Cell, Vol. 101, 613–623, June 9, 2000, Copyright ©2000 by Cell Press

6S RNA Regulates *E. coli* RNA Polymerase Activity

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Summary

The *E. coli* 6S RNA was discovered more than three decades ago, yet its function has remained elusive. Here, we demonstrate that 6S RNA associates with RNA polymerase in a highly specific and efficient manner. UV crosslinking experiments revealed that 6S RNA directly contacts the σ^{70} and β/β' subunits of RNA polymerase. 6S RNA accumulates as cells reach the stationary phase of growth and mediates growth phase–specific changes in RNA polymerase. Stable association between σ^{70} and core RNA polymerase in extracts is only observed in the presence of 6S RNA. We show 6S RNA represses expression from a σ^{70}-dependent promoter during stationary phase. Our results suggest that the interaction of 6S RNA with RNA polymerase modulates σ^{70}-holoenzyme activity.

Introduction

Small RNAs (sRNAs) in *E. coli* were first described in 1967. To date, more than ten sRNAs in addition to tRNAs and rRNAs are known to be encoded by the *E. coli* genome (reviewed in Wassarman et al., 1999). These stable, abundant, untranslated sRNAs act by multiple mechanisms utilizing RNA–RNA basepairing, RNA–protein interactions, and intrinsic RNA activity. The sRNAs regulate such diverse cellular functions as RNA processing, mRNA stability, translation, protein stability, and secretion. Interestingly, however, the function of the first sequenced sRNA (Brownlee, 1971), *E. coli* 6S RNA, has been unknown.

The 6S RNA was first detected as an abundant RNA on polyacrylamide gels of in vivo labeled total RNA (Hindley, 1967). It is transcribed as part of a dicistronic message that contains the gene encoding 6S RNA (*ssrS*) at the 5′ end and an open reading frame (*ygfA*) at the 3′ end (Hsu et al., 1985). The mechanism of processing the mature, 184 nt 6S RNA from its precursor has not been characterized. The function of the putative YgfA protein also is not known, although the deduced amino acid sequence exhibits some similarity to 5,10-methenyltetrahydrofolate synthetase of higher eukaryotes. The lack of a reported phenotype for either 6S RNA null mutants (Lee et al., 1985) or 6S RNA overexpression (Hsu et al., 1985) has precluded finding a function for the 6S RNA. Here, we show that the 6S RNA forms a complex with RNA polymerase.

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E. coli RNA polymerase (RNAP) is a multisubunit enzyme that also has been under investigation for several decades. RNAP exists in two major forms. Core RNAP contains α, β, and β' subunits and carries out transcription elongation. The holoenzyme form (core + σ subunit) is required for promoter recognition and transcription initiation. The σ subunit confers promoter binding and specificity to RNAP; thus, the regulated exchange of different σ factors is one critical mode of transcriptional regulation (reviewed in Gross et al., 1992; Helmann, 1994; Ishihama, 1999). There are seven known σ subunits in *E. coli*; σ^{70} is essential for normal growth, σ^S is utilized during stationary phase as well as other environmental stresses, and the other σ subunits are used under various growth conditions. The activity of individual σ subunits is often regulated at the level of expression. Differential affinity for core RNAP binding also contributes to differential utilization of each σ subunit. In addition, anti-sigma factors regulate σ activity, in part, by binding to free σ subunit and preventing its association with core RNAP (reviewed in Hughes and Mathee, 1998; Helmann, 1999).

Gram-negative bacteria enter stationary phase upon nutrient limitation (reviewed in Hengge-Aronis, 1999; Ishihama, 1999). Stationary phase cells undergo morphological and physiological changes which impart the ability to survive prolonged starvation and to resist many environmental stresses. σ^S, encoded by the *rpoS* gene, is an important regulator during the transition from exponential to stationary phase as well as for the maintenance of stationary phase. σ^S levels are at least three times lower than σ^{70} levels in stationary phase (Jishage and Ishihama, 1995), and σ^S affinity for core polymerase is significantly lower than σ^{70} affinity for the core enzyme (Kusano et al., 1996). Nevertheless, σ^S is preferentially utilized over σ^{70} during stationary phase. Hence, the relative utilization of σ^S and σ^{70} must be regulated to maintain appropriate gene expression during stationary phase. Here, we show that 6S RNA binds to the σ^{70}-holoenzyme form of RNAP and acts to reduce its activity in stationary phase.

Results

6S RNA Cosediments with RNAP

Gradient fractionation previously showed that 6S RNA in *E. coli* cell extracts migrates at ~11S while deproteinized 6S RNA migrates at 6S (Lee et al., 1978). The larger apparent size of 6S RNA in extracts suggests the sRNA binds to other molecules. To elucidate 6S RNA function, we set out to identify 6S RNA interacting factors. Extracts from late exponential phase *E. coli* K12 cells were fractionated on glycerol gradients. Total RNA was isolated from each gradient fraction and analyzed by Northern hybridization to determine the location of 6S RNA in the gradient. As shown in Figure 1A, 6S RNA is found predominantly in a peak which most likely corresponds to the previously reported 11S form (lanes 11–14). A second, minor peak of 6S RNA is found near the top of the gradient (lanes 19–20).

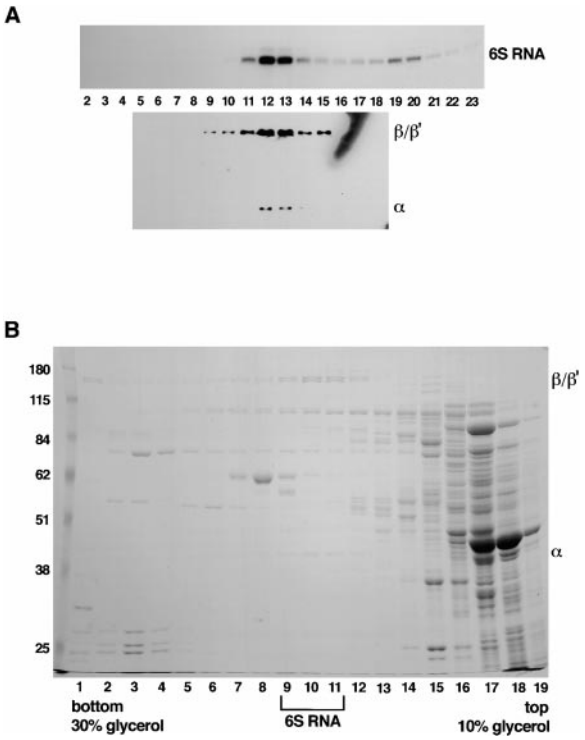

Figure 1. 6S RNA Cofractionates with RNAP in Glycerol Gradients

(A) 100 μl of extract from K12 cells at OD_{600} = 0.8 was separated on a 10%–30% glycerol gradient (24 fractions). Each fraction was divided and analyzed by Northern analysis for 6S RNA (top panel) or Western analysis with core antibodies (bottom panel).

(B) 200 μl of extract from K12 cells at OD_{600} = 0.6 was separated on a 10%–30% glycerol gradient (20 fractions). Total protein was precipitated with TCA followed by analysis on 9% SDS-PAGE and staining with Coomassie brilliant blue. Approximate molecular weights are from prestained protein markers (Life Technologies).

Total protein from glycerol gradient fractions was analyzed (Figure 1B). The composition of the 11S region was surprisingly simple. We detected an ~40 kDa protein that fractionated with a peak profile similar to 6S RNA (lanes 9–11 and data not shown). Mass spectrometry analysis identified this protein as the α subunit of RNA polymerase (data not shown). A doublet of ~150 kDa comigrated with the 6S RNA; the molecular weight of these proteins suggested the presence of β and β′ subunits of RNA polymerase. We confirmed the identity of the large RNAP subunits by Western analysis using an antiserum which recognizes core RNAP subunits (Figure 1A, bottom panel). Thus, 6S RNA cofractionates with the α, β, and β′ subunits of RNAP.

