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ppGpp and DksA likely regulate the activity of the extracytoplasmic stress factor σ^E in *Escherichia coli* by both direct and indirect mechanisms

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Summary

One of the major signalling pathways responsible for intercompartmental communication between the cell envelope and cytoplasm in *Escherichia coli* is mediated by the alternative sigma factor, σ^E. σ^E has been studied primarily for its role in response to the misfolding of outer membrane porins. This response is essentially reactionary; cells are stressed, porin folding is disrupted, and the response is activated. σ^E can also be activated following starvation for a variety of nutrients by the alarmone ppGpp. This response is proactive, as σ^E is activated in the absence of any obvious damage to the cell envelope sensed by the stress signalling pathway. Here we examine the mechanism of regulation of σ^E by ppGpp. ppGpp has been proposed to activate at least two alternative sigma factors, σ^N and σ^S, indirectly by altering the competition for core RNA polymerase between the alternative sigma factors and the housekeeping sigma factor, σ^{70}. *In vivo* experiments with σ^E are consistent with this model. However, ppGpp and its cofactor DksA can also activate transcription by Eσ^E *in vitro*, suggesting that the effects of ppGpp on σ^E activity are both direct and indirect.

Introduction

One of the hallmarks of bacteria is their remarkable ability to adapt to different environmental conditions and survive a wide range of cellular stresses. This ability relies on a sophisticated array of stress responses, many of which work by altering transcription so that genes required to combat a particular stress are induced, and those that may be deleterious under the stress conditions are repressed. These stress responses allow the cell to remodel its physiology to meet whatever conditions are encountered.

The bacterial RNA polymerase (RNAP) has five subunits, α, β, β', ω and σ. The catalytic core of the enzyme (E) consists of the $\beta\beta'\alpha_2\omega$ subunits and is capable of transcription elongation and termination. The sigma subunit is required for promoter binding and specific transcription initiation. All bacteria have a primary sigma subunit, known as σ^{70} in *Escherichia coli*, which directs the bulk of cellular transcription. Most bacteria also have one or more alternative sigma factors that direct transcription of specific subsets of genes. In most cases, transcription in bacteria is regulated in response to environmental and cellular cues by repressors or activators, proteins that bind to DNA near specific promoters and modulate the activity of RNAP at that promoter, or by alternative sigma factors, proteins that reprogram gene expression by replacing σ^{70} and redirecting RNAP to promoters specific for that particular sigma factor. *E. coli* has six alternative sigma factors, σ^S, σ^H, σ^N, σ^E, σ^F and σ^{FecI}, which each respond to different cellular stresses (Gruber and Gross, 2003). These two types of transcriptional controls are not mutually exclusive. Repressors or activators can regulate transcription by holoenzymes containing alternative sigma factors, thereby integrating multiple environmental signals at a particular promoter to modulate gene expression appropriately.

Alternative sigma factors are regulated by dedicated signal transduction pathways that are activated by particular stresses, conditions, or developmental programs. For example, in *E. coli* the extracytoplasmic stress factor σ^E is activated by degradation of the σ^E-specific anti-sigma factor RseA in response to conditions that interfere with the folding of outer membrane porin proteins (Alba *et al.*, 2002; Kanehara *et al.*, 2002). The activity of the nitrogen-responsive sigma factor, σ^N, is regulated by the signal-dependent activation of enhancer-binding proteins,

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which are required for transcription initiation by $E\sigma^N$ (Shingler, 1996). Some alternative sigma factors can respond to several different stresses and are regulated by multiple regulators, utilizing a variety of mechanisms. A classic example is σ^S, whose activity is modulated in response to an assortment of stresses including osmotic shock, acid stress, cold shock, and entry into stationary phase, by regulators that affect transcription of the *rpoS* gene, translation of the *rpoS* mRNA, stability of the σ^S protein, and activity of the σ^S protein (Hengge-Aronis, 2002). Regulation of stress responses by these signalling pathways is essentially reactionary; damage or stress occurs, is sensed, and then the cell responds.

