserved TGn immediately upstream of the −10 element (consensus TGnTATAAT) (Voskuil *et al.*, 1995; Bown *et al.*, 1997). Extended −10 promoters make additional contacts with σ^{70} within region 3.0, which facilitate relatively high levels of transcription initiation even in the absence of conserved −35 elements or in the absence of σ^{70} region 4 (Kumar *et al.*, 1993; Barne *et al.*, 1997). Previously identified promoters inhibited by 6S RNA contain extended −10 elements suggesting this group of promoters might be universally inhibited by 6S RNA (Trotochaud and Wassarman, 2004; 2006). Here we investigate the contribution of specific promoter elements in determining if a promoter is regulated by 6S RNA. We show that the relative match to consensus of the −35 element is an important determinant in promoters inhibited by 6S RNA; promoters with weak −35 elements are sensitive to 6S RNA while those with strong −35 elements are not. In addition to the −35 element, the presence of an extended −10 element also contributes to 6S RNA regulation, resulting in a graded effect. We also show that region 4.2 of σ^{70} is required for 6S RNA binding to Eσ^{70} suggesting this region is a primary contact point for 6S RNA that contributes critical strength and specificity to the 6S RNA interaction with Eσ^{70}. Together, these data support a model in which 6S RNA and promoter DNA compete for binding to region 4.2 of σ^{70}, thereby resulting in the observed promoter specificity of 6S RNA regulation.

Results

The strength of the −35 element determines 6S RNA-sensitive promoters

Although the vast majority of Eσ^{70} is bound to 6S RNA in late stationary phase (Wassarman and Storz, 2000), 6S RNA inhibition of σ^{70}-dependent transcription is promoter specific (Trotochaud and Wassarman, 2004). 6S RNA interactions with Eσ^{70} block promoter DNA binding to RNAP *in vitro* (Wassarman and Saecker, 2006; Gildehaus *et al.*, 2007), suggesting transcription initiation at sensitive promoters is inhibited by direct competition between 6S RNA and promoter DNA binding to RNAP. Several promoters involved in amino acid metabolism require higher concentrations of Eσ^{70} than control promoters for maximal transcription (Barker *et al.*, 2001a,b), suggesting they might be inefficient for competitive binding to RNAP. To test if these promoters are subject to 6S RNA regulation, expression from promoter–*lacZ* reporter genes was compared in wild-type and 6S RNA null (*ssrS1*) strain backgrounds in late stationary phase (Fig. 2). These reporters contain limited sequences downstream of the transcription start site (+1 to +35, see Table 2) and, except for *argI–lacZ*, do not include sequences known to be recognized by transcription factors or known to be involved in other forms of regulation (e.g. attenuation) (Barker *et al.*, 2001b). The *argI–lacZ* contains ArgR binding sites; therefore promoter–*lacZ* activity of this promoter was monitored in a Δ*argR* background (Barker *et al.*, 2001b).

These amino acid reporter genes did not all respond similarly to 6S RNA; *livJ*, *argI* and *hisG* increased 1.7- to 1.8-fold in the absence of 6S RNA (*ssrS1*) compared with expression in a wild-type background, while *lysC*, *pheA* and *thrABC* were unchanged or minimally affected by 6S RNA. For comparison, expression from *gal*P2, an extended −10 promoter, was 3.3-fold higher in the absence of 6S RNA, while *lacUV5* was unchanged as previously reported (Fig. 2) (Trotochaud and Wassarman, 2004). Although changes in expression from these reporter genes are modest, they are very reproducible.
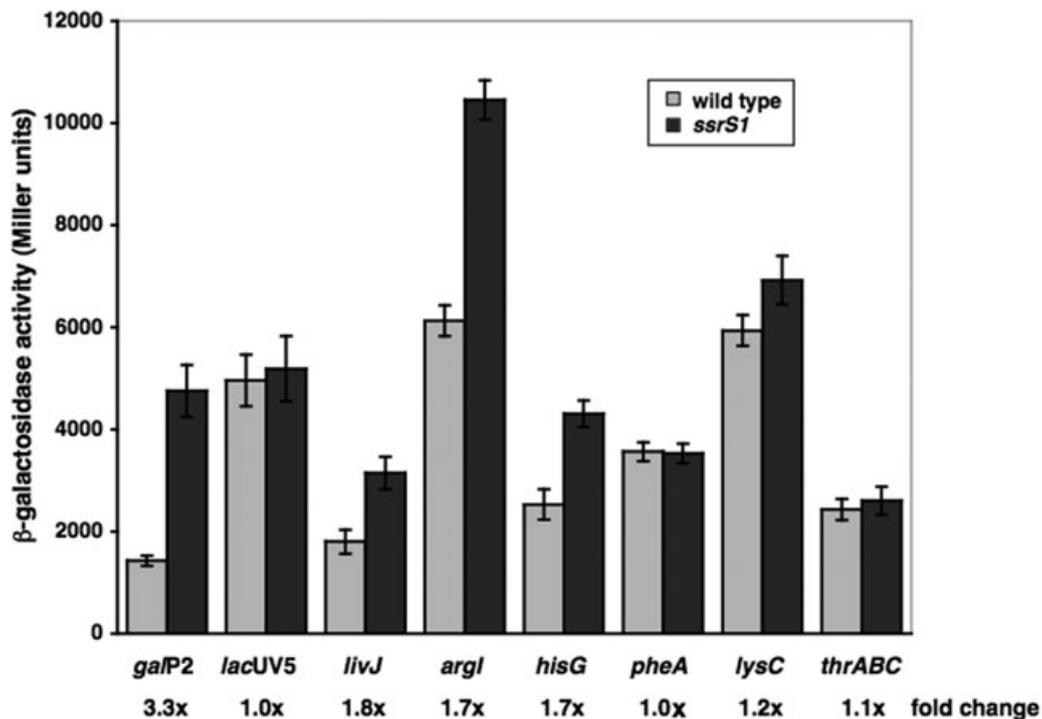
Fig. 2. β-Galactosidase activity of various promoter–*lacZ* reporter genes. Wild-type (light grey bars) and 6S RNA null (*ssrS1*; dark grey bars) strains containing chromosomal copies of the promoters indicated were grown to late stationary phase (24 h) in M9-glucose medium at 37°C, except for *hisG*–*lacZ*-containing strains, which were grown at 30°C as required for these 'system I' lysogens. The promoter–*lacZ* fusions contain minimal core promoter sequences (see Table 2) that do not include known regulatory elements, except for *argl*, which was tested in a Δ*argR* background to eliminate effects of this regulator. Fold change is the β-galactosidase activity in *ssrS1*/β-galactosidase activity in wild-type backgrounds. Data shown are an average of at least three independent experiments with three duplicate samples per experiment. Error bars correspond to ±standard deviations from the averages.

We have shown that this level of change is biologically relevant, as the 6S RNA-dependent change in *pspF* expression in stationary phase is sufficient to alter cell survival at high pH (Trotochaud and Wassarman, 2006). Here, we will consider promoters with ≥ 1.6-fold higher expression in cells lacking 6S RNA (*ssrS1* or *ssrS3*) compared with wild type to be 6S RNA 'sensitive', and those with < 1.2-fold change to be 'insensitive'. The fold change for each reporter gene is determined by the average of several independent experiments (at least three), each containing three replicate cultures, to facilitate examination of these levels of change. In addition, all experiments here examine expression in late stationary phase (16–24 h of growth, see *Experimental procedures*) as we have demonstrated previously that β-galactosidase activity correlates well with mRNA levels at this time as measured on reporter genes by primer extension (Trotochaud and Wassarman, 2004; 2006), and on endogenous genes by RNase protection and by microarray analyses (Wassarman and Storz, 2000; see below).

