# Regulation of σ factor competition by the alarmone ppGpp

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**Many regulons controlled by alternative σ factors, including $\sigma^S$ and $\sigma^{32}$, are poorly induced in cells lacking the alarmone ppGpp. We show that ppGpp is not absolutely required for the activity of $\sigma^S$-dependent promoters because underproduction of $\sigma^{70}$, specific mutations in *rpoD* (*rpoD40* and *rpoD35*), or overproduction of Rsd (anti-$\sigma^{70}$) restored expression from $\sigma^S$-dependent promoters in vivo in the absence of ppGpp accumulation. An in vitro transcription/competition assay with reconstituted RNA polymerase showed that addition of ppGpp reduces the ability of wild-type $\sigma^{70}$ to compete with $\sigma^{32}$ for core binding and the mutant $\sigma^{70}$ proteins, encoded by *rpoD40* and *rpoD35*, compete less efficiently than wild-type $\sigma^{70}$. Similarly, an in vivo competition assay showed that the ability of both $\sigma^{32}$ and $\sigma^S$ to compete with $\sigma^{70}$ is diminished in cells lacking ppGpp. Consistently, the fraction of $\sigma^S$ and $\sigma^{32}$ bound to core was drastically reduced in ppGpp-deficient cells. Thus, the stringent response encompasses a mechanism that alters the relative competitiveness of σ factors in accordance with cellular demands during physiological stress.**

[*Key Words*: Stringent response; σ factor competition; RpoD; RpoS; RpoH; Rsd]

Received February 14, 2002; revised version accepted April 2, 2002.

Cells of *Escherichia coli* elicit stringent control of ribosome production during the transition from exponential growth to stationary phase (Sands and Roberts 1952; Stent and Brenner 1961). The effector molecule of the stringent control modulon is the alarmone guanosine tetraphosphate, ppGpp (Cashel and Gallant 1969; Lazzarini et al. 1971; Ryals et al. 1982a,b,c; Baracchini and Bremer 1988). The production of this nucleotide is dependent on the (p)ppGpp synthetases PSI and PSII encoded by the *relA* and *spoT* genes, respectively (Xiao et al. 1991). The alarmone ppGpp binds to the β and β′ subunits of core RNA polymerase (E) (Chatterji et al. 1998; Toulokhonov et al. 2001) and thereby inhibits superfluous rRNA biosynthesis during growth inhibition (e.g., Travers 1976; Gourse et al. 1986; Ohlsen and Gralla 1992; Heinemann and Wagner 1997; Zhou and Jin 1998). Mechanisms suggested to explain this regulation of rRNA synthesis include ppGpp-dependent alterations in the initiation pathway that traps RNA polymerase (Heinemann and Wagner 1997), a reduced ability of the RNA polymerase to form an open complex (Ohlsen and Gralla 1992), and a reduction in the stability of the promoter-E$\sigma^{70}$–ppGpp open complex (Gourse et al. 1998). Because the *rrnP*1 promoters form intrinsically unstable open complexes with E$\sigma^{70}$, such promoters may be argued to be especially sensitive to the destabilizing effects of ppGpp (Gourse et al. 1998). Consistent with this idea, it has been shown that RNA polymerase (RNAP) mutants that suppress the requirement for ppGpp in vivo form unstable complexes with stable RNA promoters in vitro (Zhou and Jin 1998).

The alarmone ppGpp can also act as a positive effector of gene expression, and some $\sigma^{70}$-dependent genes require this nucleotide for their induction during growth arrest (Xiao et al. 1991; Nyström 1994; Kvint et al. 2000b). In addition, many operons encoding amino acid biosynthetic pathways require ppGpp for their transcription, and *E. coli* cells lacking ppGpp are polyauxotrophs (Xiao et al. 1991). It has been suggested that the effect of ppGpp on such promoters is linked to ppGpp-dependent changes in core availability. According to a model by Zhou and Jin (1998), the rate-limiting step of promoters that are positively regulated by ppGpp is E$\sigma^{70}$ recruitment, and it is argued that these promoters would therefore be very sensitive to the concentration of free RNA polymerase. Thus, the accumulation of ppGpp is suggested to result in the dissociation of core from stringent *rrnP*1 promoters and the consequent increased availability of core leads to elevated initiation of transcription at promoters that have a relatively poor ability to recruit E$\sigma^{70}$ (Zhou and Jin 1998). Some aspects of this model have been supported recently by in vivo and in vitro transcription assays (Barker et al. 2001a,b).

To add to the role of ppGpp in the cell, genes requiring

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Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.227902.

alternative σ factors have been shown to depend on ppGpp for their induction. For example, the inducers of the $\sigma^{54}$-dependent promoters Po and Pu are only effective when ppGpp levels are elevated (Sze and Shingler 1999; Carmona et al. 2000; Sze et al. 2002). Similarly, mutant cells with no or low levels of ppGpp exhibit an attenuated and sluggish expression of $\sigma^{32}$-dependent heat-shock genes (Grossman et al. 1984; VanBogelen and Neidhardt 1990; Jones et al. 1992). In addition, mutants lacking functional *relA spoT* or $\sigma^S$ of the general stress response have been found to exhibit similar phenotypes. The $\sigma^S$ transcription factor accumulates and directs the RNA polymerase to >50 genes upon conditions of cellular starvation and stress (Hengge-Aroni 2000). Mutants lacking $\sigma^S$ exhibit an accelerated die-off during conditions of growth arrest (Lange and Hengge-Aronis 1991), and markedly elevated levels of oxidized proteins (Dukan and Nystrom 1998, 1999). The fact that $\sigma^S$ itself requires ppGpp for its production (Gentry et al. 1993; Lange et al. 1995; Zgurskaya et al. 1997) initially appeared to fully explain the similarity between Δ*relA* Δ*spoT* and *rpoS* phenotypes. However, it was later shown that $\sigma^S$-dependent genes require ppGpp for their induction, even in the presence of wild-type levels of $\sigma^S$ (Kvint et al. 2000a). Thus, ppGpp appears to exert a dual control on the RpoS regulon by affecting both the levels of the required σ factor and its activity.

The exact role of ppGpp in alternative σ-factor function is not clear and could, conceivably, include control of promoter recognition and transcription initiation or act at the level of σ binding to core. In this work, we present evidence using (1) in vivo and in vitro competition assays and (2) quantification of $\sigma^S$ and $\sigma^{32}$ association with core so that the ability of $\sigma^S$ and $\sigma^{32}$ to compete with $\sigma^{70}$ for core binding is facilitated in the presence of ppGpp, and that ppGpp requirement can be suppressed by $\sigma^{70}$ underproduction. The data show that a ppGpp-dependent alteration in σ-factor competition for core binding is an integral part of the typical stringent response allowing alternative σ factors to operate successfully in concert with $\sigma^{70}$ during cellular stress.

## Results

### *The failure of relaxed mutants to induce the $\sigma^S$ regulon can be alleviated by underproduction of $\sigma^{70}$ or overproduction of Rsd*

As seen in Figure 1A, expression of a $\sigma^S$-dependent model gene is induced during the transition phase and reaches a new steady state (stationary phase value) about 2 h after a brake point in the growth curve can be observed. The difference between the wild-type and $ppGpp^0$ mutant is maximal at this time, whereas no significant difference can be observed during exponential growth (Fig. 1A). To facilitate easy and direct comparisons between expression levels in different strains, only the mean stationary phase values are shown in the subsequent experiments.