Reactionary stress responses, such as those described above, are not always the most effective way to respond to stress. When nutrients are scarce, the cell may not have the resources to rapidly mount a response that requires the energy-consuming processes of transcription and translation. In this case, a more general alarm that activates the individual responses in tandem could effectively preload the cell with stress factors, allowing it to combat stresses should they arise. In *E. coli* the alarmone ppGpp fulfils this role, as it can activate several alternative sigma factors independently of their dedicated signalling pathways (Magnusson *et al*., 2005; Costanzo and Ades, 2006).

ppGpp is well known as a general signal of starvation stress (Cashel *et al*., 1996). The level of ppGpp in the cell is determined by the relative activities of the ppGpp synthase RelA and the bifunctional synthase/degradase SpoT (Xiao *et al*., 1991). The best-studied cellular role of ppGpp is its involvement in balancing the protein synthetic capacity of the cell with nutrient availability. ppGpp levels rise during nutrient downshifts caused by either abrupt changes in media conditions, such as starvation for amino acids, carbon, phosphate, or nitrogen, or upon more complex growth-limiting conditions, such as entry into stationary phase (Cashel *et al*., 1996). Under steady-state growth conditions ppGpp levels are inversely correlated with the growth rate of the culture, lower in rich media and higher in nutrient-poor media (Ryals *et al*., 1982).

ppGpp, in conjunction with a cofactor DksA, can regulate transcription both negatively and positively (Paul *et al*., 2004a; 2005; Mallik *et al*., 2006). Unlike more conventional activators and repressors that regulate promoter activity by binding to sites on the DNA and contacting RNAP, ppGpp and DksA bind only to RNAP and modulate its activity directly (Paul *et al*., 2004a; Perederina *et al*., 2004). ppGpp and DksA reduce transcription of ribosomal RNA genes by $E\sigma^{70}$ and therefore the number of ribosomes in the cell (Paul *et al*., 2004a,b). In addition to inhibiting rRNA synthesis, ppGpp and DksA activate transcription of several genes by $E\sigma^{70}$, including a subset of the genes encoding enzymes required for amino acid biosynthesis (Paul *et al*., 2005). The ω subunit of RNAP, encoded by the *rpoZ* gene, also contributes to the regulation of transcription by ppGpp. RNAP lacking the ω subunit cannot respond to ppGpp *in vitro* (Vrentas *et al*., 2005). ω can also affect transcriptional regulation by ppGpp under some conditions *in vivo* (Rutherford *et al*., 2007).

ppGpp not only regulates transcription by $E\sigma^{70}$, but also has been shown to activate transcription by the alternative holoenzymes $E\sigma^S$, $E\sigma^N$ and $E\sigma^E$, during entry into stationary phase, and $E\sigma^S$ and $E\sigma^N$ in response to other growth-limiting conditions (Gentry *et al*., 1993; Sze and Shingler, 1999; Kvint *et al*., 2000; Costanzo and Ades, 2006). The mechanism by which ppGpp regulates alternative sigma factor activity has been investigated for σ^S and σ^N, but is still not fully understood (Lange *et al*., 1995; Sze and Shingler, 1999; Jishage *et al*., 2002; Laurie *et al*., 2003; Bernardo *et al*., 2006; Szalewska-Palasz *et al*., 2007). ppGpp increases both the expression and activity of σ^S in response to a variety of starvation conditions, and increases the activity, but not expression, of σ^N during entry into stationary phase (Gentry *et al*., 1993; Sze and Shingler, 1999; Kvint *et al*., 2000). As ppGpp binds to core RNAP, it can potentially regulate transcription by RNAP associated with any sigma factor. However, even though the activity of the σ^S and σ^N alternative sigma factors is clearly ppGpp-dependent *in vivo*, this activation may be entirely indirect because ppGpp has not been found to affect transcription by either $E\sigma^S$ or $E\sigma^N$ in *in vitro* transcription assays (Jishage *et al*., 2002; Laurie *et al*., 2003; Bernardo *et al*., 2006; Szalewska-Palasz *et al*., 2007). This has led to a model in which ppGpp is proposed to activate the alternative sigma factors indirectly by altering the competition among sigma factors for core RNAP such that the fraction of alternative sigma factors associated with RNAP increases (Jishage *et al*., 2002; Magnusson *et al*., 2005).