The amino acid promoters that were sensitive to 6S RNA did not correlate with the concentration of Eσ[70] necessary for maximal promoter activity *in vitro* (Barker *et al.*, 2001a); for example, *thrABC* required substantially higher levels of RNAP than *argl*, yet *thrABC* is unaffected by 6S RNA while *argl* is downregulated. Thus, there does not appear to be a simple relationship between overall promoter affinity for RNAP and 6S RNA inhibition.

Comparison of promoter sequences revealed that promoters insensitive to 6S RNA generally contained −35 elements with strong matches to consensus (Fig. 3A). To directly test whether the strength of the −35 element, as indicated by its match to consensus, is important for 6S RNA regulation, we asked if the *livJ* promoter could be converted to a 6S RNA-insensitive promoter by changing the −35 sequence element to consensus [*livJ*(−35cons)] (Fig. 3B). Overall promoter activity increased, as expected with a strong −35 element. However, *livJ*(−35cons)–*lacZ* expression was not increased in the *ssrS1* strain background indicating this promoter is no longer sensitive to 6S RNA (Fig. 3B, Table 1). Conversely, changing the relatively strong −35 element (TTtACA) in the *lacUV5* promoter to one that has no match to consensus (cactt) [*lacUV5*(−35weak)] resulted in 2.5-fold higher expression in the *ssrS1* strain background compared with wild type (Fig. 3B, Table 1). Therefore, the relative strength of the −35 element plays a significant role in determining promoter specificity for 6S RNA inhibition of transcription.

A

		-35		-10		+1	fold change
1	*rsd*P2	GCTC TTGCAC	TACCTTTGCATCACTGG	CATGTT	TAACAT	G	3.4x
	*gal*P1	GTCA CACTTT	TCGCATCTTTTTTATGC	TATGGT	TATTTC	A	3.2x
	*gal*P2	TCCA TGTTAC	ACTTTTCGCATCTTTGT	TATGCT	ATGGTT	A	3.3X
	*pspF*P1	CTGA TTGAAG	AATCAACAGCAACATGC	CAGGAT	GAGTT	A	2.6x
	*hupB*P2	GGTG TTGTAA	GGGGATGGCTGGCCTGA	TATAAC	TGCTG	C	2.5X
2	*livJ*	GCTA TTCCAA	TATCATAAAAATCGGGA	TATGTT	TTAGC	A	1.8x
	argI	AGAC TTGCAA	ATGAATAATCATCCATA	TAAATT	GAATTTT	A	1.7x
	hisG	GTTC TTGCTT	TCTAACGTGAAAGTGGTT	TAGGTT	AAAAGAC	A	1.7x
	pheA	TTTG TTGACA	GCGTGAAAACAGTACGGG	TACTGT	ACTAA	A	1.0x
	lysC	ATCG TTGACA	ACCGCCCGCTCACCCTT	TATTTA	TAAAT	G	1.2x
	thrABC	TTTA TTGACT	TAGGTCACTAAATACTT	TAACCA	ATATAGGC	A	1.1x
3	*lacUV5*	AGGC TTTACA	CTTTATGCTTCCGGCTCG	TATAAT	GTGTGGA	A	1.0x
	*rrnB*P1	CCTC TTGTCA	GGCCGGAATAACTCCC	TATAAT	GCGCCACC	A	0.9x
	RNAI	GTTC TTGAAG	TGGTGGCCTAACTACGGC	TACACT	AGAAGG	A	1.0x
	λPR	CGTG TTGACT	ATTTTACCTCTGGCGGT	GATAAT	GGTTGC	A	1.1x

B

		-35		-10		+1	fold change
livJ	wt	TAGCTA TTCCAA	TATCATAAAAATCGGGA	TATGTT	TTAGC	A	1.8x
	-35cons	TAGCTA TTGACA	TATCATAAAAATCGGGA	TATGTT	TTAGC	A	1.0x
	ext-10	TAGCTA TTGACA	TATCATAAAAATCGTGA	TATGTT	TTAGC	A	2.2x
lacUV5	wt	CCAGGC TTTACA	CTTTATGCTTCCGGCTCG	TATAAT	GTGTGG	A	1.0x
	-35weak	CCAGGC CACTTT	CTTTATGCTTCCGGCTCG	TATAAT	GTGTGG	A	2.5x
*hupB*P2	wt	GGGGTG TTGTAA	GGGGATGGCTGGCCTGA	TATAAC	TGCTGC	G	2.5x
	TT	GGGGTG TTGTAA	GGGGATGGCTGGCCTTA	TATAAC	TGCTGC	G	1.3x
	-35cons	GGGGTG TTGACA	GGGGATGGCTGGCCTGA	TATAAC	TGCTGC	G	1.0x
	-35cons,TT	GGGGTG TTGACA	GGGGATGGCTGGCCTTA	TATAAC	TGCTGC	G	1.0x
	weak-35	GGGGTG CACTTT	GGGGATGGCTGGCCTGA	TATAAC	TGCTGC	G	2.9x
	-35weak,TT	GGGGTG CACTTT	GGGGATGGCTGGCCTTA	TATAAC	TGCTGC	G	1.7x

Fig. 3. Alignment of σ[70] promoters tested for 6S RNA inhibition.
A. Natural promoter sequences separated into three groups: 1: tested extended −10 promoters, 2: amino acid biosynthetic promoters tested here, 3: additional tested promoters that are not inhibited by 6S RNA. All promoters shown have been tested for 6S RNA sensitivity by comparing expression of promoter–*lacZ* reporter genes in the presence (wild-type cells) or absence (*ssrS1* cells) of 6S RNA, and fold change is the β-galactosidase activity in *ssrS1*/β-galactosidase activity in wild-type backgrounds in late stationary phase. In addition, mRNA levels from reporter genes or endogenous genes have been examined by primer extension for *rsd*, *pspF* and *hupB* with similar results. Transcription start sites (+1) are indicated as reported at http://Ecocyc.org (Keseler *et al.*, 2005). −10 and −35 promoter elements are indicated, extended −10 element regions are underlined, and red signifies match to consensus. Note that *gal*P2 is shown with a 17 bp spacer (Bown *et al.*, 2000), but with an 18 bp spacer, the −35 element has a 3 out of 6 match to consensus. For other promoters, the −35 element match to consensus is not improved from those shown when spacer regions of 16–18 bp are allowed.
B. Promoters with indicated changes made to test the relative importance of the −35 and an extended −10 elements in 6S RNA regulation. Fold change is the β-galactosidase activity in *ssrS1*/β-galactosidase activity in wild-type backgrounds when grown in M9-glucose (*livJ* and *lacUV5*) or LB (*hupB*P2).

Table 1. β-Galactosidase activity of promoter–*lacZ* fusions in the presence and absence of 6S RNA.