To elucidate whether there is an absolute requirement

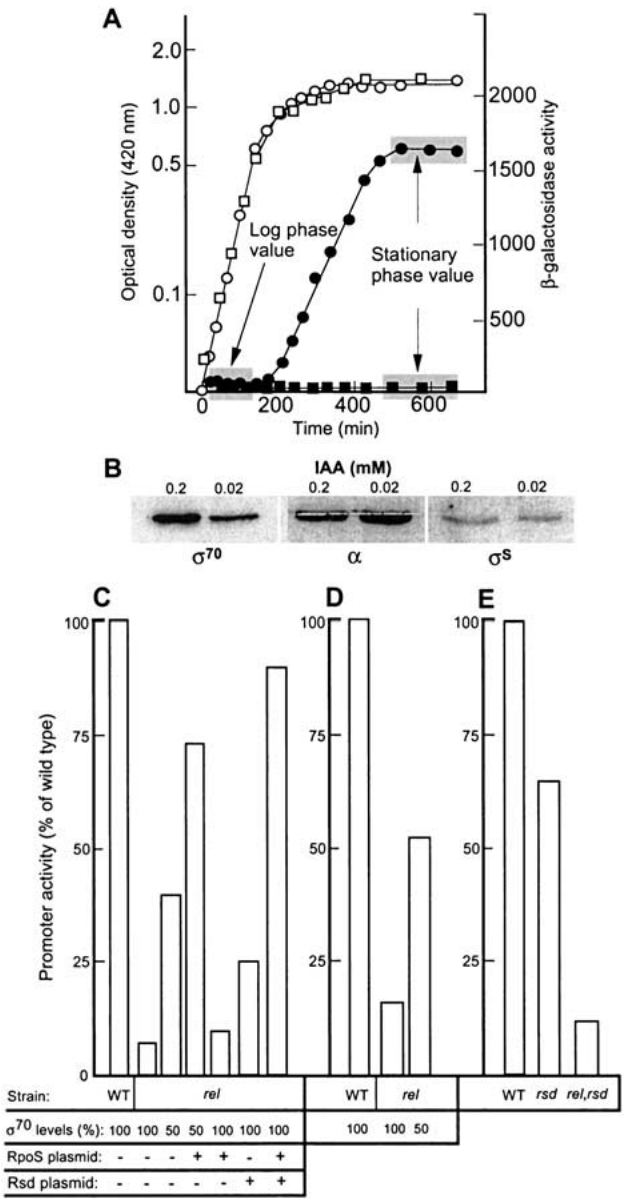


**Figure 1.** Effects of $\sigma^{70}$ underproduction and Rsd overproduction on $\sigma^S$-dependent gene expression. (*A*) P*uspB–lacZ* expression (closed symbols) and cell density (open symbols) in the wild-type (AF633, circles) and $ppGpp^0$ (KK180, boxes) strains during growth and stationary phase. (*B*) Western blot immunoassay for detection of RpoD, RpoS, and α-subunit of RNAP in cells grown in different amounts of IAA as indicated (the levels of the β subunit were, like α, unaffected by 0.2 mM IAA; data not shown). (*C*) Expression of P*uspB–lacZ* in wild-type and relaxed strains (*relA1* Δ*spoT*) underproducing RpoD, and/or overproducing RpoS and Rsd as indicated. The strains used are MJ429, MJ442, TN324, KK317, KK318, and KK316 (see Table 1 for details). (*D*) Expression of P*katE–lacZ* in wild-type and relaxed strains (*relA1* Δ*spoT*) underproducing RpoD. The strains used are TN321 and MJ432. (*E*) Expression of P*uspB–lacZ* in wild type (AF633), *rsd*::Km (MJ234), and *rsd*::Km Δ*spoT* (TN325). The data show expression levels after 1 h of glucose starvation. The phenotype *rel* (*relA1* Δ*spoT*::Km) indicated in the figure implies that the strains fail to accumulate ppGpp during the glucose starvation condition used.

of ppGpp for $\sigma^S$ function or whether ppGpp facilitates $\sigma^S$ competition for core binding, we determined the effect of $\sigma^{70}$ underproduction on the expression from the $\sigma^S$-dependent promoters, P*uspB* and P*katE*, in relaxed cells that fail to elevate ppGpp levels during carbon starvation (e.g., Kvint et al. 2000a). To approach this question, we used a genetic system for underproduction of $\sigma^{70}$ in which expression of this σ factor is regulated by the *trp* promoter and can be controlled by the levels of IAA (indole-3-acrylic acid; an antagonist of the Trp repressor; Lonetto et al. 1998). We first determined the concentration of IAA (0.2 mM) that generated $\sigma^{70}$ levels corresponding to wild-type $\sigma^{70}$ levels and resulted in the correct growth rate in the growth medium used (data not shown). We also confirmed that the kinetics and magnitude of induction of $\sigma^S$ and $\sigma^{70}$-dependent genes were indistinguishable from that of wild type during entry of cells in glucose starvation-induced stationary phase by use of this concentration of IAA (data not shown). Growing cultures were diluted and split into two, such that one received 0.2 mM IAA (wild-type levels of $\sigma^{70}$), whereas the other received 0.02 mM IAA (underproduction of $\sigma^{70}$; see Fig. 1B) and the effect of this underproduction of $\sigma^{70}$ on the expression of $\sigma^S$-dependent genes was determined. Note that underproduction of $\sigma^{70}$ did not change the levels of the α subunit (Fig. 1B) or the β subunit (data not shown) of core, nor did $\sigma^S$ levels change appreciably (Fig. 1B). In the experiment shown, we used the *relA1* and Δ*spoT*::Km alleles rather than Δ*relA*::Km and Δ*spoT*::Cm because the P*trp–rpoD* fusion is linked to the Cm marker. However, we have confirmed that the behavior of the double Δ*relA* Δ*spoT* deletion mutant and the *relA1* Δ*spoT*::*Km* mutant is indistinguishable with respect to the regulation of the promoters studied during the glucose starvation conditions used. As seen in Figure 1, C and D, underproduction of $\sigma^{70}$ partly suppressed the lack of induction of P*uspB* and P*katE* upon entry of relaxed cells into glucose starvation-induced stationary phase. It should be noted that the expression levels reached are clearly below those of wild-type cells. This result would be anticipated as $\sigma^S$ levels are much lower in the relaxed mutant, and consequently $\sigma^{70}$ underproduction alone could not be expected to accomplish a full suppression of $\sigma^S$-dependent expression. However, a stronger suppression was achieved when $\sigma^{70}$ underproduction was performed with a strain carrying *rpoS* on a high copy number plasmid (Fig. 1C). Consistent with previous data (Kvint et al. 2000a), no effect was observed with the *rpoS* plasmid alone (Fig. 1C). As expected, $\sigma^{70}$ underproduction did not up-regulate a $\sigma^{70}$-dependent promoter (P*uspA*) requiring ppGpp for induction (data not shown).