The extracytoplasmic stress factor, σ^E, is activated during entry into stationary phase and this activation is dependent upon ppGpp, not the dedicated cell envelope stress-sensing pathway (Costanzo and Ades, 2006). In this paper, we further investigate the correlation between σ^E activity and ppGpp levels, and the mechanism of regulation of σ^E-dependent transcription by ppGpp. In theory, the model that ppGpp alters the competition among sigma factors for RNAP could be sufficient to explain activation of σ^E by ppGpp. However, *in vitro* experiments demonstrate that ppGpp and DksA can also directly activate $E\sigma^E$-dependent transcription. Therefore, we propose that the positive regulation of σ^E-dependent promoters by ppGpp and DksA has two components: direct regulation of σ^E-dependent transcription and indirect regulation by increasing the amount of $E\sigma^E$ in the cell through negative regulation of transcription of rRNA promoters. This is the

first reported example of direct activation of alternative sigma factor-dependent transcription by ppGpp and DksA.

Results

ppGpp regulates σ^E activity

Previous work demonstrated that the activity of the alternative sigma factor, σ^E, was regulated with respect to growth phase (Costanzo and Ades, 2006). In wild-type cells growing in rich media, σ^E activity can be divided into three parts. σ^E activity is low in early exponential phase ($OD_{600} < 0.3$), then increases fourfold during mid-exponential phase (OD_{600} from 0.4 to 1.8) and 19-fold during entry into stationary phase ($OD_{600} > 1.8$) compared with its activity in early exponential phase. The low activity of σ^E during early exponential phase is not due to recovery from stationary phase and is likely to be a function of growth in fresh media (Costanzo and Ades, 2006). The increase in activity during entry into stationary phase required the alarmone ppGpp, suggesting that ppGpp is a positive regulator of σ^E-dependent promoters. Because these experiments were performed with cultures grown in a rich medium, Luria–Bertani (LB) broth, and entry into stationary phase is complex in rich medium owing to both depletion of nutrients and accumulation of secondary metabolites, all of which can affect gene regulation; it is possible that signals in addition to ppGpp are required for regulation of σ^E activity. To further explore the connection between ppGpp and σ^E activity, we examined σ^E activity under several conditions in which ppGpp levels are known to increase.

To determine if σ^E activity increased during entry into stationary phase under more defined conditions caused by the depletion of a specific nutrient, σ^E activity was monitored in cultures grown in media containing limiting concentrations of glucose (0.02%) or phosphate (0.13 mM). ppGpp levels are known to increase when cells enter stationary phase as either nutrient is depleted (Spira *et al*., 1995; Cashel *et al*., 1996). Transcription by Eσ^E was assayed by measuring β-galactosidase activity produced from a chromosomally encoded reporter in which the σ^E-dependent *rpoH* P3 promoter directs transcription of the *lacZ* gene (Mecsas *et al*., 1993). This reporter contains the σ^E-dependent promoter from the *rpoH* gene and has been used extensively to monitor σ^E activity under a wide range of conditions in a variety of strain backgrounds. σ^E activity increased when cell growth slowed owing to glucose or phosphate depletion, indicating that additional signals accompanying entry into stationary phase in rich media are not required for the regulation of σ^E activity (Fig. 1A and B). The increase in σ^E activity following depletion of a specific nutrient is not a property unique to the *rpoH* P3 promoter. The σ^E-dependent *fkpA* promoter, previously shown to be activated by ppGpp during entry into stationary phase in rich media (Costanzo and Ades, 2006), was activated during entry into stationary phase following phosphate depletion as well (data not shown).