Promoters	β-Galactosidase activity		Fold change (*ssrS1*/wild-type activity)
	+6S RNA	−6S RNA	
livJ	1 802 ± 235	3 147 ± 320	1.8×
livJ(−35cons)	11 561 ± 834	11 681 ± 819	1.0×
livJ(ext-10)	4 195 ± 368	9 022 ± 572	2.2×
lacUV5	5 834 ± 371	5 950 ± 293	1.0×
lacUV5(−35weak)	200 ± 18	498 ± 48	2.5×
*hupB*P2	1 522 ± 196	3 782 ± 343	2.5×
*hupB*P2(TT)	379 ± 33	505 ± 37	1.3×
*hupB*P2(−35cons)	12 238 ± 845	12 035 ± 1282	1.0×
*hupB*P2(−35cons,TT)	12 181 ± 756	12 524 ± 565	1.0×
*hupB*P2(−35weak)	368 ± 47	1069 ± 63	2.9×
*hupB*P2(−35weak,TT)	86 ± 8	149 ± 9	1.7×

In contrast to the −35 element, a weak −10 element did not appear to correlate with 6S RNA sensitivity. For example, *lacUV5*(−35weak) contains a consensus −10 element, yet it is inhibited by 6S RNA, and the amino acid promoters that are inhibited by 6S RNA generally have slightly stronger −10 elements as measured by the number of nucleotides matching consensus or by generating a score based on the sum of the frequency of nucleotides at each position (see *Experimental procedures*). We also generated two additional *lacUV5*-derived promoters [*lacUV5*(−10 thrABC) and *lacUV5*(−10 pheA)] containing −10 elements with the same sequences as *thrABC* or *pheA* and found that they were not sensitive to 6S RNA (data not shown).

The extended −10 element further contributes to 6S RNA inhibition

Previous work revealed that several extended −10 promoters were inhibited by 6S RNA, as measured by expression of promoter–*lacZ* reporter genes *in vivo* and mRNA levels from several endogenous genes driven by extended −10 promoters (see Fig. 3A) (Trotochaud and Wassarman, 2004; 2006). However, most naturally occurring promoters with extended −10 elements also have −35 elements with weaker matches to consensus. Examination of 100 extended −10 promoters compiled by Mitchell *et al.* (2003) revealed that none contained a consensus −35 element, 11 had a 5 out of 6 match, 19 had a 4 out of 6 match, while the remaining 70 promoters had weaker matches when potential −35 elements were assigned based on the best possible match to consensus allowing a 16–18 bp spacer region (Table S1). These observations led us to question whether the extended −10 element directly contributes to 6S RNA inhibition in the tested promoters, or whether weak −35 elements in extended −10 promoters were responsible.

To test the relative impact of the −35 and extended −10 elements on 6S RNA inhibition of transcription, we generated a series of promoters based on the *hupB*P2 promoter (see Fig. 3B) (Claret and Rouviere-Yaniv, 1996; 1997). The *hupB*P2 promoter was chosen as an example of an extended −10 promoter with a moderate −35 element (4/6 match to consensus) so that both stronger and weaker matches to consensus of the −35 element in the same promoter context could be tested. Fis and CRP regulate expression of *hupB*; however, the Fis binding sites are at −208, −185, −152 and +77 and the CRP site is at −146.5 relative to the start site of transcription at P2 (Claret and Rouviere-Yaniv, 1996; Keseler *et al.*, 2005) and are well outside the minimal promoter region used here (−42 to +5). β-Galactosidase activity from wild-type *hupB*P2–*lacZ* was 2.5-fold higher in *ssrS1* cells relative to wild-type cells, confirming that the *hupB*P2 promoter responds to 6S RNA similarly to other extended −10 promoters (Fig. 3B, Table 1). Primer extension analysis examining mRNA from the endogenous *hupB* gene showed a similar increase in expression in the *ssrS1* strain background compared with wild type in late stationary phase (data not shown) indicating that our reporter gene is representative of endogenous 6S RNA regulation of expression from *hupB*P2.

To test whether the relative strength of the −35 element is important for 6S RNA regulation in the context of an extended −10 promoter, we next examined *hupB*P2(−35cons) and *hupB*P2(−35weak) in wild-type compared with *ssrS1* strain backgrounds (Fig. 3B, Table 1). Changing the −35 element to consensus resulted in a loss of 6S RNA inhibition, even in the presence of the extended −10 element. Similar loss of 6S RNA inhibition was observed when the −35 element was changed to consensus in *galP1* and *pspFP1* promoter–*lacZ* fusions (data not shown). Expression from all the promoters with consensus −35 and extended −10 elements was very high; therefore mRNA was examined by primer extension and similar levels of *hupB*–*lacZ* mRNA were observed in wild-type and *ssrS1* cells carrying *hupB*P2(−35cons)–*lacZ* (data not shown). Thus, an

extended −10 element does not specify 6S RNA regulation at promoters with consensus −35 elements, although naturally occurring extended −10 promoters containing consensus −35 elements have not been identified. *hupB*P2(−35weak)–*lacZ* was inhibited by 6S RNA to a similar extent as the wild-type *hupB*P2 promoter containing a 4 out of 6 match to consensus, suggesting weak to moderate −35 elements are similarly regulated by 6S RNA in the presence of an extended −10 element.

Next, we examined the importance of the extended −10 element in this series of promoters (Fig. 3B). With a weak −35 element, the removal of the extended −10 element did not eliminate 6S RNA inhibition [compare *hupB*P2(−35weak) with *hupB*P2(−35weak,TT)], although the fold change between *ssrS1* and wild-type strains is reduced from 2.9 to 1.7 demonstrating that the extended −10 element does contribute to 6S RNA sensitivity (Fig. 3B, Table 1). Similarly, introduction of an extended −10 element into the *livJ* promoter results in increased sensitivity to 6S RNA [2.2-fold for *livJ*(ext-10), see Fig. 3B and Table 1]. For the wild-type *hupB*P2 that contains a −35 element with a 4 out of 6 match to consensus, the presence of the extended −10 element is required for notable 6S RNA inhibition, although the *hupB*P2(TT) may still be slightly inhibited by 6S RNA as the 1.3-fold change in the absence of 6S RNA is reproducible and also observed when the extended −10 element was mutated to TT in the *pspF*P1 promoter that has a −35 element with a 4 out of 6 match to consensus (data not shown). However, other promoters with −35 elements containing a 4 out of 6 match to consensus are not sensitive to 6S RNA, such as the RNAI promoter that has the same −35 element sequence as *pspF*P1 (see Fig. 3A), indicating other sequences or factors must influence these small changes.

6S RNA regulates transcription of hundreds of genes in stationary phase

Our results suggest that the presence of an extended −10 element or a weak −35 element defines promoters that are inhibited by 6S RNA in stationary phase. We used these two features to predict whether mapped σ^{70}-dependent promoters (Mitchell *et al.*, 2003) would be inhibited by 6S RNA (Table S1). Nineteen percent (100 out of 523) of these promoters contain an extended −10 element (see also Burr *et al.*, 2000), and as none of these extended −10 promoters have a consensus −35 element we predict that most of them will be sensitive to 6S RNA regulation. For promoters lacking an extended −10 element (423 out of 523), 55% have −35 elements with 0–3 out of 6 match to consensus, suggesting that they will be sensitive to 6S RNA regulation as well if they are expressed in stationary phase.