Lee et al. (1978) described the 6S RNA complex as 11S. RNAP has been described as a 14S–15S complex (Iwakura et al., 1974), but RNAP sedimentation behavior is dependent on many variables including ionic strength (Burgess, 1971). We clearly observe comigration of 6S RNA and RNAP and will refer to this region of the gradient as 11S.

6S RNA Coimmunoprecipitates with RNAP

To determine whether cofractionation on glycerol gradients represents a physical interaction between the 6S RNA and RNAP, we carried out immunoprecipitation experiments with antibodies recognizing core RNAP subunits. 6S RNA coimmunoprecipitates with RNAP-specific antibodies, but not with control antibodies or protein A Sepharose alone (Figure 2A, compare lanes 6–8 with lanes 3–5). Binding of 6S RNA to RNAP is very efficient. Under conditions in which RNAP is effectively depleted from extracts, represented by a reduction of RNAP from supernatants, the 6S RNA is also substantially diminished from the supernatants (Figure 2A, lane 14).

Next, we determined the specificity of 6S RNA binding to RNAP by evaluating the binding of another abundant small RNA, 5S rRNA. No 5S rRNA was detected upon immunoprecipitation of RNAP (Figure 2A). To further assess the specificity of the 6S RNA–RNAP interaction, total immunoprecipitated RNA was radiolabeled at the 3′-end so all RNA species could be visualized (Figure 2B). 6S RNA is the predominant RNA immunoprecipitated with RNAP antibodies (Figure 2B, lanes 5–7). No RNAs are specifically immunoprecipitated with core antibodies from *ssr1* mutant cells which lack 6S RNA (data not shown). Thus, 6S RNA binding to RNAP is highly specific.

The antibodies used above immunoprecipitate core enzyme as well as RNAP holoenzyme (D. Jin, personal communication; also see Figure 2A). To determine whether 6S RNA associates with core RNAP or holoenzyme, we carried out coimmunoprecipitation experiments with antibodies specific for the σ^{70} subunit. 6S RNA efficiently and specifically coimmunoprecipitates with σ^{70}-specific antibodies (data not shown, see below). Together with the glycerol gradient comigration at 11S, these data strongly support an interaction of the 6S RNA with the σ^{70}-holoenzyme form of RNAP, but do not preclude possible 6S RNA interactions with core RNAP as well, or with other forms of RNAP.

To determine if 6S RNA is present in purified RNAP preparations, we carried out the first steps of the standard biochemical procedure for RNAP purification (Burgess and Jendrisak, 1975). The 6S RNA and RNAP coprecipitate with polyethylenimine, but only trace amounts of 6S RNA are recovered together with RNAP from the pellet along with similar amounts of 5S rRNA (data not shown). Thus, specific 6S RNA association with RNAP is lost in the first step of the standard RNAP purification.

RNAP Coselection with a 6S RNA Antisense Oligonucleotide

To purify 6S RNA-bound RNAP, we employed an RNA antisense selection approach. We were not able to target the endogenous *E. coli* 6S RNA with antisense oligonucleotides. Secondary structure predictions (Figure 3A) and the observation that 6S RNA in extracts is relatively resistant to nuclease digestion (data not shown) suggest the *E. coli* 6S RNA is highly structured and does not contain single-stranded regions accessible for binding an exogenous oligonucleotide. However, there is a 6S-like RNA in the genomic sequence of *Haemophilus influenzae* (Brosius, 1996) that has an insertion of 13 nt at the end of the predicted stem of the *E. coli* 6S RNA (Figure 3B). We targeted this region with a biotinylated

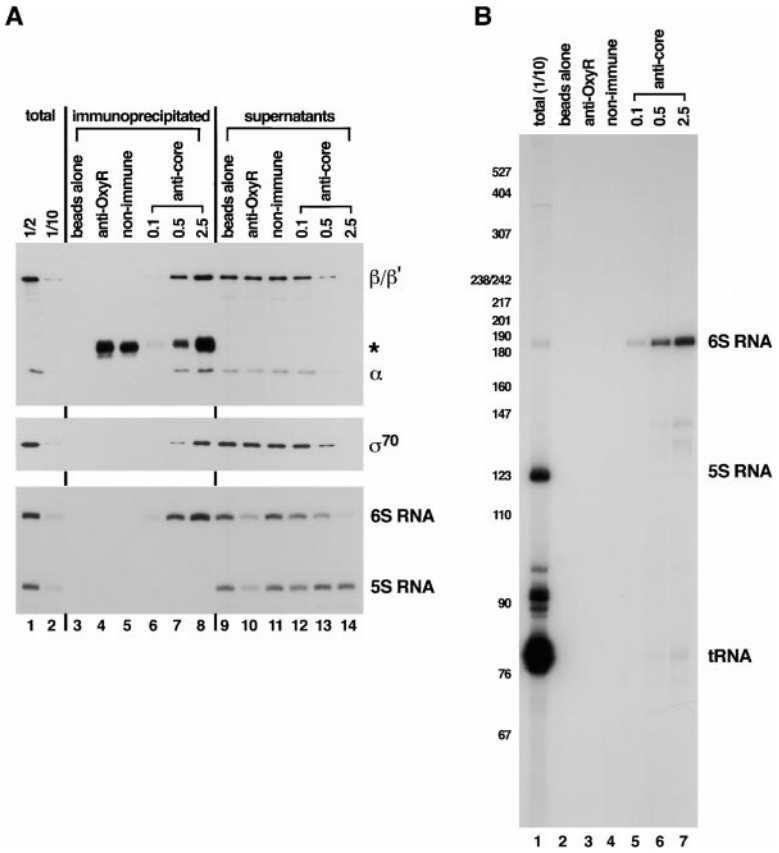

Figure 2. 6S RNA Coimmunoprecipitates with RNAP

(A) Immunoprecipitation using an extract from K12 cells after 24 hr of growth. Lanes 3–8 contain selected samples and lanes 9–14 are 1/2 the total supernatant after selection. Immunoprecipitations were done using no antibody (lanes 3 and 9), 2.5 μl of OxyR antisera (lanes 4 and 10), 2.5 μl of nonspecific rabbit serum (lanes 5 and 11), or 0.1, 0.5, or 2.5 μl of core antisera (lanes 6 and 12, 7 and 13, or 8 and 14, respectively). Lanes 1 and 2 contain total extract equivalent to 1/2 or 1/10 the amount used in immunoprecipitations. Top panel is Western analysis with core antibodies. Middle panel is Western analysis with σ^{70} antibodies. Bottom panel is Northern analysis using 6S RNA and 5S rRNA specific probes. Asterisk (*) indicates antibody heavy chain from the immunoprecipitation.

(B) Immunoprecipitated RNA using no antibody (lane 2), 2.5 μl of OxyR antisera (lane 3), 2.5 μl of nonspecific rabbit serum (lane 4), or 0.1, 0.5, or 2.5 μl of core antisera (lanes 5, 6, or 7, respectively) was 3′-end labeled and examined on an 8% polyacrylamide denaturing gel. Lane 1 contains total 3′-end labeled RNA from 1/10 the amount of extract used in selection. 5′-end labeled MspI-digested pBR322 DNA was used as molecular weight size markers.

antisense oligonucleotide (Hi6SA oligo) and were able to efficiently select the *H. influenzae* 6S RNA after expression in *E. coli* (data not shown, see below). The *H. influenzae* RNA coimmunoprecipitates with RNAP antibodies (data not shown), binds to RNAP (see below), and is able to rescue 6S RNA null phenotypes (discussed below), indicating that *H. influenzae* 6S RNA is a functional homolog of the *E. coli* 6S RNA.

To determine whether other proteins are present in the 6S RNA–RNAP complex, we carried out antisense selection via the *H. influenzae* 6S RNA. Stationary phase *ssr1* cells expressing the *H. influenzae* 6S RNA were labeled with ^{35}S. Extracts then were used for antisense selection (Figure 3C). α, β, β′, and σ^{70} subunits of RNAP are specifically selected with the Hi6SA oligo from cells containing the *H. influenzae* 6S RNA, demonstrating that the 6S RNA–RNAP complex can be selected through the RNA (Figure 3C, compare lanes 4 and 7). Western analysis confirmed the identities of α, β, β′, and σ^{70} subunits and revealed that at least 30% of α, β, and β′ and 50%–75% of σ^{70} could be selected via the *H. influenzae* 6S RNA (data not shown and Figure 3D). The presence of σ^{70}, α, β, and β′ confirms 6S RNA binds to the σ^{70}-holoenzyme.