The cellular level of ppGpp in exponential phase varies inversely with the growth rate of a culture, such that ppGpp levels are high in nutrient-poor media supporting slow growth rates, and low in nutrient-rich media supporting high growth rates. Many genes that are subject to regulation by ppGpp also show growth rate-dependent regulation (Cashel *et al*., 1996; Sze and Shingler, 1999; Paul *et al*., 2004b). We monitored transcription by Eσ^E during early exponential phase in cultures grown in media that support different growth rates. σ^E activity was regulated with respect to growth rate, increasing as the growth rate decreased (Fig. 1C).

The above experiments demonstrate that under conditions in which ppGpp levels are known to increase, σ^E activity also increases. However, each of these conditions is accompanied by physiological adaptations to changes in nutrient availability, in addition to the production of ppGpp. This raises the possibility that Eσ^E could be sensing another signal that is coincident with nutrient depletion or culture conditions. If ppGpp were sufficient to regulate transcription by Eσ^E by itself, then σ^E activity should increase when ppGpp is made during exponential phase in rich medium. To test this hypothesis a truncated variant of the *relA* gene that constitutively produces ppGpp without associating with ribosomes was overexpressed from the plasmid pALS13 (Svitil *et al*., 1993). When this variant *relA* gene was overexpressed, σ^E activity increased (Fig. 1D). σ^E activity did not increase upon overexpression of a catalytically inactive variant of the truncated RelA protein that cannot synthesize ppGpp (Fig. 1D). These results demonstrate that additional signals accompanying nutrient depletion are not required for induction of σ^E activity by ppGpp. Because cell growth slows when ppGpp production increases, we cannot formally eliminate the possibility that σ^E activity increases owing to a separate event associated with the transition to slower growth.

If ppGpp is required for increasing σ^E activity, then eliminating the ability of RNAP to respond to ppGpp should eliminate the increase in σ^E activity observed in cells that can make ppGpp. As both DksA and ω have been shown to affect the ability of ppGpp to function in positive and negative regulation *in vitro* and *in vivo* (Vrentas *et al*., 2005; Rutherford *et al*., 2007), we constructed a Δ*dksA* Δ*rpoZ* strain and tested the effects on transcription by Eσ^E. Growth phase-dependent regulation of σ^E activity was disrupted in the Δ*dksA* Δ*rpoZ* strain. Transcription by Eσ^E in this strain was low throughout the growth curve,