To test these predictions, we compared mRNA levels from genes containing mapped promoters in cells lacking 6S RNA (*ssrS3*) and wild-type cells in late stationary phase by microarray analysis. Several of these genes contained multiple mapped promoters; therefore we assigned a single prediction for each gene (as opposed to each promoter) to facilitate comparison with the microarray results. We made an assignment of a 'primary' promoter for each gene based on published data when available, based on the promoter with the strongest −35 element based on match to consensus, or with an extended −10 element. In addition, we eliminated genes from the predictions that were not expressed in either cell type, or that were not represented on the microarrays (i.e. tRNA genes). This left 312 genes to examine further, and 199 that are predicted to be sensitive to 6S RNA based on the presence of a primary promoter with a weak −35 element or an extended −10 element (Table S2).

Seventy-seven genes were predicted to be inhibited by 6S RNA based on the presence of an extended −10 promoter (Table S1). We observed that 59 out of 77 (77%) were increased (> 1.6-fold) in *ssrS3* cells compared with wild-type cells, suggesting that most are sensitive to 6S RNA in agreement with predictions (Table S2). We also observed good agreement between many changes observed in microarrays and promoter–*lacZ* reporter genes. For example, *hupB* was 2.3-fold increased in cells lacking 6S RNA as measured by microarray analysis compared with 2.5-fold change of the *hupB*P2–*lacZ* reporter.

For the 235 promoters without an extended −10 element, we found that 40% (94 out of 235) were increased in *ssrS3* cells relative to wild-type cells overall (Table S2). Only 16% (18 out of 113) of promoters with strong −35 elements (4–6 out of 6 match to consensus) were increased, 73% (35 out of 48) of promoters with very weak −35 elements (0–2 out of 6 match) were increased, and 55% (41 out of 74) of promoters with −35 elements with a 3 out of 6 match to consensus were increased. These observations agree quite well with our predictions, although the last set of promoters agreed less than the others, so we looked further at the features of these promoters.

First, we assigned scores to the −35 element based on the frequency of nucleotides found in each position (Hawley and McClure, 1983), rather than a simple match or mismatch to consensus (see *Experimental procedures*). However, these weighted scores did not distinguish 6S RNA-sensitive and -insensitive promoters in this set any better (Table S2). In addition, the frequency of specific nucleotides at each position in the −35 element was similar between promoters sensitive and insensitive to 6S RNA suggesting no individual position was able to determine sensitivity to 6S RNA.

Next, we asked whether the strength of the −10 element was a factor. We generated frequency scores for the −10

element and found no correlation between the strength of the −10 element and 6S RNA-sensitive promoters of this group (−35 elements with 3 out of 6 match to consensus), nor in the full set (see *Experimental procedures*). Even a consensus −10 element was not sufficient to make a promoter with a weak −35 element 6S RNA insensitive [e.g. *gltA* and see *lacUV5*(−35weak)], nor does a very weak −10 element correlate with 6S RNA sensitivity (e.g. *malT* and *thrABC*).

Finally, we asked whether overall expression levels correlated with 6S RNA inhibition, and found no relationship between 6S RNA regulation and expression levels (see *Experimental procedures*). In fact, the gene with the highest expression observed on the microarray [*cspA* expressed at 62 679 average intensity units (AI units)] is sensitive to 6S RNA, while many genes with very low expression (e.g. *recA* and *adiA* at 57 and 95 AI units respectively) are insensitive. Similarly, we have several minimal promoter–*lacZ* reporter genes with quite low expression (200–400 Miller units) that are not inhibited by 6S RNA (data not shown), while other reporters with high activity are sensitive to 6S RNA (e.g. *argI*–*lacZ* at 6000 Miller units, see Fig. 2). Although mRNA and β-galactosidase levels are not simple measures of transcription initiation frequency as they may be subject to post-transcriptional regulation, altogether these data suggest 6S RNA regulation is not limited to weak promoters.

σ^{70} region 4.2 is required for 6S RNA binding to $E\sigma^{70}$

Next we investigated which regions of σ^{70} might be important for 6S RNA interactions by examining a 6S RNA–σ^{70} cross-linked species generated by UV irradiation of cell lysate (Wassarman and Storz, 2000). We were able to map the cross-link to the C-terminal half of σ^{70} (containing regions 2+3+4) by proteolytic mapping (data not shown), but it became clear that there were multiple cross-link sites between 6S RNA and σ^{70}, preventing further mapping with this approach. We chose to focus first on region 4.2 of σ^{70} as it contains a helix–turn–helix motif that mediates DNA binding to the −35 element (Lonetto *et al.*, 1992; Campbell *et al.*, 2002).

We tested the importance of region 4.2 of σ^{70} for binding to 6S RNA by examining mutant σ factors. All σ factors discussed here were able to saturate core RNAP at similar levels of input σ, as measured by co-immunoprecipitation with core-specific antibodies (data not shown, see *Experimental procedures*), suggesting they are not defective in core binding as also reported previously for all except $\sigma^{70:S}$ (Lonetto *et al.*, 1998; Minakhin and Severinov, 2003; Ross *et al.*, 2003; Geszvain *et al.*, 2004; Nechaev and Geiduschek, 2006). Binding of RNAP to 6S RNA and the inactive 6S(M5) RNA was determined from the level of ^{32}P-RNAs

that co-immunoprecipitated with RNAP. The level of 6S RNA binding was determined by the amount of 6S RNA selected, and the specificity of the binding was tested by examining 6S(M5) RNA, which does not bind wild-type $E\sigma^{70}$ (Wassarman and Storz, 2000; Trotochaud and Wassarman, 2005) nor any of the variant holoenzymes examined here (see Fig. 4B). In addition, holoenzymes containing variant σ factors were active for transcription as they were able to initiate transcription on extended −10 promoters (data not shown), which do not require region 4.2 for transcription initiation (Kumar *et al.*, 1993; Minakhin and Severinov, 2003; Nechaev and Geiduschek, 2006).

First, we tested whether removal of region 4.2 would alter 6S RNA binding by examining $\sigma^{70}(1–565)$, which contains amino acids 1 through 565 of σ^{70} such that region 4.2 and the C-terminal region are deleted (see Fig. 4A) (Minakhin and Severinov, 2003; Nechaev and Geiduschek, 2006). $E\sigma^{70}(1–565)$ was severely defective for binding 6S RNA; < 8% 6S RNA co-immunoprecipitated with $E\sigma^{70}(1–565)$ relative to wild-type $E\sigma^{70}$ (Fig. 4B). Assessing 6S RNA:RNAP complex formation by native gel electrophoresis gave similar results (data not shown).

To further test the contribution of region 4.2 for 6S RNA binding, we generated $\sigma^{70:S}$, a protein containing regions 1–4.1 of σ^{70} and region 4.2 and the C-terminal end from σ^S (see Fig. 4A). $E\sigma^S$ does not form stable complexes with 6S RNA *in vivo* or *in vitro* (Wassarman and Storz, 2000; Trotochaud and Wassarman, 2005), yet region 4.2 is highly conserved between σ^{70} and σ^S suggesting this swap might still allow 6S RNA binding. However, $E\sigma^{70:S}$ also was defective for 6S RNA binding as measured by co-immunoprecipitation or native gel assays (Fig. 4B and data not shown).