The *E. coli* Rsd protein binds free $\sigma^{70}$ and has been suggested to act as an anti-σ factor with a role in curtailing $\sigma^{70}$-dependent transcription by blocking the access of $\sigma^{70}$ to core RNA polymerase upon entry of cells into stationary phase (Jishage and Ishihama 1998). Because the *rsd* gene is induced in stationary phase in a ppGpp-dependent fashion (Jishage and Ishihama 1999), we entertained the idea that the reduced levels of Rsd observed in relaxed cells may thus reduce the ability of $\sigma^S$ to compete for core binding. We tested this idea by elucidating the effect of overproducing Rsd on $\sigma^S$-dependent transcription in relaxed cells. As shown in Figure 1C, Rsd overproduction alone had a very small effect on *uspB* expression in the relaxed mutant. However, the effect was significantly enhanced when more RpoS was provided from the pMMKatF2 plasmid (Fig. 1C). Also, induction of the *uspB* gene was attenuated in the *rsd* mutant, but not to the same extent as in the ppGpp$^0$ mutant (Fig. 1E). Thus, we conclude that the Rsd protein may be an important member of the stringent control modulon that allows $\sigma^S$ to compete more successfully for core binding. However, the poor induction of the RpoS regulon in relaxed cells cannot be solely explained by the diminished concentration of Rsd in these cells.

### *The* rpoD40 *and* rpoD35 *suppressor mutations partially restore $\sigma^S$-dependent promoter activity in a ppGpp$^0$ mutant*

Two new *rpoD* mutations, called *rpoD35* and *rpoD40* (see Table 1), have been identified recently, which suppress the ppGpp requirement of the $\sigma^{54}$-dependent Po promoter. With respect to suppression of Po, the *rpoD40* allele is a markedly better suppressor than the *rpoD35* allele, and this phenotype has been shown to be attributable to reduced ability to bind and compete for core RNA polymerase (A. Laurie and V. Shingler, unpubl.). We transduced these *rpoD* suppressor alleles into KK358 (P*uspB–lacZ* Δ*relA* Δ*spoT*). Using tetrazolium lactose plates, it became immediately obvious that the *rpoD40* (Fig. 2A) and *rpoD35* (data not shown) mutations also restored expression from the $\sigma^S$-dependent P*uspB* promoter in a ppGpp$^0$ strain. Quantification of the promoter activity (Fig. 2B) showed that the *rpoD40* allele is a better suppressor than *rpoD35*, and thus that the hierarchy observed with the $\sigma^{54}$-dependent Po promoter is maintained at the $\sigma^S$-dependent P*uspB* promoter. We also introduced plasmid pMMKatF2 (overproducing $\sigma^S$) into the suppressor strains, and noted that the activity of P*uspB* was further elevated by $\sigma^S$ overproduction (Fig. 2B).

The mutations in the *rpoD* gene, such as *rpoD*40 and *rpoD*35, are not expected to directly affect promoters dependent on alternative σ factors. However, it is possible that these mutations, by reducing the ability of the $\sigma^{70}$ to bind core, may thus also reduce their ability to compete for core. If this is the case, the underlying reason that these *rpoD* mutations restore expression from $\sigma^S$-dependent promoters in a ppGpp$^0$ background may be similar to that of $\sigma^{70}$ underproduction or Rsd overproduction, namely, a reduced potential to compete with alternative σ factors. To approach this notion, we set up a mixed in vitro transcription assay to directly analyze σ-factor competition.

### *ppGpp does not influence E$\sigma^{32}$ transcription from P*dnaK *in vitro*

To set up a reliable in vitro transcription-competition (IVT) system, we used a $\sigma^{32}$-dependent promoter

**Table 1.** *Bacterial strains and plasmids*

| Strain | Relevant genotype | Source/reference |
|---|---|---|
| AF633 | MC4100 λφ (*uspB–lacZ*) | Farewell et al. 1998b |
| AF634 | MC4100 λφ (*uspA–lacZ*) | Farewell et al. 1996 |
| EC2922 | MG1655 Δ*lac relA*::Km *spoT*::Cm *aer*-3075::Tn10 *rpoD*-Y571H, (*rpoD*35) | V. Shingler |
| EC2871 | MG1655 Δ*lac relA*::Km *spoT*::Cm *aer*-3075::Tn10 *rpoD*-∇DSA (536–538), (*rpoD*40) | V. Shingler |
| KK153 | AF633/pMMKatF2 | This work |
| KK180 | AF633 Δ*relA*::Km Δ*spoT*::Cm | Kvint et al. 2000a |
| KK315 | AF633 *relA1 spoT*::Km | This work |
| KK316 | KK315/pRsd/pMMKatF2 | This work |
| KK317 | KK315/pRsd | This work |
| KK318 | KK315/pMMKatF2 | This work |
| KK357 | AF634 Δ*relA* Δ*spoT* | This work |
| KK358 | AF633 Δ*relA* Δ*spoT* | This work |
| KK373 | KK358 *rpoD*-∇DSA (536–538), (*rpoD*40) | This work |
| KK374 | KK358/pMMKatF2 | This work |
| KK375 | KK373/pMMKatF2 | This work |
| KK384 | KK358 *rpoD*-Y571H, (*rpoD*35) | This work |
| Kk385 | KK384/pMMKatF2 | This work |
| KK390 | AF634/pKV1278 | This work |
| KK391 | KK357/pKV1278 | This work |
| MC4100 | F-*araD*139 Δ'*arqF–lac*' U169 *rpsL* 150 *relA1 flb*B5301 *deo*C1 *pts*F25 *rbs*R | Lab. stock |
| MJ234 | AF633 *rsd*::Km | This work |
| MJ265 | MC4100 λφ(mmp*uspA*4b–*lacZ*) | This work |
| MJ271 | MC4100 λφ (*katE–lacZ*) | This work |
| MJ285 | MJ265 Δ*relA*::Km Δ*spoT*::Cm | This work |
| MJ321 | MJ265/pMMKatF2 | This work |
| MJ325 | MJ265/pKV1278 | This work |
| MJ342 | AF633/pKV1278 | This work |
| MJ352 | MJ265/pKVQ805 | This work |
| MJ381 | MJ271/pKV1278 | This work |
| MJ382 | SP887/pKV1278 | This work |
| MJ429 | AF633 Ω(Cam$^R$) *Ptrp–rpoD* | This work |
| MJ432 | MJ271 Ω(Cam$^R$) *Ptrp–rpoD* | This work |
| MJ440 | MJ285/pKV1278 | This work |
| MJ442 | MJ429 *spoT*::Km | This work |
| MJ500 | MJ285/pMMKatF2 | This work |
| MO1000EL | λφ (*katE–lacZ*) | Ohnuma et al. 2000 |
| TN321 | MJ432 *spoT*::Km | This work |
| TN322 | MC4100 λφ(*fadD–lacZ*)/pMMKatF2 | This work |
| TN323 | TN322 Δ*relA*::Km Δ*spoT*::Cm/pMMKatF2 | This work |
| TN324 | MJ442/pMMKatF2 | This work |
| TN325 | MJ234 Δ*spoT*::Cm | This work |
| TN326 | MC4100 λφ(*fadD–lacZ*)/pKV1278 | This work |
| TN327 | TN326 Δ*relA*::Km Δ*spoT*::Cm/pKV1278 | This work |

| Plasmid | Vector | Gene to be expressed | Source/reference |
|---|---|---|---|
| pAT153 | | | L-O. Hedén |
| phis173 | | | Joo et al. 1997 |
| pJET40 | | P*dnaK, rna* P1 | C. Gross |
| pKV1278 | pTrc99A | *rpoH* | M. Kanamori, unpubl. |
| pKVQ805 | pTrc99A | *rpoH*173 (Q80R) | This work |
| pMJ261 | pTL61T | *uspA* promoter | This work |
| pMMKatF2 | pAT153 | *rpoS* | Mulvey et al. 1988 |
| pRsd | pACYC184 | *rsd* | Jishage and Ishihama 1999 |
| pTL61T | | | Linn and St. Pierre 1990 |