A few other proteins are seen after selection, but they also are present with an unrelated oligonucleotide (OxyS oligo) and from cells that do not contain 6S RNA, indicating they are nonspecific (Figure 3C, see lanes 4, 5, and 8). The lack of other specific bands suggests there are no other proteins present at stoichiometric levels in the 6S RNA–RNAP complex. To directly examine whether 6S RNA interacts with RNAP associated with the stationary phase sigma factor, σ^S, Hi6SA oligo-selected samples were probed with a σ^S antibody (Figure 3D). Selection was done from extracts of stationary phase *ssr1* cells expressing the *H. influenzae* 6S RNA (Figure 3D, lane 4) or no 6S RNA (Figure 3D, lane 3). No σ^S was detected after selection under conditions in which significant levels of σ^{70} are selected. Together, these results indicate the interaction between 6S RNA and σ^{70}-holoenzyme is specific and direct.

6S RNA Crosslinks to RNAP

We used a crosslinking approach to analyze which subunit of RNAP is binding 6S RNA directly. Extracts from stationary phase *ssr1* cells expressing *H. influenzae* 6S RNA were irradiated with UV light, and the 6S RNA–RNAP complex was selected using the Hi6SA oligo. Crosslinked proteins were visualized by radiolabeling of bound RNA (Figure 3E). As controls, extracts from *ssr1* cells, which do not contain any 6S RNA (lanes 1 and 2), and extracts that had not been UV irradiated (lanes 1 and 3) also were examined. Three 6S RNA-dependent crosslinked bands (lane 4) and one 6S RNA-independent crosslinked band (lane 2, marked by asterisk) were detected. The largest band was sometimes observed in the absence of 6S RNA, but always was strongly enhanced by 6S RNA (Figure 3E and data not shown). The two smaller bands were absolutely dependent on the 6S RNA (Figure 3E, compare lanes 2 and 4). The sizes of the crosslinked proteins suggest the larger band corresponds to β/β′ and the smaller band to σ^{70}.

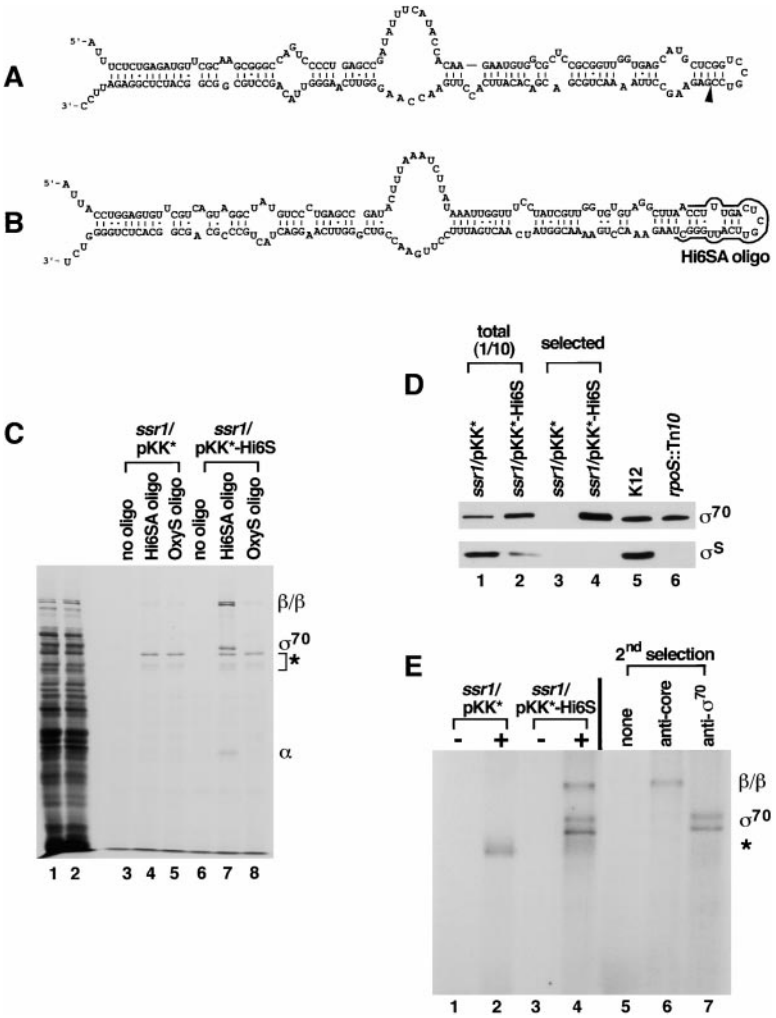

Figure 3. 6S RNA Crosslinks to RNAP

(A) *E. coli* 6S RNA sequence in a computer predicted secondary structure (modified from Vogel et al., 1987). Arrowhead indicates location of insertion in *H. influenzae* 6S RNA.

(B) *H. influenzae* 6S RNA sequence in an analogous secondary structure. The 5′ and 3′ ends of the *H. influenzae* 6S RNA have been estimated by comparison to the *E. coli* 6S RNA. The bar represents region complementary to the Hi6SA oligo.

(C and D) Antisense selection of RNAP. (C) Extracts from ^{35}S-labeled *ssr1* cells carrying the control vector pKK* (lanes 1 and 3–5) or the *H. influenzae* 6S RNA expressing vector pKK*-Hi6S (lanes 2 and 6–8) were incubated with no oligonucleotide (lanes 3 and 6), Hi6SA oligo (lanes 4 and 7), or OxyS oligo (lanes 5 and 8). Lanes 1 and 2 contain total protein from 1/10 the amount of extract used in selections. (D) Extracts from *ssr1* cells carrying the control vector pKK* (lanes 1 and 3) or the *H. influenzae* 6S RNA expressing vector pKK*-Hi6S (lanes 2 and 4) were used for selection with the Hi6SA oligo. Selected samples (lanes 3 and 4), total protein from 1/10 the amount of extract used in selection (lanes 1 and 2), or protein from K12 or *rpoS*::Tn*10* cells were analyzed by Western hybridization with σ^{70} antisera (top panel) or σ^{S} 1RS1 antibody (bottom panel).

(E) UV crosslinking followed by antisense selection and immunoprecipitation. Extracts from *ssr1* cells carrying the control vector pKK* (lanes 1 and 2) or *ssr1* cells expressing the *H. influenzae* 6S RNA from pKK*-Hi6S (lanes 3–7) were UV irradiated (lanes 2 and 4–7) or not irradiated (lanes 1 and 3), and antisense selected using the Hi6SA oligo. Samples were RNase treated, radioactively labeled, and analyzed on a 9% SDS PAGE directly (lanes 1–4) or submitted to heat denaturation and a second selection (lanes 5–7). Second selection was immunoprecipitation with no antibody (lane 5), core antisera (lane 6), or σ^{70} antisera (lane 7). Asterisk (*) indicates nonspecific bands in (C) and (E).

To confirm the identity of the crosslinked proteins, they were subjected to a second selection by immunoprecipitation with core or σ^{70} antibodies. Samples were denatured prior to the second selection such that proteins are immunoprecipitated only through direct interaction with antibody and not through higher order complexes. The largest band is immunoprecipitated by core RNAP antibodies confirming it contains β and/or β′ (Figure 3E, lane 6). The smaller band is immunoprecipitated with σ^{70} antibodies confirming it is σ^{70} (Figure 3E, lane 7). Interestingly, the middle size band also contains σ^{70} (Figure 3E, lane 7). The difference in apparent size of the two bands corresponding to σ^{70} could be due to crosslinking to different size RNA fragments remaining after the RNase treatment during the labeling procedure, or due to formation of multiple crosslinks between σ^{70} and 6S RNA.