In addition to deletion of region 4.2, the $\sigma^{70}(1–565)$ is missing the C-terminal tail of σ^{70} (residues 600–613, see Fig. 4A), and the $\sigma^{70:S}$ contains the C-terminal tail from σ^S. To test whether the C-terminal region of σ^{70} might be important for 6S RNA binding, we examined $E\sigma^{70}(1–608)$ in which only the last five amino acids of σ^{70} have been deleted (Geszvain *et al.*, 2004). $E\sigma^{70}(1–608)$ had an intermediate binding activity with ~50% of wild type binding levels (Fig. 4B). One C-terminal residue known to be important for interaction with the α-subunit or *trans*-acting factors during promoter binding, R603 (Lonetto *et al.*, 1998; Ross *et al.*, 2003), was not required for 6S RNA binding as $E\sigma^{70}(R603A)$ bound 6S RNA as well as wild-type $E\sigma^{70}$ (Fig. 4B).

Discussion

Here we demonstrate that two core promoter features are important for the specificity of 6S RNA regulation of transcription at σ^{70}-dependent promoters. First, the relative

Fig. 4. Activity of various σ-subunits for 6S RNA binding.
A. A CLUSTAL alignment of region 4 and C-terminal tail regions for *E. coli* σ70 and σS. The location of the helix–turn–helix motif in region 4.2 is indicated by schematic, and numbering for amino acid residues of interest is shown. Black arrows mark the junction region in the σ70:S fusion protein.
B. 6S RNA binding to Eσ reconstituted from core and excess σ was examined. An equimolar mixture of ^{32}P-labelled 6S RNA and 6S(M5) RNA was incubated with core RNAP (lane 1) or various forms of reconstituted Eσ as indicated, followed by immunoprecipitation with core-specific antisera. A loading control (^{32}P-5S RNA) was included in subsequent steps of RNA isolation prior to separation on 8% denaturing polyacrylamide gel. Lane 7 contains 50% of input RNA carried through all post-immunoprecipitation steps. These experiments have been repeated at least three times with similar results; a representative experiment is shown.

strength of the −35 element, as measured by match to consensus, is one feature determining sensitivity to 6S RNA, and second, the presence of an extended −10 element further contributes to 6S RNA inhibition. The combination of the effects of these two core promoter elements results in modulation of 6S RNA-dependent changes in expression at different promoters. We have demonstrated that 6S RNA is a global regulator as expression of hundreds of genes is altered in stationary phase in a 6S RNA-dependent manner. In addition, we have determined that region 4.2 of σ70 is necessary for stable interactions between 6S RNA and Eσ70. Recognition of the −35 element in promoter DNA also is mediated through direct binding to region 4.2 of σ70 (Dombroski *et al.*, 1992); therefore, we propose that the mechanism for 6S RNA inhibition of promoters with weak −35 elements involves competition with promoter DNA for binding to region 4.2 of σ70 within Eσ70.

A competition model for 6S RNA inhibition of transcription

Promoters with strong −35 elements are insensitive to 6S RNA while those with weaker matches to consensus (0–3 out of 6 match) are generally downregulated by 6S RNA. 6S RNA-insensitive (*lacUV5*) and -sensitive (*livJ*) promoters can be interconverted by changing only the −35 element sequence (see Fig. 3B, Table 1), indicating that the promoter context of the −35 element is fairly unimportant except when an extended −10 element in present (see below). Although genes containing promoters with −35 elements with 3 out of 6 match to consensus were not as well predicted as the others, 55% of these genes were sensitive to the presence of 6S RNA indicating a weak −35 element is an important feature for many 6S RNA-sensitive promoters. We suggest these intermediate strength −35 elements are more sensitive to the impact of other factors, including sequences within the promoter and interactions with *trans*-acting factors. However, the strength of the −10 element does not appear to be a determining feature, either specifically within this group of promoters or in determining 6S RNA sensitivity in general. A strong −10 element is not sufficient to make a promoter insensitive to 6S RNA, nor does a weak −10 element correlate with promoters sensitive to 6S RNA.

The importance of the −35 element in determining 6S RNA sensitivity, together with data demonstrating the importance of region 4.2 of σ^{70} for 6S RNA binding to Eσ^{70} (see below), suggests there is competition between 6S RNA and promoter DNA binding to σ^{70} region 4.2. In this model, promoters with weaker −35 elements are at a disadvantage for competition with 6S RNA, and subsequently are transcribed less often during stationary phase when 6S RNA is abundant.

Extended −10 promoters and 6S RNA regulation

The extended −10 element also is a determinant for 6S RNA-regulated promoters in that it causes promoters with moderate −35 elements to be subject to 6S RNA regulation, and those with weak −35 elements to be further downregulated by 6S RNA. For example, promoters without a recognizable −35 element are inhibited to a greater extent with an extended −10 element than without [compare the 2.9-fold for *hupB*P2(−35weak) with the 1.7-fold for *hupB*P2(−35weak,TT) promoters] (see Fig. 3B, Table 1). Promoters with moderate −35 elements are weakly or not affected by 6S RNA in the absence of an extended −10, but are still strongly regulated by 6S RNA when an extended −10 element is present [compare wild-type *hupB*P2 with *hupB*P2(TT)] (see Fig. 3B, Table 1). Globally, ~20% of mapped promoters contain extended −10 elements (Burr *et al.*, 2000; Mitchell *et al.*, 2003) and

we have observed that 77% of those expressed in stationary phase are subject to 6S RNA regulation, indicating this large group of promoters are generally sensitive to 6S RNA.

The molecular mechanisms underlying the contributions of the extended −10 element to the promoter specificity of 6S RNA regulation are not yet clear. One possibility is that the interaction between region 3.0 of σ^{70} and the extended −10 element alters the strength of region 4.2 interactions with the −35 element, making them preferentially subject to 6S RNA competition. Region 4.2 of σ^{70} is dispensable for transcription at extended −10 promoters (Kumar *et al.*, 1993; data not shown), and it has been suggested that extended −10 promoters do interact with region 4.2 of σ^{70} differently from non-extended −10 promoters (Minakhin and Severinov, 2003), although RNAP must still contact the −35 element as transcription from an extended −10 promoter can be enhanced by strengthening the −35 element. Alternatively, 6S RNA binding to Eσ^{70} could block σ^{70} region 3.0 from binding the extended −10 element in a manner independent of and distinct from our proposed competition for region 4.2 on non-extended −10 promoters. Although we have demonstrated a key role for region 4.2 of σ^{70} in binding to 6S RNA, 6S RNA also resides within the active site of Eσ^{70} quite distant from this interaction (Wassarman and Saecker, 2006; Gildehaus *et al.*, 2007), and it is likely that there are many contacts between 6S RNA and RNAP that add additional strength and/or specificity to 6S RNA binding. More details about 6S RNA and Eσ^{70} interactions, understanding the dynamics of the interaction between these molecules as well as a kinetic understanding of 6S RNA and DNA binding to Eσ^{70} under *in vivo* conditions are needed to help distinguish between such models.

6S RNA–σ^{70} interactions

We have discovered that region 4.2 of σ^{70} is critical for Eσ^{70} binding to 6S RNA, suggesting direct contacts between this region of σ^{70} and 6S RNA contribute significantly to the overall strength of the 6S RNA–Eσ^{70} interaction. The location of the single-stranded region of 6S RNA at the active site of Eσ^{70} and the identity of the pRNA template region within 6S RNA orients 6S RNA relative to Eσ^{70} (Wassarman and Saecker, 2006; Gildehaus *et al.*, 2007) and allows us to speculate that an 'upstream' region of 6S RNA is positioned for likely contacts with σ^{70} region 4.2 by analogy to promoter DNA binding (see Fig. 1). Interestingly, this region of 6S RNA is not well conserved at a primary sequence level and secondary structure analyses suggest this region of the RNA is not fully double-stranded (Barrick *et al.*, 2005; Trotochaud and Wassarman, 2005). Therefore, the precise

contacts between this region of 6S RNA and σ^{70} may diverge from those made at DNA promoters, although the molecular details await further investigation.