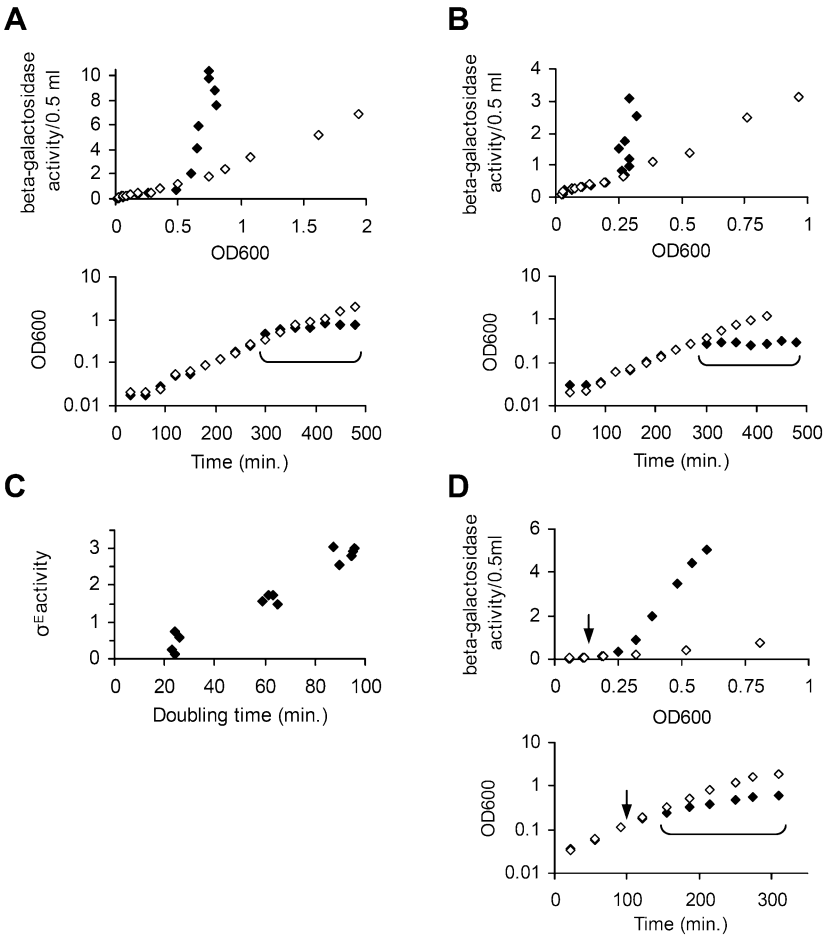

Fig. 1. σ^E activity increases under conditions in which ppGpp levels are known to increase. (A, B, D) Differential rate plots (top) displaying the accumulation of β-galactosidase produced from the σ^E-dependent *rpoH* P3::*lacZ* reporter in SEA001 as a function of the growth of a culture (see *Experimental procedures* for an explanation of differential rate plots) and corresponding growth curves (bottom). β-Galactosidase activity is the amount of *o*-nitrophenol formed, as measured by OD_{420}, divided by the reaction time for each 0.5 ml sample (see *Experimental procedures*). The samples with increased σ^E activity are noted with a bracket on the growth curves.
A–B. Cultures were grown in MOPS minimal media (Teknova) with limiting concentrations of either phosphate or glucose: (A) 0.13 mM phosphate (filled diamonds) or 1.3 mM phosphate (open diamonds), and (B) 0.02% glucose (filled diamonds) or 0.2% glucose (open diamonds).
C. σ^E activity in strain SEA001 was measured in cultures grown in media supporting different growth rates (see *Experimental procedures* for media composition). σ^E activity was determined in early exponential phase ($OD_{600} < 0.3$), before effects from increases in ppGpp levels in late exponential phase occurred.
D. σ^E activity was measured from the *rpoH* P3::*lacZ* reporter following gratuitous induction of ppGpp. IPTG was added (indicated by the arrow) to a final concentration of 20 μM to induce active *relA'* from pALS13 in SEA2025 (filled diamonds) or the catalytically inactive form of *relA* from pALS14 in SEA2026 (open diamonds). The slight increase in σ^E activity seen in the strain with pALS14 reflects the growth phase-dependent increase of σ^E activity in mid-exponential phase (Costanzo and Ades, 2006).
In A, B and D representative data sets are shown. Variation between data sets was less than 10%. In C data from four independent experiments at each growth rate are shown.

increasing only slightly between exponential and stationary phase (Fig. 2A and C).

σ^E activity was altered in the single-mutant Δ*rpoZ* strain, suggesting a role for ω in σ^E-dependent transcription. In wild-type cells, σ^E activity increased fourfold from early exponential phase to mid-exponential phase, while in the Δ*rpoZ* strain σ^E activity only increased twofold at this point in the growth curve (Fig. 2A and C). The regulator responsible for the increase in σ^E activity between early and mid-exponential phase has not been identified, but these results suggest that ω contributes to this regulation. This is one of the few phenotypes that have been detected from deletion of the *rpoZ* gene. It is possible that the effects of the *rpoZ* deletion are in part due to polar effects on the *spoT* gene immediately downstream. However, the *rpoZ* allele used in this study has only a weak effect on SpoT expression (Gentry *et al.*, 1991).