To ensure that observed crosslinks were not unique to the plasmid-encoded *H. influenzae* 6S RNA, crosslinking experiments also were carried out with extracts from wild-type K12 cells. Very similar patterns of crosslinked bands were immunoprecipitated with core antibodies from wild-type cells expressing endogenous *E. coli* 6S RNA and cells expressing *H. influenzae* 6S RNA, but not from *ssr1* cells that do not express 6S RNA (data not shown).

To confirm that 6S RNA is the RNA component in each crosslinked species, UV-irradiated extracts from *ssr1* cells expressing the *H. influenzae* 6S RNA were heated in the presence of SDS prior to selection with the Hi6SA oligo (see Figure 3E). Any proteins selected under these conditions must be directly crosslinked to a 6S RNA molecule. The same three 6S RNA-dependent crosslinks were selected (data not shown). In addition, experiments in which UV irradiation was performed after immunoprecipitation, under conditions when the 6S RNA is the only detectable RNA selected (see Figure 2B), yielded the same three crosslinked species (data not shown). Therefore, 6S RNA crosslinks directly to σ^{70} and β/β′.

6S RNA Accumulates throughout Growth

To determine when the 6S RNA may be acting, we quantitated the levels of 6S RNA during different stages of growth. The levels of 6S RNA and 5S rRNA from K12

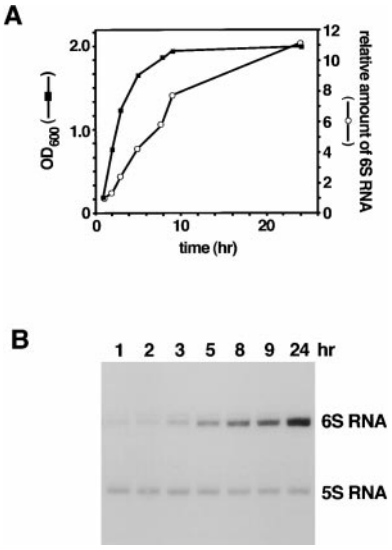

Figure 4. 6S RNA Accumulates with Growth

(A) Wild-type K12 cells were diluted 1:100 in LB medium and grown at 37°C for times indicated.

(B) RNA was isolated from an equal number of OD_{600} units, and 5 μg of RNA at each time point was analyzed by Northern hybridization using probes specific for 6S RNA and 5S rRNA. 6S RNA and 5S rRNA signals were quantitated, 6S RNA signal normalized to 5S rRNA signal, and 6S RNA levels are given relative to the 1 hr time point.

cells at different times after dilution in LB medium were examined by Northern hybridization analysis (see Figure 4). 5S rRNA was used as an internal reference to correct for efficiencies of RNA isolation and gel loading. The relative amount of 6S RNA at each time point was calculated as the ratio of 6S RNA to 5S rRNA signal and then normalized to the 1 hr time point. Clearly, 6S RNA accumulates significantly from 1 to 24 hr after dilution (Figure 1B, compare lanes 1 and 7). By comparison to in vitro transcribed RNA, we estimate 6S RNA levels to change from 1,000 to 10,000 copies per cell (see Experimental Procedures and data not shown). The accumulation of 6S RNA in stationary phase does not change in an *rpoS*::Tn*10* mutant background indicating the *ssr1* promoter is not regulated by σ^S (data not shown).

6S RNA Is Required for RNAP Changes throughout Growth

We next examined the 6S RNA–RNAP complex from wild-type K12 cells throughout growth. The vast majority of 6S RNA migrates in the 11S form from 1.5 to 72 hr after dilution in LB medium even as 6S RNA is accumulating during stationary phase (see Figure 1A and data not shown). In contrast to the unchanging 6S RNA profile, we observe a shift in migration of RNAP at different phases of growth (Figure 5A). During exponential growth (2 hr), some RNAP is in the 11S region of the gradient, but most RNAP is in heavier, heterologous complexes. As cells progress into stationary phase (6 hr), increasing amounts of RNAP are found in the 11S region of the gradient. By 24 hr of growth, most of the RNAP is present at 11S. The proportion of RNAP present in the 11S region of the gradient correlates with 6S RNA levels (see Figure 4).

Glycerol gradient fractionation of extracts from *ssr1* cells revealed 6S RNA is essential for the changes in sedimentation behavior of RNAP during stationary phase (Figure 5A). Specifically, RNAP in *ssr1* cells is present predominantly in the heavy form through all phases of growth and does not shift appreciably to the 11S form in stationary phase. Expression of either *E. coli* or *H. influenzae* 6S RNA from a plasmid in *ssr1* cells is able to rescue normal RNAP migration on gradients (data not shown). Therefore, 6S RNA is not only present in a complex with RNAP, but mediates growth-dependent changes in the physical properties of RNAP.

The size and heterogeneity of the heavy RNAP form suggested it may be bound to DNA. Extracts from exponentially growing wild-type cells were treated with DNase I prior to fractionation on glycerol gradients. The majority of core RNAP subunits were shifted to the 11S peak of RNAP, strongly supporting the conclusion that the heavy form of RNAP is DNA bound (data not shown).

We also analyzed the fractionation pattern of σ^{70} in wild-type cells (data not shown). At all stages of growth, the majority of σ^{70} sediments in the 11S region of the gradient indicating σ^{70}-holoenzyme migrates at 11S. Some σ^{70} is in a lighter peak that does not contain other RNAP subunits. The free σ^{70} and the light form of 6S RNA both migrate near the top of the gradients, but their peaks do not coincide. Thus, we do not detect an interaction of 6S RNA and free σ^{70} (data not shown).

6S RNA Is Required for Stable σ^{70} Association with Core RNAP in Extracts

Gradient fractionation revealed that the amount of free σ^{70} subunit is increased in *ssr1* cells at all times examined (data not shown). Even more dramatic 6S RNA-dependent changes in levels of RNAP-bound σ^{70} were seen in immunoprecipitation experiments. No σ^{70} subunit is detected when core antibodies were used to immunoprecipitate RNAP from *ssr1* cells (Figure 5B, lanes 4–6). In wild-type cells, there is a substantial increase in σ^{70} coimmunoprecipitation with core RNAP in stationary phase compared to exponential growth, coincident with increased levels of 6S RNA during stationary phase (Figure 5B, lanes 1–3). Therefore, stable association of σ^{70} with RNAP is only observed in the presence of high levels of 6S RNA that normally occur in stationary phase.

6S RNA Inhibits σ^{70}-Holoenzyme Activity during Stationary Phase

Since 6S RNA interacts specifically with σ^{70}-holoenzyme, and since 6S RNA is required for observed changes in RNAP sedimentation during stationary phase, we examined whether 6S RNA alters utilization of σ^{70}-holoenzyme compared to the stationary phase specific σ^S-holoenzyme. To quantitate σ^{70} RNAP activity relative to σ^S RNAP activity, we analyzed transcription of the endogenous *rsd* gene using an RNase protection assay. During stationary phase, the *rsd* gene is transcribed from two promoters: one that requires σ^S (P1) and a second that requires σ^{70} (P2) (Jishage and Ishihama, 1999) (see Figure 6A). In wild-type cells (K12), both P1 and P2 are active during stationary phase (Figure 6B,