(P*dnaK*), rather than a $\sigma^S$-dependent promoter, to monitor effects of σ-factor competition. This choice was based on the fact that a $\sigma^{70}$-programmed holoenzyme has been shown to be able to initiate transcription from $\sigma^S$-dependent promoters in vitro (e.g., Kusano et al. 1996), an in vitro phenomenon we have also observed with P*uspB* and P*fic* as templates (data not shown). In addition, by using a $\sigma^{32}$-dependent promoter, we avoided possible promoter interference, as $E\sigma^{32}$ and $E\sigma^{70}$ recognizes markedly different promoters, and no cross activity has

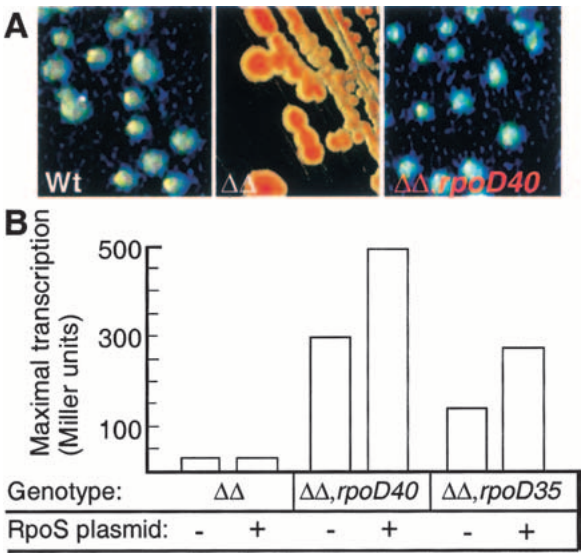


**Figure 2.** Effects of RpoD mutations on P*uspB–lacZ* expression. (*A*) Colonies of wild type (AF633), Δ*relA* Δ*spoT* (KK358), and Δ*relA* Δ*spoT rpoD*40 (KK373) grown on tetrazolium lactose plates. (*B*) Maximal transcription of P*uspB–lacZ* in ppGpp$^0$ strains carrying different *rpoD* alleles. The strains used are Δ*relA* Δ*spoT* (KK358), Δ*relA* Δ*spoT*/pMMKatF2 (KK374), Δ*relA* Δ*spoT rpoD*40 (KK373), Δ*relA* Δ*spoT rpoD*40/pMMKatF2 (KK375), Δ*relA* Δ*spoT rpoD*35 (KK384), and Δ*relA* Δ*spoT rpoD*35/pMMKatF2 (KK385). (ΔΔ) The Δ*relA* Δ*spoT* genotype.

been observed between these systems in vitro (e.g., Gross et al. 1992).

To test the IVT system, we added a fixed amount of supercoiled DNA template (P*dnaK* and P*rna1*; 1.25 nM) and core (10 nM) with increasing amounts of $\sigma^{32}$ in multiple round transcription reactions. As seen in Figure 3A, no transcription of the E$\sigma^{70}$-dependent promoter *rna1* was observed using E$\sigma^{32}$. A saturation curve for this assay is shown in Figure 3B. To ensure that purified E$\sigma^{70}$ would not initiate transcription from the P*dnaK* promoter of the template, we repeated the experiment with reconstituted core RNAP and each of the individual $\sigma^{70}$ (wild type, RpoD35, and RpoD40). No transcript derived from P*dnaK* could be detected with wild-type $\sigma^{70}$ (Fig. 3C) or the two mutant $\sigma^{70}$ (data not shown). In addition, we tested whether increasing amounts of ppGpp changed the transcriptional activity of P*dnaK* using E$\sigma^{32}$. We detected no direct effects of ppGpp (0 to 644 μM) on the transcriptional activity from the *dnaK* promoter (Fig. 3D).

### *The $\sigma^{70}$ mutant proteins compete poorly for RNAP core in vitro*

To elucidate the relative competitiveness (inhibition of transcription from P*dnaK*) of the wild-type and mutant $\sigma^{70}$s, we added increasing amounts of the different $\sigma^{70}$s to our IVT reaction mix that contained a fixed concentration of $\sigma^{32}$ (250 nM) that saturated P*dnaK*, and measured the relative level of the *dnaK* transcript. Increasing amounts of wild-type $\sigma^{70}$ drastically inhibited transcription from *dnaK* (Fig. 4A). Inhibition by the RpoD35 protein was significantly lower than by wild-type $\sigma^{70}$ and the RpoD40 protein was the least effective in inhibiting transcription from the *dnaK* promoter (Fig. 4A). In addition, we performed a similar experiment, but added increasing amounts of $\sigma^{32}$ to a fixed amount of $\sigma^{70}$ (wild-type RpoD at 10 nM, and RpoD35 and RpoD40 at 80 nM). The concentration of the different $\sigma^{70}$s used in this experiment were chosen on the basis of the concentration of the σ that saturated the *rna1* promoter (data not shown). Again, we found that the wild-type $\sigma^{70}$ was much more competitive compared with the mutant σs and that RpoD35 was better than RpoD40 (Fig. 4B). Note also that the *rpoD40* allele was more effective than *rpoD35* in restoring in vivo $\sigma^S$-dependent expression in ppGpp$^0$ strains (Fig. 2B).