Truncation of the C-terminal tail [σ^{70}(1–608)] also resulted in reduction of 6S RNA binding, but to a lesser extent than the larger deletion [σ^{70}(1–565)] (see Fig. 4). The C-terminal tail has been implicated in positioning region 4.2 on the β-flap of RNAP to maintain spacing appropriate for efficient recognition of both −35 and −10 elements in promoter DNA (Kuznedelov *et al.*, 2002; Murakami *et al.*, 2002; Geszvain *et al.*, 2004), as well as to influence early elongation events (Nickels *et al.*, 2005; 2006). We propose that the decreased binding of Eσ^{70}(1–608) may be due to loss of region 4.2 positioning rather than due to direct contacts between 6S RNA and the C-terminal tail of σ^{70}. Appropriate spacing and positioning of σ^{70} region 4.2 relative to the rest of Eσ^{70} is expected to be important for efficient 6S RNA binding within the active site. However, it is possible that direct contacts between 6S RNA and the C-terminal tail of σ^{70} or the β-flap itself may contribute to the strength of 6S RNA binding in addition to the likely interactions with region 4.2 that are responsible for the drastic decrease in binding of the Eσ^{70}(1–565).

It is intriguing that E$\sigma^{70:S}$ does not bind 6S RNA as σ^S region 4.2 is quite similar to σ^{70} region 4.2 (see Fig. 4A), and Eσ^S binds DNA with similar, although not identical, preferences to Eσ^{70} (Gaal *et al.*, 2001; Weber *et al.*, 2005). σ^S C-terminal tail appears to make stronger contacts with the β-flap of core RNAP (Kuznedelov *et al.*, 2002), which might account for differential spacing or positioning of σ^S region 4.2 that does not allow 6S RNA binding. It also is possible that residues in region 4.2 of σ^{70} important for 6S RNA binding will not correspond to those important in DNA binding that are conserved between σ^{70} and σ^S, especially given the rather poor apparent mimic the upstream region of 6S RNA is for the −35 region in promoter DNA (see above). In either case, it suggests that region 4.2 and potentially the C-terminal tail of σ^{70} may be important in mediating the specificity of 6S RNA binding to Eσ^{70} over other holoenzymes.

Large-scale regulation of transcription by 6S RNA in stationary phase

We have defined features of promoters that make them sensitive to 6S RNA regulation by testing the effects of mutating specific promoter features on their expression in cells with and without 6S RNA. We have applied these results to predictions of which endogenous genes will be subject to 6S RNA regulation in stationary phase and found a 68% agreement between expressed genes that are sensitive to 6S RNA and our predictions. Expression of hundreds of genes are altered in a 6S RNA-dependent

manner; therefore, 6S RNA has a major impact on gene expression changes globally, even as the specific changes in expression of individual genes are rather modest (~1.6- to 4.0-fold). However, we have shown that even these modest changes in a single gene can be physiologically relevant as 6S RNA-dependent changes in *pspF* expression are sufficient to alter cell survival at elevated pH (Trotochaud and Wassarman, 2006).

Many of the promoters affected by 6S RNA also are regulated by *trans*-acting factors that often act to determine the overall timing and extent of transcription. We suggest the role of 6S RNA regulation is to dampen expression of many genes during stationary phase, rather than to regulate on–off decisions between exponential- and stationary-phase expression. For example, expression of *pspF* is downregulated by 6S RNA in stationary phase, but 6S RNA does not alter the ability of this promoter to respond to *trans*-acting factors that upregulate expression in response to stress (Trotochaud and Wassarman, 2006). In fact, many of the promoters inhibited by 6S RNA direct transcription of genes that are normally expressed and important during stationary phase, including *hupB* encoding HU-β (Williams and Foster, 2007). Whether specific 6S RNA-dependent changes in gene expression will directly contribute to general cell survival in a manner similar to the primary role of PspF in mediating altered survival during growth at high pH, or whether optimal cell survival results from the overall dampening of transcription at many genes, perhaps for nutrient conservation, remains to be determined as the impact of individual gene changes on cell survival in stationary phase is examined.

Experimental procedures

Strains

Escherichia coli strains (Table 2) were grown in LB Lennox broth (LB) or M9 minimal salts supplemented with 0.2% glucose, 0.002% vitamin B₁ and trace minerals (M9-glucose) (Balch *et al.*, 1979; Miller, 1992), as indicated. Chromosomal promoter–*lacZ* fusions were λ-phage lysogens generated as described elsewhere (Rao *et al.*, 1994). All were 'system II' lysogens except for RLG4418 and KW359 carrying *hisG–lacZ* which is a 'system I' lysogen. Promoter regions for new constructs described here were generated by annealing oligonucleotides containing the entire promoter region and cloning into the EcoRI and HindIII sites in pMSB1 (Rao *et al.*, 1994). *livJ*(−35cons), *livJ*(ext-10) and *lacUV5*(−35weak) were generated by site-directed mutagenesis of parent promoters in pMSB1 using quikchange according to manufacturer protocols (Stratagene). See Table S3 for all oligonucleotide sequences. The extent of the promoter regions included in reporter genes is indicated as the number of nucleotides upstream and downstream of the +1 transcription start site (Table 2). All plasmid intermediates and the chromosomal

Table 2. Bacterial strains.