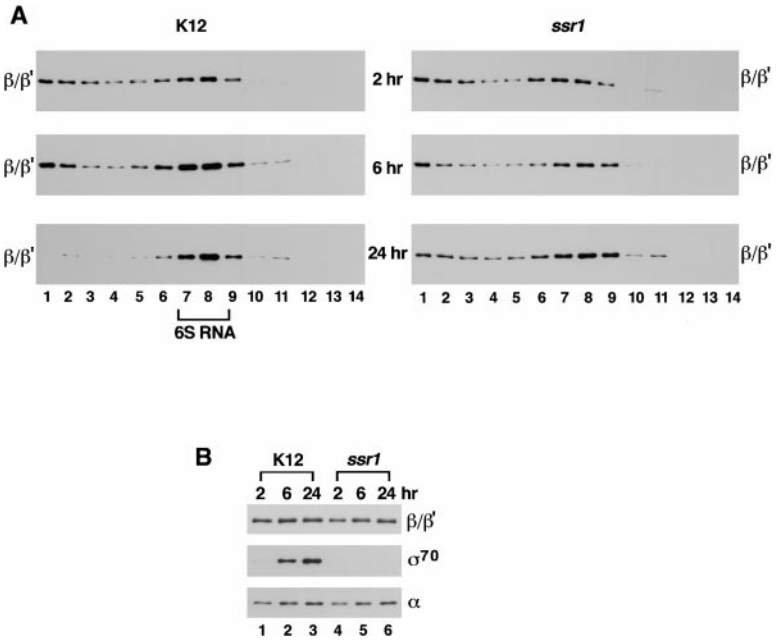

Figure 5. 6S RNA Mediates Growth Phase–Specific Changes in RNAP

(A) Gradient fractionation (15 fractions) using 100 μl of extract from K12 or *ssr1* cells grown at 37°C for 2 (top panel, OD_{600} = 0.5), 6 (middle panel, OD_{600} = 2.0), or 24 (bottom panel, OD_{600} = 2.0) hr after dilution. The 11S peak of 6S RNA was located in fractions 7–9 as determined by Northern analysis (data not shown). 6S RNA-dependent changes in RNAP sedimentation throughout growth were reproducible and observed in four independent experiments.

(B) Immunoprecipitation with core antibodies using extracts from K12 cells (lanes 1–3) or *ssr1* cells (lanes 4–6) after growth at 37°C for 2 (lanes 1 and 4), 6 (lanes 2 and 5), or 24 (lanes 3 and 6) hr. Western analysis was done using core antibodies (A and B) and σ^{70} antibodies (B).

lane 1). In *rpoS*::Tn*10* mutant cells lacking σ^S, transcription from P1 is nearly eliminated (data not shown). In cells without 6S RNA (*ssr1*), utilization of P2 is increased while there is no change in use of P1, suggesting σ^{70}-holoenzyme is more active (Figure 6B, lane 2). We also constructed plasmids to examine the effects of 6S RNA overexpression. pKK*-6S and pKK*-6S+Y express the wild-type 6S RNA approximately 2-fold and 10-fold above endogenous levels, respectively, at 12 and 24 hr of growth (data not shown). A pKK*-6S+Y derivative (M5) in which nucleotides 42 to 58 were replaced with a CAC trinucleotide was generated as a control. The mutant 6S RNA expressed from pKK*-6S+Y(M5) is present at levels at least 10-fold higher than normal at 12 and 24 hr of growth and does not affect σ^{70} binding to core RNAP in extracts (data not shown). RNase protection assays of *rsd* transcription revealed that moderate to high overexpression of wild-type 6S RNA from pKK*-6S and pKK*-6S+Y results in somewhat decreased transcription of P2 relative to P1, while high overexpression of the inactive mutant 6S RNA from pKK*-6S+Y(M5) has no affect (data not shown). Together, these results show that 6S RNA alters the utilization of the σ^{70} promoter relative to the σ^S promoter at *rsd*.

Overexpression of 6S RNA Decreases Viability

The experiments described above indicate that 6S RNA functions during stationary phase at a time when σ^S is important for cell survival. To test whether a physiological phenotype for the absence of 6S RNA could be uncovered in strains lacking σ^S, we constructed *rpoS*::Tn*10* mutants carrying the *ssr1* mutation. Comparison of *ssr1 rpoS*::Tn*10* with *rpoS*::Tn*10* cells revealed no differences in growth or viability in LB or M63 minimal media during exponential growth, stationary phase, or recovery from stationary phase (data not shown). We also transformed the *rpoS*::Tn*10* mutant with pKK*-6S and pKK*-6S+Y and determined the number of viable cells after 12 or 24 hr of growth in LB (Figure 7). For the *rpoS*::Tn*10*/pKK*-6S+Y strain, which highly overexpresses 6S RNA, there is a substantial decrease in viability after 24 hr and a partial decrease after 12 hr. Moderate overexpression of 6S RNA in *rpoS*::Tn*10*/pKK*-6S results in a decrease in viability after 24 hr of growth. High overexpression of the inactive 6S RNA mutant from pKK*-6S+Y(M5) did not cause a decrease in viability at 12 or 24 hr of growth (data not shown). The pKK*-6S+Y construct contains the open reading frame (*ygfA*) normally encoded downstream of 6S RNA. To eliminate any phenotype of potential YgfA overexpression, two mutant plasmids were generated that disrupt translation of *ygfA*. pKK*-6S+Y(M1) has a mutation in the reported *ygfA* translational start codon (Hsu et al., 1985), and pKK*-6S+Y(M2) contains a stop codon replacing another ATG codon 96 nt downstream. Both mutant constructs maintain high overexpression of 6S RNA and lead to the same decrease in viability as pKK*-6S+Y (data not shown). Thus, wild-type 6S RNA, but not mutant 6S RNA overexpression leads to reduced viability of σ^S-deficient cells during stationary phase.

Discussion

We have found that 6S RNA interacts with σ^{70}-RNAP holoenzyme. The 6S RNA–RNAP interaction is highly efficient; most of 6S RNA is bound to RNAP. The 6S RNA–RNAP interaction is also extremely specific; 6S RNA is the only RNA which is stably bound to RNAP. UV crosslinking experiments revealed that 6S RNA contacts the σ^{70} and β/β' subunits. We also found that 6S RNA accumulates in stationary phase cells and that 6S RNA mediates growth phase specific alterations in RNAP. Most intriguingly, stable association of σ^{70} with core enzyme in extracts is only observed in the presence of high levels of 6S RNA . Finally, we have demonstrated

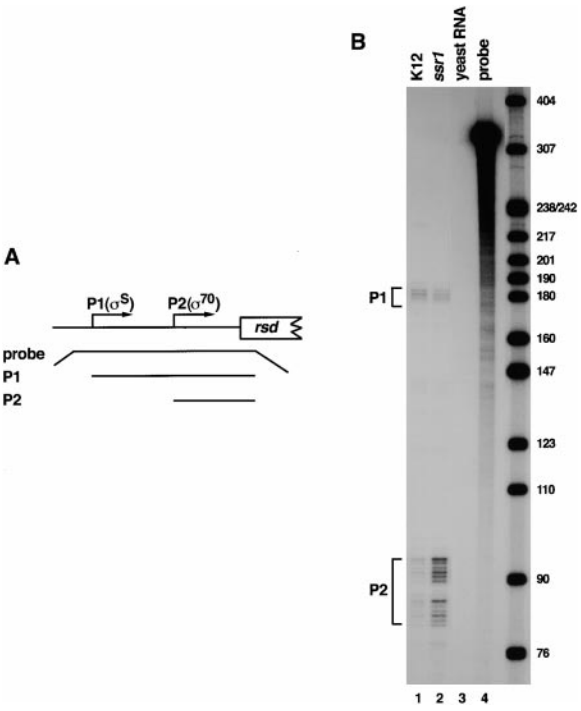

Figure 6. Utilization of a σ^{70} Promoter Is Altered in the Absence of 6S RNA

(A) Schematic diagram of *rsd* gene, probe used for RNase protection assay, and expected protected fragments.

(B) RNase protection to analyze *rsd* mRNA from 50 μg of total RNA isolated from K12 (lane 1) or *ssr1* (lane 2) cells, or yeast (lane 3). Lane 4 contains 1/50 of input probe. 5′-end labeled MspI-digested pBR322 DNA was used as molecular weight size markers. Change in relative utilization of P1 and P2 in *ssr1* cells compared to K12 cells was seen in five experiments.