### *Wild-type $\sigma^{70}$ competitiveness is reduced in the presence of ppGpp*

We next repeated the in vitro competition assay with wild-type $\sigma^{70}$ and $\sigma^{32}$ competing in the presence and ab-

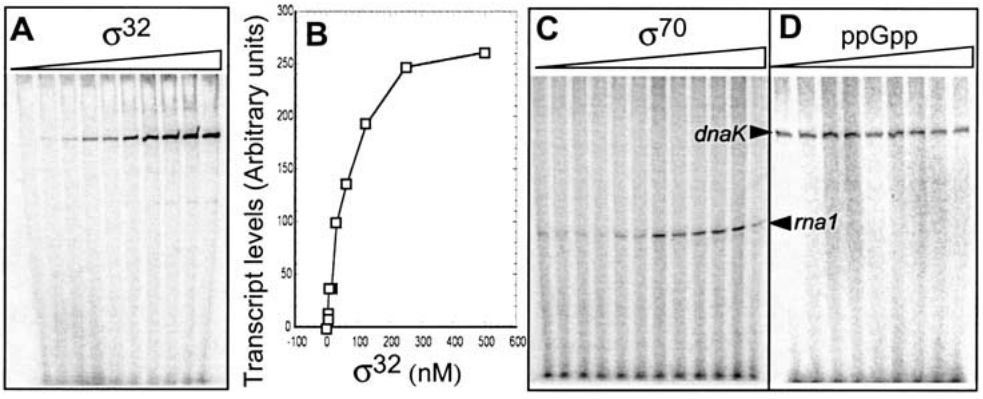


**Figure 3.** In vitro transcription (IVT) assay of P*dnaK* and *rna* P1 by $\sigma^{32}$ and $\sigma^{70}$ programmed RNAP. (*A*) Autoradiogram obtained from the transcription assay performed with increasing amounts of $\sigma^{32}$ (0–500 nM) as indicated. (*B*) Quantification of the transcripts plotted as relative units. (*C*) Autoradiogram obtained from the transcription assay with increasing amounts (0–60 nM) of wild-type RpoD as indicated. (*D*) Autoradiogram obtained from transcription assay with fixed amounts of $\sigma^{32}$ (250 nM) and increasing levels (0–644 μM) of ppGpp as indicated.

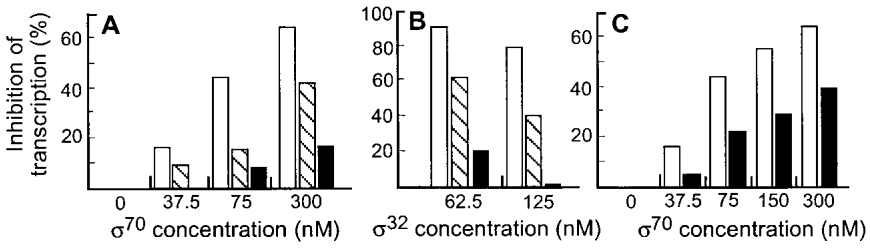


**Figure 4.** Inhibition of *dnaK* transcription by wild-type and mutant RpoD proteins in an IVT competition assay. (*A*) Transcription of *dnaK* in multiple round transcription reactions with 1.25 nM P*dnaK*-containing template (pJET40), 10 nM core RNAP, 250 nM $\sigma^{32}$, and increasing amounts of wild-type or mutant $\sigma^{70}$ proteins as indicated. The extent of inhibition (%) of *dnaK* transcription was related to transcription with no competing RpoD in the assay. (Open bars) Competition with wild-type RpoD; (hatched bars) competition with RpoD35; (solid bars) competition with RpoD40. (*B*) Same as *A* but increasing amounts of $\sigma^{32}$ (as indicated) and fixed amounts of wt RpoD (20 nM) and mutant RpoD (80 nM) proteins. (*C*) Relative inhibition *dnaK* transcription by RpoD in the presence and absence of ppGpp ($\sigma^{32}$ concentration fixed at 250 nM). (Open bars) indicate Competition in the absence of ppGpp; (solid bars) competition in the presence of 180 μM ppGpp.

sence of ppGpp. The experiment was performed as described above using a fixed amount of $\sigma^{32}$ and increasing amounts of wild-type $\sigma^{70}$, with and without ppGpp (180 μM). As seen in Figure 4C, the competitiveness of wild-type $\sigma^{70}$ was markedly reduced by the addition of ppGpp. Thus, ppGpp has a positive effect on in vitro *dnaK* transcription under conditions of competition between $\sigma^{32}$ and $\sigma^{70}$, but have no observable effect when $\sigma^{32}$ operates alone.

### *Sigma factors, $\sigma^{32}$ and $\sigma^{S}$, compete less effectively in cells lacking ppGpp*

The data presented so far suggest that the poor ability of ppGpp$^0$ mutants to induce regulons requiring alternative σ factors may be explained by a poor ability of these σ factors to compete with $\sigma^{70}$ in the absence of ppGpp. Thus, the ability to down-regulate transcription from promoters requiring $\sigma^{70}$ should be a measure of an alternative σ factor's ability to successfully bind and compete for core binding in vivo. Therefore, we used *lacZ* fusions to elucidate the ability of $\sigma^{S}$ and $\sigma^{32}$ to reduce transcription from $\sigma^{70}$-dependent genes in wild-type and ppGpp$^0$ strains. First, we noted that overproduction of $\sigma^{S}$ (fourfold overproduction measured by Western blotting; data not shown) caused such down-regulation of two model $\sigma^{70}$-dependent promoters (*uspA* and *fadD*; Fig. 5A), consistent with previous results (Farewell et al. 1998a). However, this inhibition of transcription only occurred when cells entered stationary phase (high ppGpp levels), with no repression observable during exponential growth (data not shown). Next, we repeated the same experiment in a ppGpp$^0$ mutant and noted that the same overproduction of $\sigma^{S}$ failed to reduce expression from both *uspA* and *fadD* in stationary phase cells (Fig. 5A). We obtained the same results with *relA1*, Δ*spoT*::Km mutants (data not shown). Similarly, we found that ectopic overproduction of $\sigma^{32}$ was much more effective in inhibiting expression from the *uspA* and the *fadD* promoters in the wild-type background than the ppGpp$^0$ mutant strain (Fig. 5A).

Overproduction of Q80R mutant $\sigma^{32}$ (Fig. 5B), which exhibits a drastically reduced affinity for core (Joo et al. 1997) totally failed to repress the $\sigma^{70}$-dependent promoter P*uspA* (Fig. 5C). This confirms that the observed inhibition of *uspA* and *fadD* transcription is an effect of $\sigma^{32}$ out-competing $\sigma^{70}$ for core binding.

### *Binding of $\sigma^{S}$ and $\sigma^{32}$ to core RNA polymerase is reduced in cells lacking ppGpp*

Next, we measured the concentration of $\sigma^{S}$ and $\sigma^{32}$ bound to core RNA polymerase in wild-type and ppGpp$^0$ strains during carbon starvation. The cells carried plasmid-borne copies of the *rpoS* (pMMKatF2) and *rpoH* (pKV1278) genes to ensure similar expression levels in the wild-type and ppGpp$^0$ strains. After harvesting, whole-cell lysates were fractionated by gel filtration and the concentration of σ factors and core subunits in each fraction was quantified by using monoclonal antibodies against $\sigma^{S}$, $\sigma^{32}$, β, and β′ subunits. σ factors were found in two separate portions of the fractions collected. In fractions 4–28, σ factors coeluted with the β and β′ subunits of core RNA polymerase (interpreted as bound σs), whereas fractions 54–76 contained σ factors but no traces of core subunits (Fig. 6A). It became immediately evident that a significantly larger fraction of $\sigma^{S}$ was bound to core in wild-type compared with ppGpp$^0$ cells (Fig. 6A). Quantifications of Western blots showed that the concentration of $\sigma^{S}$ and $\sigma^{32}$ recovered in the core-bound fraction was reduced by ~70% in the ppGpp$^0$ strain compared with the wild type (Fig. 6B). These results cannot be attributed to reduced $\sigma^{S}$ and $\sigma^{32}$ content, as the presence of appropriate plasmids in the parental strains ensures that these do not differ significantly in this respect (Fig. 6C).