Strain name	Genotype	Reference
KW72	Laboratory wild-type strain *E. coli* K12	Wassarman and Storz (2000)
GS075	KW72, *ssrS1* (Amp^R)	Wassarman and Storz (2000)
KW489	*E. coli* MG1655	
KW490	KW489 *ssrS3*	This work
RLG3499	MG1655 *pyrE*+ *lacI lacZ* [VH1000]	Gaal *et al.* (2001)
KW372	RLG3499 λ*rsd*P2(−149+91)−*lacZ*	Trotochaud and Wassarman (2004)
KW373	KW372 *ssrS1*	Trotochaud and Wassarman (2004)
RLG6358	RLG3499 λ*rrnB*P1(−41+1)−*lacZ*	Hirvonen *et al.* (2001)
KW238	RLG6358 *ssrS1*	Trotochaud and Wassarman (2004)
RLG6641	RLG3499 λi²¹ lambdaP_R(−40+20)−*lacZ*	Barker and Gourse (2001)
KW325	RLG6641 *ssrS1*	Trotochaud and Wassarman (2004)
RLG5079	RLG3499 λRNA1(−60+1)−*lacZ*	Trotochaud and Wassarman (2004)
KW321	RLG5079 *ssrS1*	Trotochaud and Wassarman (2004)
KW376	RLG3499 λ*gal*P2(−89+50)(−37C-T)−*lacZ*	Trotochaud and Wassarman (2004)
KW376	KW376 *ssrS1*	Trotochaud and Wassarman (2004)
KW439	RLG3499 λ*pspF*−*lacZ*(−151+33 relative to P1)	Trotochaud and Wassarman (2006)
KW440	KW439 *ssrS1*	Trotochaud and Wassarman (2006)
RLG4978	RLG3499 λ*argI*(−45+35)−*lacZ argF::fol*	Barker *et al.* (2001b)
KW347	RLG4978 *ssrS1*	This work
RLG4418	RLG3499 λ*hisG*(−60+15)−*lacZ*	Barker *et al.* (2001b)
KW359	RLG4418 *ssrS1*	This work
RLG4818	RLG3499 λ*pheA*(−73+10)−*lacZ*	Barker *et al.* (2001b)
KW351	RLG4818 *ssrS1*	This work
RLG4816	RLG3499 λ*lysC*(−59+16)−*lacZ*	Barker *et al.* (2001b)
KW355	RLG4816 *ssrS1*	This work
RLG5080	RLG3499 λ*thrABC*(−72+1)−*lacZ*	Barker *et al.* (2001b)
KW459	RLG5080 *ssrS1*	This work
KW460	RLG3499 λ*lacUV5*(−48+4)−*lacZ*	This work
KW461	KW460 *ssrS1*	This work
KW462	RLG3499 λ*lacUV5*(−*35weak*)(−48+4)−*lacZ*	This work
KW463	KW462 *ssrS1*	This work
RLG4422	^aRLG3499 λ*livJ*(−60+13)−*lacZ*	Barker *et al.* (2001b)
KW464	RLG4422 *ssrS1*	This work
KW465	^aRLG3499 λ*livJ*(−*35cons*)(−60+13)−*lacZ*	This work
KW466	KW465 *ssrS1*	This work
KW491	^aRLG3499 λ*livJ*(*ext-10*)(−60+13)−*lacZ*	This work
KW492	KW491 *ssrS1*	This work
KW467	RLG3499 λ*hupB*P2(−43+3)−*lacZ*	This work
KW468	KW467 *ssrS1*	This work
KW469	RLG3499 λ*hupB*P2(TT)(−43+3)−*lacZ*	This work
KW470	KW469 *ssrS1*	This work
KW471	RLG3499 λ*hupB*P2(−35cons)(−43+3)−*lacZ*	This work
KW472	KW471 *ssrS1*	This work
KW473	RLG3499 λ*hupB*P2(−35cons,TT)(−43+3)−*lacZ*	This work
KW474	KW473 *ssrS1*	This work
KW475	RLG3499 λ*hupB*P2(−35weak)(−43+3)−*lacZ*	This work
KW476	KW475 *ssrS1*	This work
KW477	RLG3499 λ*hupB*P2(−35weak,TT)(−43+3)−*lacZ*	This work
KW478	KW477 *ssrS1*	This work
KW479	RLG3499 λ*pspF*P1(−41+4)−*lacZ*	This work
KW480	KW479 *ssrS1*	This work
KW481	RLG3499 λ*pspF*P1(−35cons)(−41+4)−*lacZ*	This work
KW482	KW481 *ssrS1*	This work
KW483	RLG3499 λ*pspF*P1(TT)(−41+4)−*lacZ*	This work
KW484	KW483 *ssrS1*	This work
KW485	RLG3499 λ*gal*P1(−42+1)(−19G-T)−*lacZ*	This work
KW486	KW485 *ssrS1*	This work
KW487	RLG3499 λ*gal*P1(−42+1)(−35cons)(−19G-T)−*lacZ*	This work
KW488	KW487 *ssrS1*	This work

a. The *livJ* promoter sequence used here is as reported in Adams *et al.* (1990) and shown in Fig. 3. However, in the *E. coli* K12 genome sequence (Blattner *et al.*, 1997) −12A is deleted as also confirmed by PCR sequencing of the endogenous *livJ* from strains KW72 and RLG3499 (data not shown). 6S RNA regulation is similar on both forms of the promoter, with and without 12A (data not shown).

regions containing the lysogen promoters were confirmed by sequencing. P1 transductions were performed as previously described (Silhavy *et al.*, 1984) to move *ssrS1* (Lee *et al.*, 1985) into strains containing chromosomal reporter genes. The *ssrS1* allele contains a *bla* insertion into the 6S RNA gene (*ssrS*). An allele containing a precise replacement of the 6S RNA coding sequence with a tetracycline resistance cassette was made (*ssrS2*), and *ssrS3* was generated from *ssrS2* by removal of the tetracycline resistance cassette by Flp recombinase resulting in replacement of the 6S RNA coding region with a 13 bp scar (see Trotochaud and Wassarman, 2006). In all three strains (*ssrS1*, *ssrS2* and *ssrS3*), there is no detectable 6S RNA expressed, and expression of the downstream gene (*ygfA*) is the same as wild type as measured by quantitative reverse transcription polymerase chain reaction (RT-PCR) (*ssrS1*, *ssrS2* and *ssrS3*) and by microarray analysis (*ssrS3*) suggesting these mutations are non-polar on *ygfA*. In addition, 6S RNA-dependent changes in β-galactosidase activity of reporter genes and growth phenotypes are indistinguishable in these three alleles.

β-Galactosidase assays

β-Galactosidase activity was measured at 30°C as previously described (Trotochaud and Wassarman, 2004) and activity is expressed in Miller units (ΔOD_{420} per minute per OD_{600} unit) (Miller, 1972). Briefly, cultures inoculated from a single colony were grown for 24 h, diluted 1:100 in fresh medium ($OD_{600} = 0.04$), grown for an additional 18 h (LB) or 24 h (M9-glucose) to late stationary phase. Cells were lysed with sodium dodecyl sulphate and chloroform prior to β-galactosidase assays. Cells were grown at 30°C in LB or 37°C in M9-glucose, except for RLG4418 and KW359, which were grown at 30°C in both media to maintain the 'system I' lysogen. 6S RNA effects on *livJ*, *argI* and *hisG* were more pronounced in M9-glucose than LB. For all other promoters, the fold change between β-galactosidase activity in *ssrS1* compared with wild-type strains was similar in M9-glucose and LB. At least three independent cultures per strain were used per experiment, and all experiments were repeated at least three times. Although the *hupB*P2(−35cons) had very high expression, cells carrying the *hupB*P2(−35cons) were readily generated and β-galactosidase activity was stable, in contrast to our observations when trying to examine even higher expressing reporters based on an *rsd*P2 derivative. Therefore, the high expression from *hupB*P2(−35cons) is not deleterious to the cells and within a measurable range, as also supported by analysis of mRNAs by primer extension.

Primer extension

Total RNA was isolated from cells grown in LB at 37°C for 18 h using Trizol Reagent as previously described (Wassarman and Storz, 2000). Primer extension was performed using SuperscriptII (Invitrogen) according to manufacturer's protocol for first-strand synthesis, except that 10 μg of total RNA and 2 ng of oligonucleotide were used per reaction as previously described (Trotochaud and Wassarman, 2004).

Microarray analysis

RNA was isolated (Qiagen RNeasy) from wild-type (MG1655; KW489) or 6S RNA null (*ssrS3*; KW490) cells grown at 37°C for 16 h after dilution ($OD_{600} = 0.05$) into LB medium. Sixteen hours of growth was chosen as Northern analysis demonstrated that 6S RNA was maximally expressed in MG1655 (data not shown), and co-immunoprecipitation and gradient analysis (see Wassarman and Storz, 2000) demonstrated that 6S RNA was fully associated with $E\sigma^{70}$ by this time (data not shown). For microarray analysis, RNA was isolated from two independent cultures for each cell type, and 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* K12). Each RNA sample was assayed independently, so for each gene, expression levels were averaged for four data points (each chip contains two replicates of each gene, and we examined two chips per each cell type giving four replicates per gene); expression is given in AI units. 'Expressed' genes were those with > 40 AI units in either cell type. In general, standard deviation between replicates was quite low, although higher for the lowest expressing genes (see Table S2). 6S RNA effects on each gene are given as a fold change (expression in *ssrS3* cells/expression in wild-type cells) for the genes with mapped promoters (see Table S2).