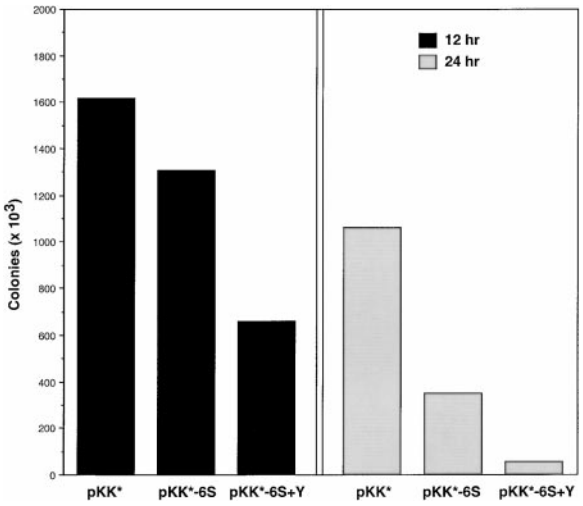

Figure 7. Overexpression of 6S RNA Causes Reduced Viability

rpoS::Tn*10* cells containing a control vector (pKK*) or 6S RNA overexpression vectors (pKK*-6S and pKK*-6S+Y) were grown for 12 hr (black) or 24 hr (gray) followed by dilution and plating on LB-agar plates. Experiment was repeated six times; a typical data set is shown. 6S RNA overexpression at all times examined also resulted in the appearance of smaller colonies at 1%–10% frequency.

that 6S RNA modulates σ^{70} promoter usage in stationary phase.

Our discovery that 6S RNA binds to and modifies RNAP activity is especially surprising in light of the fact that *E. coli* RNAP has been the focus of extensive research for over 30 years. We suggest that the 6S RNA was never detected in vitro because most experiments were carried out with RNAP which was purified or reconstituted under conditions that would exclude 6S RNA. Given our findings, it is worth considering whether additional sRNAs have been missed by standard purification schemes of other enzymatic activities.

6S RNA Interaction with RNAP

Most of the 6S RNA is in the 11S peak in gradients, and we are able to coimmunoprecipitate the majority of the 6S RNA with RNAP antibodies, indicating that nearly all 6S RNA is bound to RNAP. Up to 75% of the σ^{70} from stationary phase cells can be selected with an antisense oligonucleotide directed against 6S RNA, indicating most of the σ^{70}-holoenzyme is bound by 6S RNA. We estimate there are up to 10,000 copies of 6S RNA per cell during stationary phase. The RNAP core enzyme has been reported to be present in 1000 to 3000 copies per cell (Ishihama, 1991) and σ^{70} is estimated to be present in 700 copies per cell (Jishage and Ishihama, 1995). These numbers suggest 6S RNA is in excess of the potential σ^{70}-holoenzyme molecules and is even in surplus over core molecules. However, the number of molecules per cell, at best, are estimations, and even 2-fold deviations in the numbers could give an equimolar ratio between 6S RNA and RNAP. It also is possible more than one 6S RNA molecule is binding each σ^{70}-holoenzyme, raising the question of whether multiple 6S RNA molecules and contacts are required for the changes in RNAP.

We have observed 6S RNA crosslinking to σ^{70} and to β/β'. Since UV irradiation only can crosslink molecules across short distances, the 6S RNA is likely to be in direct contact with σ^{70} and also β/β'. We suggest several potential models for 6S RNA interaction with the σ^{70}-holoenzyme. 6S RNA may bind RNAP in a manner analogous to DNA promoter binding to RNAP. The proposed 6S RNA structure is considerably double-stranded in nature with a central bulged, single-stranded region that is reminiscent of an "open complex" found during transcription initiation. If 6S RNA binding to σ^{70}-holoenzyme is similar to DNA binding, simple competition between 6S RNA and promoter DNA for binding RNAP may account for inhibition of σ^{70}-holoenzyme activity during stationary phase. Alternatively, 6S RNA may bind σ^{70}-holoenzyme differently than promoter DNA. This type of interaction could change RNAP conformation so that the enzyme can no longer efficiently interact with promoter DNA or carry out a downstream event in transcription. In addition, 6S RNA may have more than one mode of binding and even may undergo a conformational change upon binding to RNAP.

6S RNA-Dependent Changes in RNAP

In exponentially growing wild-type cells, the major form of RNAP is bound to DNA and probably represents elongation complexes. During stationary phase in *ssr1* cells,

the preponderance of RNAP is also DNA bound. The nature of these complexes is not known. The molecules may be legitimate elongation complexes. If so, a substantial increase in overall transcription might be expected from such a dramatic increase in elongation complexes. However, we do not observe an increase in total protein levels in *ssr1* mutant cells (K. M. W. and G. S., unpublished data). The DNA-bound RNAP also could represent arrested or terminating complexes that are not actively transcribing. In both of these scenarios, the observed increase in free σ^{70} would be a direct consequence of a lack of free core enzyme.

Alternatively, the DNA-bound RNAP in *ssr1* cells could represent forms of RNA polymerase that are not transcription intermediates. Core RNAP is known to bind DNA nonspecifically. If the σ^{70}-core interaction is destabilized in the absence of 6S RNA, the resulting free core enzyme could bind to DNA. In this model, the observed binding of core RNAP to DNA would be a result of decreased association of σ^{70} with core. The direct cause of this potential σ^{70} displacement from the holoenzyme in our *ssr1* extracts is not known. Unstructured polyribonucleotides (such as rU_n), ssDNA, and tRNA have been reported to induce dissociation of σ^{70} from holoenzyme in purified systems, although only at salt concentrations below those used here (Krakow and von der Helm, 1970; Spassky et al, 1979). Our extracts contain the high levels of RNA, DNA, and other macromolecules present in vivo. Thus, it is possible 6S RNA protects against σ^{70} dissociation by binding to holoenyzme with a higher affinity than nonspecific RNA or DNA binding to core enzyme.

Potential Roles for 6S RNA

More needs to be learned about the physiological function of the 6S RNA–RNAP interaction. We propose three potential 6S RNA roles which are not mutually exclusive. First, the 6S RNA interaction with RNAP may contribute to altered promoter utilization during stationary phase. It is not understood how σ^S is preferentially utilized over σ^{70} during stationary phase. The 6S RNA–RNAP interaction could lead to a general decrease in σ^{70}-dependent transcription. A 10-fold decrease in overall σ^{70}-dependent transcription has been reported to occur upon entry into stationary phase without changes in σ^{70} levels (Ishihama, 1991; Jishage and Ishihama, 1995). 6S RNA binding to σ^{70}-holoenzyme also could lead to differential promoter recognition such that some σ^{70}-dependent promoters are preferred over others. As a consequence of decreased or modified σ^{70}-holoenzyme activity, the 6S RNA could affect the relative utilization of σ^{70}- and σ^S-dependent promoters. Our RNase protection assays support a 6S RNA role in regulating the expression from the σ^{70}-dependent *rsd* P2 promoter, and preliminary results from a genetic screen suggest other genes may be modulated by 6S RNA during stationary phase (K. M. W., B. L. Greenberg, and G. S., unpublished data). The observation that 6S RNA overexpression has a deleterious affect on the survival of stationary phase cells also is consistent with the model that 6S RNA alters σ^{70}-dependent promoter utilization. 6S RNA overexpression may exaggerate the inhibition of σ^{70}-holoenzyme activity, which may lead to abnormally low expression of at least some σ^{70}-dependent genes in stationary phase. In the absence of σ^S-holoenzyme-dependent transcription in *rpoS*::Tn*10* mutant cells, this reduction in σ^{70}-holoenzyme transcription results in reduced viability.

Second, the 6S RNA interaction with RNAP may be a mechanism for storing σ^{70}-holoenzyme during starvation. *E. coli* transcription has been reported to start rapidly after exposure to favorable growth conditions, implying a mechanism for immediate release of active σ^{70}-holoenzyme (Siegele and Guynn, 1996). Production of a small RNA is a relatively energy efficient method to regulate σ^{70}-holoenzyme. In addition, an RNA can be degraded readily and rapidly, making it an attractive molecule for regulating processes that need to be reversed quickly. As yet, we have not detected 6S RNA-dependent differences in the recovery of growth after stationary phase (K. M. W. and G. S., unpublished data). However, our laboratory conditions may not be sensitive to 6S RNA-mediated changes in the time frames examined.