## Discussion

We report here on a new function for ppGpp as a master regulator of transcription, that is, the regulation of σ-factor competition. This notion is based on data showing (1) that alternative σ factors compete significantly better against $\sigma^{70}$ in the presence of ppGpp both in in vitro and in vivo transcription assays; (2) that the fraction of both $\sigma^{S}$ and $\sigma^{32}$ bound to core is reduced in stationary phase cells lacking ppGpp; (3) that the ppGpp requirement of

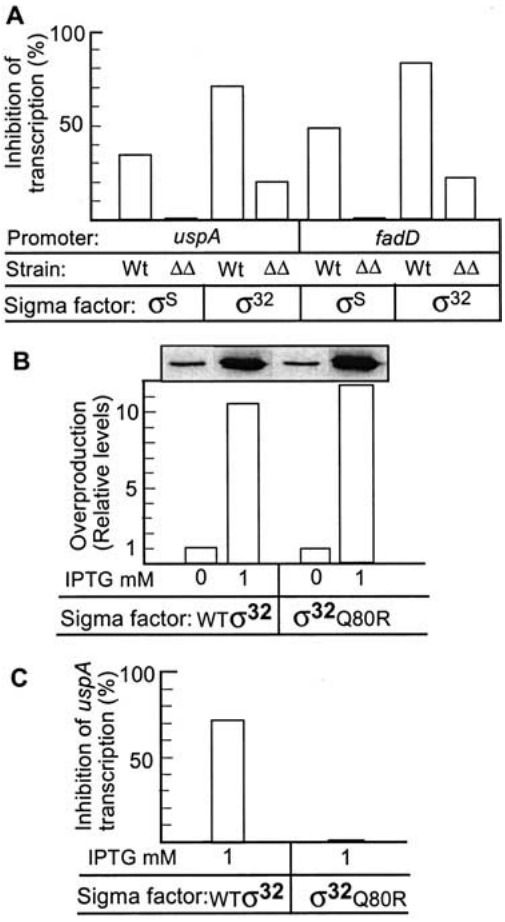


**Figure 5.** Role of ppGpp on the in vivo competitiveness of $\sigma^{32}$ and $\sigma^S$. (*A*) Relative inhibition (%) of E$\sigma^{70}$-dependent (P*uspA–lacZ* and P*fadD–lacZ*) expression by $\sigma^S$ or $\sigma^{32}$ overproduction (from pMMKatF2 or pKV1278) in a wild-type and a Δ*relA* Δ*spoT* background. Overproduction of $\sigma^{32}$ was achieved with 1 mM IPTG using the system described. The strains used for analysis of P*uspA–lacZ* expression were MJ321, MJ500, MJ325, and MJ440, and for P*fadD–lacZ* expression TN322, TN323, TN326, and TN327. (*B*) Extent of overproduction of wild-type $\sigma^{32}$ and the mutant $\sigma^{32}$Q80R by IPTG addition (1 mM). (*C*) Effects of overproducing the mutant $\sigma^{32}$Q80R on the inhibition of *uspA* expression (MJ352). The effect of overproducing wild-type $\sigma^{32}$ (strain MJ325) is shown for a comparison. (ΔΔ) The Δ*relA*::Km Δ*spoT*::Cm genotype.

genes transcribed by alternative σ factors is alleviated by specific *rpoD* alleles encoding $\sigma^{70}$ proteins with reduced ability to compete for core; (4) that there is no absolute requirement of ppGpp for $\sigma^S$-dependent promoters, as underproduction of $\sigma^{70}$ or overproduction of the anti-$\sigma^{70}$ factor Rsd rescues the promoters in the absence of ppGpp accumulation. Thus, we suggest that elevated levels of ppGpp facilitate transcription of the general stress response and the heat-shock regulon by allowing the required σ factors, $\sigma^S$ and $\sigma^{32}$, respectively, to compete more successfully for available core.

Thus, ppGpp is priming the RNA polymerase in accordance with environmental signals such that the transcriptional apparatus will be primarily occupied with transcription of $\sigma^{70}$-dependent housekeeping genes as long as the ppGpp levels are low, which signals that the nutritional status of the environment is favorable for growth. During growth arrest or growth under stress, elevated ppGpp levels allow the alternative σ factors to work in concert with $\sigma^{70}$ by shifting the relative competitiveness of the σ factors. Such a role for ppGpp has, in fact, been hypothesized previously. Travers (1985) discussed the possibility that alarmones, such as ppGpp and AppppA, may act by loosening the protein–protein interactions between $\sigma^{70}$ and core, thereby facilitating replacement of one σ by another. Alteration in the affinity of core for different σ factors was suggested also by Van-Bogelen and Neidhardt (1990) as a possible explanation for the sluggish and delayed induction of heat shock genes in ppGpp$^0$ mutants. In addition, Hernandez and Cashel (1995) showed that ppGpp drastically reduces the fraction of $\sigma^{70}$ bound to core, and put forward the idea that ppGpp may alter the competition between $\sigma^{70}$ and alternative σ factors. Our data strongly supports such an idea.

Whereas the data above supports the notion that ppGpp facilitates the competition by alternative σ factors for available core, it is important to consider that ppGpp does not cause a full σ-factor replacement per se. In parallel to genes requiring alternative σ factors, many $\sigma^{70}$-dependent genes are induced during a stringent response (e.g., Xiao et al. 1991; Cashel et al. 1996; Da Costa and Artz 1997; Diez et al. 2000; Kvint et al. 2000b). It has been proposed that the positive regulation of $\sigma^{70}$-dependent promoters by ppGpp is linked to ppGpp-dependent effects on RNA polymerase availability. The rate-limiting step of such $\sigma^{70}$-dependent promoters has been argued to be RNA polymerase recruitment and these promoters, therefore, would be very sensitive to the concentration of free RNA polymerase (Bartlett et al. 1998; Zhou and Jin 1998). The accumulation of ppGpp is suggested to result in the dissociation of RNA polymerase from stringent promoters (Bartlett et al. 1998; Zhou and Jin 1998) resulting in more RNA polymerase becoming available to initiate transcription at promoters that have a relatively poor ability to recruit RNA polymerase. Thus, despite the fact that ppGpp accumulation decreases the competitiveness of $\sigma^{70}$ during stringency, many E$\sigma^{70}$-dependent promoters may well experience an increased E$\sigma^{70}$ availability, as a large fraction of the holoenzyme is no longer sequestered in transcribing stable RNA operons. Thus, the model for the positive control of ppGpp/$\sigma^{70}$-dependent promoters (Zhou and Jin 1998) is fully compatible with the σ-factor competition model presented here. It cannot be excluded, however, that some $\sigma^{70}$-dependent promoters are directly regulated by ppGpp and examples of such positive effect of ppGpp on gene expression has been achieved using a coupled in vitro transcription translation assay (Riggs et al. 1986; Choy 2000).