To examine whether overall lower expression levels correlated with 6S RNA sensitivity, we compared the average expression of the mapped genes sensitive to 6S RNA (2224 AI units) with those insensitive to 6S RNA (1717 AI units) and the average of all the promoters (1966 AI units), and it was clear that 6S RNA-sensitive genes were not expressed lower on average. Next we divided the genes into six equal groups based on expression level (52 genes per group), and calculated the percentage of genes in each group that were 6S RNA sensitive. Thirty-three percent of Group 1 (40–233 AI units), 46% of Group 2 (243–508 AI units), 58% of Group 3 (509–898 AI units), 44% of Group 4 (909–1565 AI units), 56% of Group 5 (1580–2877 AI units) and 58% of Group 6 (2923–62698 AI units) were sensitive to 6S RNA demonstrating that there is no trend between expression level and 6S RNA sensitivity. In fact, the group of lowest expressed genes were under-represented for 6S RNA-sensitive genes. For this article we have used a cut-off of 1.6-fold change to define 6S RNA-sensitive promoters as we believe this number accurately reflects observations of changes in endogenous and reporter genes and a change that can have physiological consequences. However, the lack of a trend between expression level and 6S RNA sensitivity remains the same with a higher threshold for 6S RNA sensitivity. For instance, using a twofold change as a cut-off results in 101 out of 312 genes upregulated in *ssrS3* cells relative to wild type, and they remain distributed between the six expression groups: Group 1 = 25%, Group 2 = 33%, Group 3 = 42%; Group 4 = 23%, Group 5 = 38% and Group 6 = 33% 6S RNA-sensitive genes.

In addition to examining relative −35 and −10 element strength based on the number of nucleotide matches to consensus, we generated position-weighted scores for each element by assigning a value to each nucleotide at each position based on the frequency of finding that nucleotide in a promoter data set (Hawley and McClure, 1983).

To address whether the strength of the −10 element could be predictive of 6S RNA-sensitive promoters lacking extended −10 elements, we looked at the percentage of genes that were 6S RNA sensitive compared with the number of nucleotide match to consensus. Out of 232 genes without an extended −10 element, we found 21% of those with 6 out of 6 match to consensus were 6S RNA sensitive, 31% with 5 out of 6, 52% with 4 out of 6, 31% of 3 out of 6 and 56% with 2 out of 6 match to consensus suggesting no strong correlation between match to consensus. This conclusion was more apparent when genes were divided into six groups based on position-weighted scores for the strength of the −10 element with more equal distribution between groups (38–40 genes per group). Note that the −10 element strength decreases from Group 1 to 6: Group 1 = 20%, Group 2 = 45%, Group 3 = 50%, Group 4 = 48%, Group 5 = 37% and Group 6 = 41% sensitive to 6S RNA. These data may suggest very strong −10 elements are less likely to be 6S RNA sensitive, but in contrast to the −35 element, it is clear that a weak −10 element does not determine 6S RNA sensitivity.

RNA polymerase

pLA4 (Anthony *et al.*, 2003) was used to express wild-type σ^{70} without additional sequences or tags. pLA4 σ^{70}(1–565) was generated from pLA4 by site-directed mutagenesis (Quikchange, Stratagene). Although this method is marketed to make small mutations, large deletions also can be made by designing oligonucleotides that anneal on both sides of the region to be deleted (see Table S3). For pLA4$\sigma^{70:S}$, the σ^{70} and σ^S portions were amplified from pLA4 and pLHN30 (Nguyen and Burgess, 1996), respectively, by PCR using oligonucleotides with overlapping sequences. PCR products were gel purified and the full-length $\sigma^{70:S}$ coding sequence was generated from the annealed products by PCR, and cloned into pCRII (TOPO-TA kit, Invitrogen) to generate pCR-$\sigma^{70:S}$. The PstI–HindIII fragment in pLA4 was replaced with the PstI–HindIII fragment of pCR-$\sigma^{70:S}$, pHMKσ^{70}5ΔC (Geszvain *et al.*, 2004) and pETσ^{70}(R603A) (Ross *et al.*, 2003) to generate pLA4$\sigma^{70:S}$, pLA4σ^{70}(1–608) and pLA4σ^{70}(R603A) respectively. All generated plasmids were confirmed by sequencing. All sigma factors were purified from inclusion bodies and refolded as previously described (Arthur and Burgess, 1998; Arthur *et al.*, 2000). Core RNAP was a generous gift from R.A. Saecker and M.T. Record.

To reconstitute Eσ, core RNAP and σ were incubated in 40 mM HEPES pH 7.5, 240 mM KCl, 1 mM DTT and 7.8% glycerol (v/v) for 30 min at 37°C. To test the efficiency of σ-core binding for the purified σ factors, one- to eightfold excess σ relative to core was used in reconstitution reactions and the level of σ binding was measured by co-immunoprecipitation as previously described (Wassarman and Storz, 2000) but using core-specific sera (WI-153) for immunoprecipitation and σ^{70}-specific sera (WI-166) for western analysis. In all cases, σ binding reached maximum levels and was comparable to wild-type σ^{70} binding at approximately three- to fourfold excess σ^{70} over core (data not shown). In addition, activity of the reconstituted Eσ were confirmed by their ability to carry out *in vitro* transcription on an extended −10 promoter (data not shown). However, *in vitro* transcription from test promoters was strongly decreased in the presence of any added RNA (5S RNA, tRNA, 6S RNA), so it was not possible to address the specific effects of 6S RNA on regulating transcription of Eσ^{70} using a purified transcription assay, as previously discussed (Trotochaud and Wassarman, 2005; Wassarman and Saecker, 2006; Wassarman, 2007). In all RNA binding experiments, fivefold excess σ relative to core was used.

RNA–RNAP binding assays

Reconstitution of 6S RNA:Eσ^{70} complexes was performed as previously described (Wassarman and Saecker, 2006). Basically, *in vitro* transcribed ^{32}P-6S RNA and ^{32}P-6S(M5) RNAs (final concentration of 20 nM each) were incubated with Eσ^{70} (final concentration of 40 nM input core and fivefold excess σ, see above) for 15 min at 37°C in 20 mM HEPES, pH 7.5, 120 mM KCl, 0.5 mM MgCl$_2$, 5% glycerol and 1 mM DTT. Heparin was added to 100 μg ml^{-1}. Immunoprecipitation reactions were as previously described (Wassarman and Storz, 2000) using rabbit sera specific to *E. coli* core RNAP (WI-153). RNAs were recovered by phenol:chloroform: isoamyl alcohol extraction and ethanol precipitation in the presence of a constant amount of *in vitro* transcribed ^{32}P-5S RNA as an internal control for recovery. For native gel electrophoresis, samples containing 6S RNA or 6S(M5) RNA independently were incubated for 2 min at room temperature after addition of heparin, followed by separation on 5% polyacrylamide, 5% glycerol, 0.5× TBE as previously described (Trotochaud and Wassarman, 2005).

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Supplementary material

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