Third, 6S RNA may affect RNAP release from DNA and possibly σ^{70} reassociation with core enzyme, directly or indirectly. This function would be required throughout growth, including in exponentially growing cells when 6S RNA levels are still greater than 1000 copies per cell. The amount of coimmunoprecipitation of σ^{70} with core RNAP is decreased in the absence of 6S RNA, even during exponential phase, although the effects of the *ssr1* mutation are more dramatic in stationary phase (see Figure 5B). A potential 6S role in RNAP recycling is supported by the observation that more RNAP remains bound to DNA in the absence of 6S RNA.

Additional experiments are needed to further elucidate the mechanism of 6S RNA action and to fully understand the role of the 6S RNA–RNAP interaction. In vitro transcription assays with purified components will greatly aid these studies, but preliminary results indicate that precautions must be taken to ensure that the in vitro system replicates the specific 6S RNA activities observed in vivo. Nucleic acids have nonspecific effects on purified RNA polymerase, and in vitro transcription systems often do not retain specific dependence on σ^{70} or σ^S. Nevertheless, further studies of the 6S RNA interaction with RNA polymerase will undoubtedly lead to increased understanding of important steps in the transcription process.

Experimental Procedures

Strains and Plasmids

Strains were grown at 37°C in Luria-Bertani (LB) medium or M63 minimal medium supplemented with 0.2% glucose and 0.002% vitamin B1 (Silhavy et al., 1984). Ampicillin (Ap) (50 μg/ml), tetracycline (10 μg/ml), or chloramphenicol (Cm) (25 μg/ml) was added where appropriate. *Escherichia coli* K12 was the parent for all strains used in this study. *rpoS*::Tn*10* (GSO15) was described previously (Altuvia et al., 1994). P1 transductions were carried out as described (Silhavy et al., 1984) to move the *ssr1* mutation (Ap^r, Lee et al., 1985) into K12 and GSO15, generating *ssr1* (GSO75) and *ssr1 rpoS*::Tn*10* (GSO76), respectively. Northern blot analysis confirmed that the 6S RNA is not expressed in GSO75 and GSO76.

All overexpression vectors marked with an asterisk (*) contained Cm resistance (Cm^r) and were constructed by insertion of Cm^r amplified by PCR from pACYC184 into a unique PvuI site in parent vectors. pKK* (pGSO84, Altuvia et al., 1997) was the control vector for all

experiments in this study and is pKK177-3 carrying Cm^r. All DNA manipulations were carried out using standard procedures. The sequence of all PCR-generated fragments was confirmed.

pKK*-6S (pGSO106)

To generate pKK-6S(Ap^r) and pKK*-6S, the 6S fragment was amplified by PCR (GGCCGAATTCATTTCTCTGAGATGTTCGCAAG and GGCCGGATCCGAATCTCCGAGATGCCGCC) from K12 genomic DNA, digested with BamHI (end-filled with T4 DNA polymerase) and EcoRI, and cloned into the HindIII (Filled in) and EcoRI corresponding sites in pKK177-3.

pKK*-6S+Y (pGSO107), pKK*-6S+Y(M1) (pGSO108), pKK*-6S+Y(M2) (pGSO109), and pKK*-6S+Y(M5) (pGSO121)

To generate pKK-6S+Y(Ap^r), pKK*-6S+Y, pKK*-6S+Y(M1), pKK*-6S+Y(M2), and pKK*-6S+Y(M5) the 6S+Y fragment was amplified by PCR (GGCCGAATTCATTTCTCTGAGATGTTCGCAAG and GGC CGAATTCAAGCTTACCACTCCCAGACTTTCGACG) from K12 genomic DNA, digested with EcoRI and HindIII, and cloned into the corresponding sites in pKK177-3. The pKK*-6S+Y(M1) (CAAGAAATCCG CAAAATATTCGGCAACGTCG and CGACGTTGCCGAATATTTTGCGG ATTTCTTG), pKK*-6S+Y(M2) (CCCGGTGGTGTGATCACATACGGTCG and CGACCGTATGTGATCACACCACCGGG), and pKK*-6S+Y(M5) (GGGCCAGTCCCCTGAGCCCACGAATGTGGCGCTCCGCGGTTGG and CCAACCGCGGAGCGCCACATTCGTGGGCTCAGGGGACTGG CCC) were generated from pKK*-6S+Y using the QuikChange site-directed mutagenesis kit (Stratagene).

pKK*-Hi6S (pGSO110) and pBS-Hi6S (pGSO116)

To construct pTA-Hi6S, the *H. influenzae* 6S sequence was amplified by PCR (GGCCGAATTCATTACCTGGAGTGTTCGTCAG and GGCCGA ATTCAAGCTTGAAGACCCCAGAGTGCCGCTG) from GHIBR82 DNA (TIGR/ATCC Microbial Genome Special Collection), and cloned into the pCR2.1 vector using the Original TA Cloning kit (Invitrogen). pTA-Hi6S was digested with EcoRI and HindIII and the 6S fragment was cloned into corresponding sites in pKK177-3 to generate pKK-Hi6S(Ap^r) and pKK*-Hi6S, or pBluescript II SK^+ to generate pBS-Hi6S.

pBS-6S (pGSO112)

To construct pBS-6S, the 6S fragment was amplified by PCR (GGCCGAATTCAAAATTTCTCTGAGATGTTCGC and GGCCGGAT CCGAATCTCCGAGATGCCGCC) from K12 genomic DNA, digested with EcoRI and BamHI and cloned into corresponding sites in pBluescript II SK^+ (Stratagene).

pBS-rsd1 (pGSO117)

To construct pTA-rsd1, the *rsd* fragment was amplified by PCR (GCAAGCTTCGAAATTTGCCCGTTCGCCATATGGC and GGTTAAG CATGATTGACTCCGC) from K12 genomic DNA and cloned into the pCR2.1 vector using the Original TA cloning kit (Invitrogen). pTA-rsd1 was digested with HindIII and EcoRI and the *rsd* fragment was cloned into corresponding sites in pBluescript II SK^+ (Stratagene) to generate pBS-rsd1.

RNA Analysis

RNA Isolation

After gradient fractionation, immunoprecipitation, or RNA-antisense selection, RNA was isolated by extraction with phenol:chloroform:isoamyl alcohol (50:50:1)(PCA) followed by ethanol precipitation. For analysis of 6S RNA levels (Figure 4) and analysis of *rsd* mRNAs (Figure 6), RNA was isolated directly from cultured cells. Cells were pelleted and resuspended in 10 mM Tris (pH 7.5) and 1 mM EDTA. Lysozyme was added to 0.5 mg/ml and samples were subjected to three freeze-thaw cycles. RNA was isolated with Trizol reagent according to manufacturer protocols (Life Technologies) except that 1 ml of Trizol reagent was used for 3 OD_{600} units of cells (Figure 4) and 1.75 ml of Trizol reagent was used for 20 OD_{600} units of cells (Figure 6). RNA for RNase protection assays was further purified by an additional Trizol extraction followed by ethanol precipitation.

End Labeling of RNA

RNA was 3′-end labeled with pCp 5′-[α-^{32}P]triphosphate and RNA ligase (Roche Biochemicals). RNA samples were resuspended in 1× RNA ligase buffer (50 mM Tris [pH 7.5], 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, and 30 μg/ml BSA), heated at 95°C for 3 min followed by addition of 20 μCi of pCp and incubation for 1 hr at 37°C. End-labeled RNA was ethanol precipitated prior to electrophoresis through 8% polyacrylamide urea gels.