Likewise, our data does not exclude the possibility that ppGpp may have additional effects at the level of promoter recognition by E$\sigma^S$ and E$\sigma^{32}$ or a subsequent

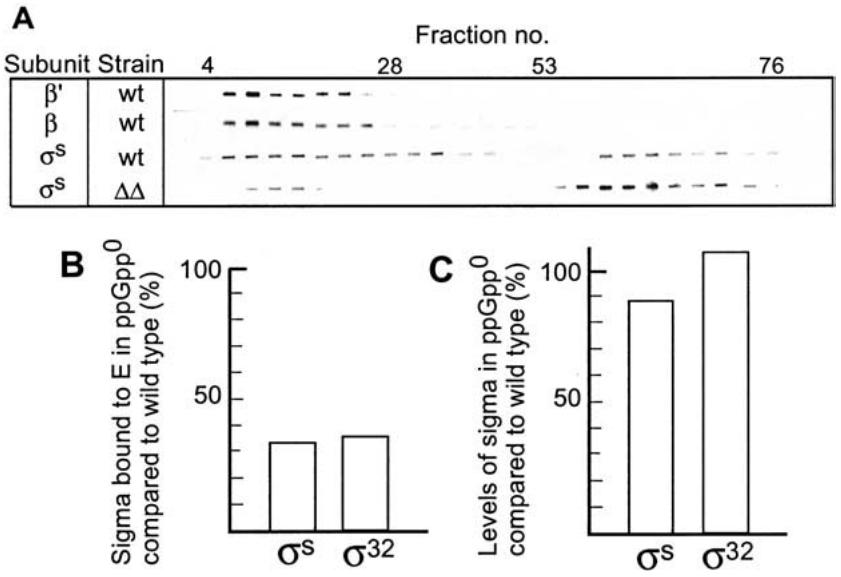


**Figure 6.** Analysis of σ-core RNA polymerase interactions in Δ*relA* Δ*spoT* and wild-type strains. (*A*) Whole-cell lysates from wild-type and ppGpp$^0$ cells were fractionated by gel filtration and the relative concentration of RNA polymerase subunits was subsequently analyzed in the fractions by using monoclonal antibodies against the $\sigma^S$, $\sigma^{32}$, β, and β′ subunits. As indicated, $\sigma^S$ coeluted with β, β′ in early fractions (bound σ), and was also recovered in later fractions with no traces of core subunits (free σ). (*B*) Fraction (%) of $\sigma^S$ and $\sigma^{32}$ that coeluted with core (β and β′ subunits) in ppGpp$^0$ cells compared with wild-type cells, which was assigned a value of 100. (*C*) Levels (%) of $\sigma^S$ and $\sigma^{32}$ in ppGpp$^0$ compared with wild type measured by Western blot analysis. Samples were taken 2 h into stationary phase and equal amounts of crude cell extracts were subjected to SDS-PAGE followed by Western blotting. Strains used were KK153, KK374, KK390, and KK391.

step in transcription initiation. So far, however, we have not detected any direct effects of ppGpp on the transcription of E$\sigma^S$- or E$\sigma^{32}$-dependent promoters in in vitro transcription assays. In addition, we cannot exclude the possibility that the antagonism between σ factors $\sigma^S$ and $\sigma^{70}$ may also be linked also to promoter interference (Landini and Busby 1999). This concept is based on the argument that, in vivo, E$\sigma^{70}$ may be able to bind a $\sigma^S$-dependent promoter in a stable but nonproductive way and thereby inhibit binding of E$\sigma^S$. Such a mechanism may work in concert with σ factor competition between $\sigma^{70}$ and $\sigma^S$ but should not apply to the antagonism between $\sigma^{70}$ and $\sigma^{32}$, as the latter two σ factors recognize vastly different promoter structures. Moreover, the framework of the promoter interference model cannot explain the drastically reduced concentration of $\sigma^S$ bound to core in ppGpp$^0$ strains.

Finally, this work clearly supports the previous suggestion that RNA polymerase is limiting for transcription and that activation of the RpoS and RpoH regulons is critically dependent on the concentration of the housekeeping σ factor in vivo (e.g., Osawa and Yura 1981; Malik et al. 1987; Gross et al. 1992; Farewell et al. 1998a; Maeda et al. 2000). Similarly, recent work in the laboratory of V. Shingler has shown that the activity of $\sigma^{54}$ is critically dependent on ppGpp and the concentration of $\sigma^{70}$ in vivo. Thus, it appears that stringency is, in part, a general mechanism harnessed by the cell to alter the relative efficiency of σ factor binding to the core polymerase such that low ppGpp levels (conditions favorable for rapid growth) favor housekeeping functions, whereas high ppGpp levels allow alternative σ factors to operate in concert with $\sigma^{70}$. In this respect, it is interesting that the *E. coli* anti-$\sigma^{70}$ factor Rsd is itself under positive control by ppGpp (Jishage and Ishihama 1999). However, as shown here, the effects of ppGpp deficiency on $\sigma^S$ activity (expression of $\sigma^S$-dependent genes) exceeds that of Rsd deficiency. Moreover, ppGpp alone clearly affected competition in vitro with reconstituted holoenzyme and competing σ factors. Future analysis, for example, using a plasmon resonance approach, may clarify the exact effects of ppGpp and Rsd on the kinetics of σ factor binding to core RNA polymerase and whether ppGpp acts by weakening $\sigma^{70}$ core interaction and/or strengthening $\sigma^S/\sigma^{32}/\sigma^{54}$ core interaction.

## Materials and methods

### *Bacterial strains and growth conditions*

*E. coli* strains used in this work are listed in Table 1. Strain MO1005EL (Ohnuma et al. 2000) containing a transcriptional *lacZ* fusion to *katE*, was used to create the strain MJ271. The *uspA* promoter, containing the promoter and 4 bp upstream of the –35 region (mm*uspA*4b), fused to *lacZ* on pMJ261 was recombined onto phage λRS45, and recombinants were used to lysogenize MC4100 as described previously (Simons et al. 1987). For $\sigma^{70}$ underproduction, the (CamR) P*trp–rpoD* (Lonetto et al. 1998) construct was introduced into different strains by phage P1-mediated transduction. EC2922 and EC2871 carrying the *rpoD*35 and *rpoD*40 mutations, respectively, were originally isolated as suppressors that restored Po-controlled transcription of a tetracycline resistance gene (and thus Tc resistance) in a Δ*relA* Δ*spoT* ppGpp$^0$ strain. The mutant *rpoD* alleles were subsequently linked to a Tn10 marker by P1-mediated transduction (A. Laurie and V. Shingler, unpubl.). The mutant isolation procedure will be published elsewhere. The strains KK357 and KK358 carrying deletions of the *relA* and *spoT* genes without resistance markers were essentially made as described earlier (Datsenko and Wanner 2000). The primer pairs, 3′-GTAGATA CAGTATATATCAATCTACATTGTAGATACGAGCAAATTT CGGCGTGTAGGCTGGAGCTGCTTC-5′, 3′TAGTTGCGATT TGCCGATTTCGGCSGGTCTGGTCCCTAAAGGAGAGGACGC ATATGAATATCCTCCTTAGT-5′ (for deletion of *relA*) and 3′-CCGTTACCGCTATTGCTGAAGGTCGTCGTTAATCACAAAG CGGGTCGCCCGTGTAGGCTGGAGCTGCTTC-5′, 3′-CTGG CGAGCATTTCGCAGATGCGTGCATAACGTGTTGGGTTCAT AAAACACATATGAATATCCTCCTTAGT-5′ (for deletion of

*spoT*) were chosen so that the coding sequence was deleted for both genes. The elimination is leaving behind the (GTGTAG GCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAAT AGGAACTTCGGAATAGGAACTAAGGAGGATATTCATATG) sequence from pKD3 in place of the disrupted gene(s) (Datsenko and Wanner 2000). Deletions were confirmed with sequencing. Cells were grown at 37°C aerobically in Lysogeny Broth (LB) or minimal M9 medium supplemented with glucose (0.08%), thiamine (10 mM) and all 20 amino acids in excess. When appropriate, the medium was supplemented with carbenicillin (50 μg/mL), chloramphenicol (30 μg/mL), tetracycline (20 μg/mL), or kanamycin (50 μg/mL).