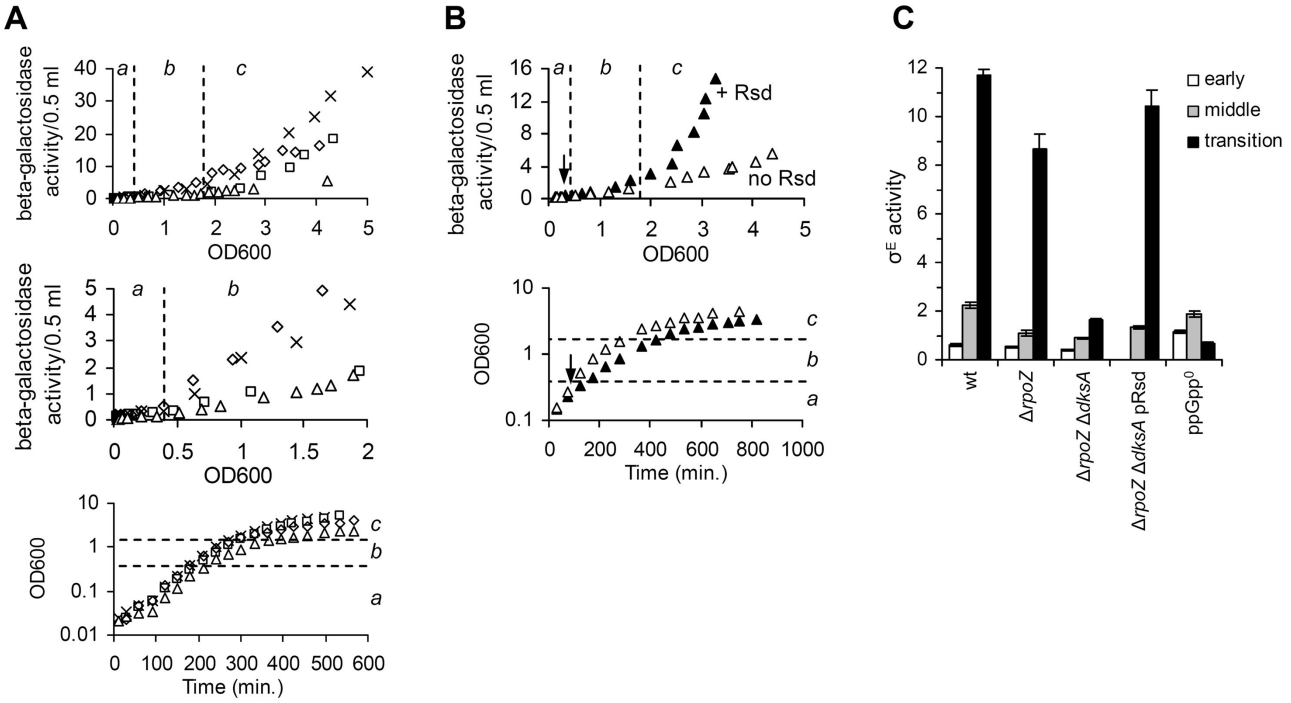

Fig. 2. Growth phase-dependent regulation of σ^E activity is shown for strains lacking proteins required for RNAP to respond to ppGpp.
A. Differential rate plots display the accumulation of β-galactosidase activity in 0.5 ml samples (*o*-nitrophenol min^{-1}) produced from the σ^E-dependent *rpoH* P3::*lacZ* reporter as a function of the growth of a culture for WT (crosses), Δ*dksA* (diamonds, SEA2051), Δ*rpoZ* (squares, SEA6017) and Δ*dksA* Δ*rpoZ* (triangles, SEA6028) strains. The top graph displays data for the entire growth curve, whereas the middle graph displays data only for exponential phase to show the lower activity in strains lacking *rpoZ* during mid-exponential phase. The corresponding growth curves are shown in the lower graph. Data from the Δ*dksA* mutant (Costanzo and Ades, 2006) are shown for comparison with the Δ*dksA* Δ*rpoZ* mutant.
B. Differential rate plot (top) and corresponding growth curve (bottom) displaying σ^E activity measured with the *rpoH* P3::*lacZ* reporter in Δ*dksA* Δ*rpoZ* strains with and without overexpression of *rsd*; pRsd + IPTG (closed triangles, SEA6145), empty vector + IPTG (open triangles, SEA6142), arrow indicates the time of IPTG addition.
The portions of the plots in A and B corresponding to early exponential phase (a, $OD_{600} < 0.4$), mid-exponential phase (b, OD_{600} from 0.4 to 1.8), the transition into stationary phase and early stationary phase (c, $OD_{600} > 1.8$) are indicated on both the differential rate plots and growth curves. For A and B, representative data sets are shown and variation between data sets was less than 10%.
C. σ^E activity, determined from the slope of the line on the differential rate plot, for each part of the growth curve is shown. Slopes were determined using compiled data from at least two experiments, and error bars represent the standard error of the slope. No value is shown for σ^E activity in early exponential phase for the pRsd Δ*dksA* Δ*rpoZ* strain, as *rsd* overexpression was not induced until the end of this phase of growth.