Northern Analysis

Northern blot analyses were performed as previously described (Wassarman and Steitz, 1992) except RNA was fractionated on 8% polyacrylamide urea gels and Hybond-N membrane (Amersham) was used. Probes against 6S RNA and 5S rRNA were made from pBS-6S and pGEM-5S (pG5019; Altuvia et al., 1997) plasmids, respectively, by in vitro transcription according to manufacturer protocols (Life Technologies). Probes were purified over G-50 microspin columns (Pharmacia) prior to use. For quantitation, in vitro transcribed 6S RNA and 5S rRNA were generated from pBS-6S and pGEM-5S (pGSO19) plasmids, respectively, and full-length RNAs were gel purified. The signal intensity of defined amounts of in vitro transcribed 6S RNA or 5S rRNA compared to endogenous 6S RNA or 5S rRNA was quantitated on a phosphorimager (Molecular Dynamics). Numbers of 6S RNA molecules per cell were calculated based on the amount of 6S RNA isolated from a known number of OD_{600} units with a conversion of 10^9 cells per OD_{600} unit. The total amount of RNA isolated per OD_{600} unit was relatively constant for the 1 to 20 hr samples, suggesting rRNA levels in general and 5S rRNA specifically do not change dramatically. The amount of total RNA isolated per OD_{600} unit for the 24 hr time sample was usually reduced to ~80% of the amount isolated at earlier time points, indicating rRNA levels may be decreasing. Given that stationary phase cells are smaller than exponential phase cells, the number of cells per OD_{600} unit also may change somewhat with growth. Thus, 6S RNA levels during stationary phase, specifically at 24 hr, could be overestimated here. Nevertheless, we suggest our numbers are within 3-fold of actual 6S RNA levels. Analogous calculations for 5S rRNA levels in our RNA samples estimate ~50,000 copies of 5S rRNA per cell from 1 to 24 hr of growth.

Protein Analysis

For Western analysis of gradient fractions, 1/5 volume of gradient load dye (0.36 M Tris [pH 6.8], 3.6% SDS, 0.75 M 2-mercaptoethanol, and bromphenol blue dye) was added to each fraction. For analysis of total protein from glycerol gradients, protein was precipitated with 1/3 volume of trichloroacetic acid and resuspended in 2× SDS load dye (125 mM Tris [pH 6.8], 20% glycerol, 2% SDS, 0.28 M 2-mercaptoethanol, and bromphenol blue dye). Immunoprecipitated proteins and antisense selected proteins were removed from beads by heating to 95°C in 2× SDS load dye. All protein samples were analyzed on 9% SDS PAGE. For Western analysis, gels were electroblotted to nitrocellulose and probed with 1:5000 (core subunit antibodies) (Sukhodolets and Jin, 1998), 1:2000 (σ^{70} antibodies) (Sukhodolets and Jin, 1998), or 1:1000 (σ^S antibody, 1RS1) (Nguyen et al., 1993). Antibodies were visualized by chemiluminesence (Super Signal kit, Pierce) after binding an HRP-conjugated secondary antibody (1:10,000, Amersham).

Extract Preparation

E. coli cell pellets from 10 OD_{600} units were resuspended in 200 μl of lysis buffer (20 mM Tris [pH 8.0], 150 mM KCl, 1 mM $MgCl_2$, and 1 mM DTT). 200 μl of 0.1 mm glass beads was added, and the mixture was vortexed for 5 min total time in 30 s bursts with 15 s cooling on ice in between. Samples were diluted with an additional 200 μl lysis buffer and centrifuged at 20,800 g for 10 min at 4°C to remove beads and insoluble material.

Glycerol Gradients

100–200 μl of extract was loaded on 10%–30% glycerol gradients containing 20 mM Tris [pH 8.0], 150 mM KCl, and 1 mM $MgCl_2$. Gradients were centrifuged in a SW41 rotor at 36,000 rpm for 16 hr at 4°C. Gradients were fractionated into 24, 20, or 15 fractions (Figures 1A, 1B, or 5, respectively). For gradients shown in Figures 1A and 5, each gradient fraction was divided; 80% was used for RNA analysis and 20% was used for protein analysis. For the gradient shown in Figure 1B, 20% of each fraction was used for RNA analysis and 80% for protein analysis.

RNAP Purification

RNAP was purified from stationary phase K12 cells by polyethylenimine precipitation followed by ammonium sulfate precipitation as

previously described (Burgess and Jendrisak, 1975) except the protocol was downsized to use 2 g of cells.

Immunoprecipitation

Immunoprecipitations were carried out as previously described (Lerner et al., 1981) using rabbit antisera against core RNAP, σ^{70}, or OxyR (Storz et al., 1990). Typically, 20 μl of extract, 0.1–5 μl of serum, and 2–5 mg of protein A Sepharose (CL-4B, Pharmacia) were used and extract was incubated with protein A Sepharose antibody pellets for 2 hr at 4°C. After immunoprecipitation, samples were divided; 80% was used for RNA analysis and 20% for protein analysis.

RNA Antisense Selection

2′OMe RNA/DNA hybrid oligonucleotides containing four biotin-TEG residues at the 5′ end (Keck Biotechnology Center, New Haven, CT) were used: Hi6SA oligo (AGCCCAAUGAACGAGUCAAAGGdU) and OxyS oligo (dAdGdAdCdCdTdGdTdTdAGCGGAUCCUGGAGAUdC). Antisense selection experiments were performed as previously described (Wassarman and Steitz, 1991) except ultralink immobilized neutravidin plus (Pierce) was used instead of streptavidin agarose, and sodium azide was omitted from all buffers. Typically, 15 μl of extract, 0.7 μg of oligonucleotide, and 10 μl of ultralink beads were used in selections.

For selection of radioactively labeled proteins, an overnight culture was diluted 1:50 in 5 ml of M63-glucose medium and grown at 37°C for 8 hr. 25 μl of trans ^{35}S-label (ICN) was added and cells were grown for an additional 16 hr before harvesting, extract preparation, and antisense selection.

UV Crosslinking

30–50 μl of extract was spotted onto parafilm on ice and UV irradiated for 15 min with 254 nm light in a UV crosslinker (Hoeffer) at a distance of approximately 2 cm from the light source. Crosslinked extracts were subjected to antisense oligonucleotide selection using the Hi6SA oligo. RNA/protein complexes were removed from ultralink beads by treatment with RNase T1 in 70 mM Tris (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$, and 5 mM dithiothreitol for 30 min at 37°C. After removal of beads by centrifugation, samples were radioactively labeled with [^{32}P]γ-ATP and T4 polynucleotide kinase for 60 min at 37°C. Samples were purified over G-50 microspin columns (Pharmacia). For samples to be analyzed directly, an equal volume of 2× SDS load buffer was added. For samples to be reselected, 1/10 volume of 100 mM Tris (pH 7.5), 1 M NaCl, and 10 mM EDTA was added after G-50 column purification. Samples were denatured by addition of SDS to 1% final concentration and heating to 85°C for 15 min. After cooling to room temperature, samples were immunoprecipitated as described above.

RNase Protection Assay

RNase protection assays were performed according to manufacturer protocols (RPA III kit, Ambion) using 50 μg of total RNA, and 10^5 cpm of *rsd* probe. The *rsd* probe was generated from pBS-rsd(1) by in vitro transcription according to manufacturer protocols (Life Technologies).

Viability Assay

For each cell type, a fresh transformant was picked into 5 ml LB Cm and grown at 37°C for times indicated. Cultures were serially diluted in M63 salts and several concentrations were plated. The number of viable cells in each culture was determined by counting the number of colonies formed on LB Cm plates. 24 hr was the maximum time cells could be grown with pKK*-6S and pKK*-6S+Y without significant plasmid loss as assayed by increasing colony formation on LB plates compared to LB Cm plates.

Acknowledgments

We thank R. Burgess and D. Jin for antibodies, C. Lee for the *ssr1* strain, and A. Yergey and J. Kowalak for the mass spectrometric analysis. We also appreciate the experimental advice of S. Gottesman, D. Jin, R. Kamakaka, P. Wade, and R. Weisberg and the editorial comments of R. Burgess, S. Gottesman, H. Nash, B. Peculis, D. Wassarman, and C. Wu. This work was supported by the intramural program of the National Institute of Child Health and Human Development.

Received February 22, 2000; revised May 1, 2000.

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