*Plasmids*

Plasmids used in this work are listed in Table 1. To create the mutant $\sigma^{32}$ (Q80R) expression plasmid pKVQ805, the *Nco*I–*Pst*I fragment isolated from phis173 (Joo et al. 1997) was cloned into pKV1278 by fragment exchange. Primers *uspA*-top (5′-AATTC CGGATTGACGGATCATCCGGGTCGCTATAAGGTAAGG ATGGTCC-3′; *Eco*RI site underlined) and *uspA*-bottom (5′-TC GAGGACCATCCTTACCTTATAGCGACCCGGATGATCC GTCAATCCGG-3′; *Xho*I site underlined) were annealed to generate the fragment containing the *uspA* promoter region sequence from –38 to +5 (called mm*uspA*4b) and the fragment was cloned into pTL61T (Linn and St. Pierre 1990) between *Eco*RI and *Xho*I to create pMJ261.

*General methods*

Gel electrophoresis was carried out using 11.5% SDS–polyacrylamide gels. Immunoblotting was performed according to standard procedures using mouse monoclonal antibodies specific for the relevant protein as primary antibody (Neoclone). For detection, we used the ECL-plus blotting kit (Amersham) using alkaline phosphatase-conjugated anti-mouse IgG as secondary antibody (Sigma). Blots were then quantified using the FUJI FILM LAS-1000 device and software.

*In vitro synthesis and purification of ppGpp*

Preparative-scale synthesis and purification of ppGpp was essentially as described previously (Carmona et al. 2000). In brief, synthesis of ppGpp was performed at 30°C using a His-tagged RelA protein (~0.2 mg/mL) in a 5-mL reaction containing 2 mM ATP and GDP, and protease inhibitors (complete, Boehringer Mannheim) in buffer RB (50 mM Tris-acetate at pH 8.0, 15 mM magnesium acetate, 60 mM potassium acetate, 30 mM ammonium acetate, 0.2 mM EDTA, 15% methanol). The reaction was terminated after 12 to 16 h by addition of ice-cold formic acid to 1 M, and followed by centrifugation at 8000 rpm at 4°C for 15 min. The supernatant was diluted (1:6) with 50 mM triethylamine acetate (pH 7.7) and applied to a 25-ml DEAE-Bio-Gel column (Bio-Rad) equilibrated with the same buffer. The column was batch eluted with 50 mM (25 mL), 100 mM (25 mL), 150 mM (25 mL), 200 mM (200 mL), and 350 mM (200 mL) triethylamine acetate (pH 7.7) and collected as 12.5-mL fractions. Peak fractions of pure ppGpp were pooled, lyophilized, and stored at –80°C until used. Purity of the preparations was monitored by thin-layer chromatography on polyethyleneimine cellulose plates (Merck), using 1.5 M $KH_2PO_4$ (pH 3.4) as chromatographic buffer. Concentrations of ppGpp were determined spectrophotometrically at $A_{260}$ using the molar extinction coefficient of 13,700.

β-*galactosidase activity*

Relative β-galactosidase levels were assayed according to the protocol of Miller (1972) with modifications (Albertson and Nyström 1994). The activity is expressed as Miller units; $1000 \times A_{420}nm/(A_{420}nm \times \text{reaction time} \times \text{volume})$. All experiments were repeated several times in to ensure reproducibility and the variation was <10%.

*In vitro transcription assays*

Multiple round transcriptions were performed at 37°C essentially as described previously (Claverie-Martin and Magasanik 1992). Reactions were performed (20 μL) in a transcriptional buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 0.275 mg of BSA/mL. The $\sigma^{70}$ and the $\sigma^{32}$ proteins were overproduced and purified as described by Fujita and Ishihama (1996). Different amounts of wild-type or mutant $\sigma^{70}$ and/or $\sigma^{32}$ were premixed for 5 min with RNAP core (10 nM; Epicentre Technologies) for holoenzyme formation. When ppGpp was used, RNAP core and ppGpp were premixed for 5 min prior to the addition of σ factor/s. Circular DNA template (pJET40, 0.125 μg) containing P*dnaK* was added, and the incubation was continued for 20 min to allow open complex formation. Multiple round transcription was started by adding a mixture of ATP, GTP, and CTP (final concentration, 0.4 mM [each]), as well as UTP (final concentration, 0.06 mM), and [α-$^{32}$P]UTP (5 μCi at >3000 Ci/mmole). After an additional 5 min at 37°C, heparin (0.1 mg/mL) was added, and 5 min later, the reactions were terminated by adding 4 μL of 6X stop buffer (150 mM EDTA, 1.05 M NaCl, 14 M Urea, 3% glycerol, 0.075% xylene cyanol, and 0.075% bromophenol blue). Samples were then analyzed on a 7-M urea-5% acrylamide sequencing gel and quantified using a Bio-Rad PhosphorImager.

*Determination of $\sigma^S$ and $\sigma^{32}$ associated with core RNAP*

Strains were grown in minimal M9 medium supplemented with glucose (0.08%), thiamine (10 mM), and all 20 amino acids in excess. Two hours into stationary phase, 50 mL of the cells was spun, down washed, and resuspended in 3-mL reconstitution buffer (10 mM Tris-HCl [pH 7.6 at 4°C], 0.1 mM DTT, 0.1 mM EDTA, 200 mM NaCl, and 5% glycerol [Maeda et al. 2000]). Crude cell extract were obtained using a 20 K French Pressure Cell (Spectronic Instruments). The extracts were subsequently centrifuged for 3 min at 14,000 rpm. A total of 500 μL of the supernatant was subjected to gel filtration through a HiLoad Superdex 200 prep grade column (bed volume 120 mL) with a smart system (Pharmacia biotech). Elution with reconstitution buffer was performed at a flow rate of 1 mL/min at 4°C into fractions of 0.7 mL. Aliquots (50 μL) of elution fractions were dot-blotted onto PVDF membranes and RNAP subunits were detected with specific antibodies as described above.

## Acknowledgments

We thank C. Gross, M. Kanamori, L-O. Hedén, A. Ishihama, B. Wanner, and A. Farewell for their contribution of plasmids and strains essential to this work. We thank G. Björk for valuable discussions. This work was supported by grants from the Swedish Natural Science Research Council and the European Commission DG XII Framework IV Programme on Cell factories, Project BIO4-CT98–0167 to T.N. and from Swedish Natural Science Research Council and the SSF to V.S.

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