ppGpp affects the activity, not production of σ^E

How does ppGpp regulate σ^E activity? ppGpp could affect the production of σ^E, transcription by Eσ^E, or both production and activity. If ppGpp activates transcription by Eσ^E by increasing the production of σ^E itself, then the level of σ^E in the cell during entry into stationary phase should increase concomitantly with the increase in Eσ^E activity, and the increase in σ^E levels should be dependent on ppGpp. To address this issue, σ^E levels were examined by Western blotting in wild-type strains and in strains unable to produce ppGpp (ppGpp^o) owing to deletion of the *relA* and *spoT* genes. σ^E levels were similar in both the wild-type and ppGpp^o strains (Fig. 3). Furthermore, the levels of σ^E did not increase following gratuitous production of ppGpp owing to overexpression of the constitutive *relA* variant from pALS13 (data not shown). Therefore, the ppGpp-dependent increase in σ^E activity is not caused by an increase in the overall amount of σ^E in the cell.

To further demonstrate that ppGpp regulates transcription by Eσ^E and not the transcription of the *rpoE* gene, we constructed a strain in which the chromosomal copy of the *rpoE* operon (*rpoE*, *rseA*, *rseB* and *rseC*) was deleted and the *rpoE* gene was expressed from a plasmid under the control of the σ^{70}-dependent pTrc promoter. The pTrc promoter is repressed by the Lac repressor, but is somewhat leaky. The uninduced level of σ^E expression was sufficient to maintain cell viability (*rpoE* is essential). σ^E activity was higher in this strain than in a wild-type strain, because the anti-sigma factor, *rseA*, was deleted as part of the *rpoE* operon. However, σ^E activity was still regulated with respect to growth phase and changes in σ^E levels cannot account for this regulation, indicating that transcriptional regulation of the *rpoE* operon promoter is not required for

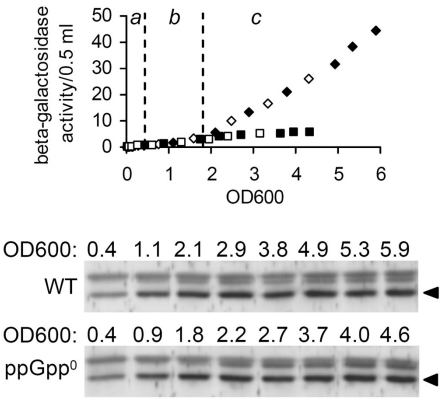

Fig. 3. ppGpp does not affect the steady-state level of σ^E
Differential rate plot displaying σ^E activity measured with the *rpoH* P3::*lacZ* reporter in wild-type (diamonds, SEA001) and ppGpp° (squares, SEA2010) strains as a function of the growth of the culture is shown. At the time points indicated by filled symbols, samples were removed and used for the Western blotting analysis to determine the steady-state level of σ^E. The portions of the plots corresponding to early exponential phase (a), mid-exponential phase (b), and the transition into stationary phase and early stationary phase (c) are indicated as described for Fig. 2. Western blots probed with anti-σ^E antibody are displayed below. Equal amounts of protein extracts were loaded in each lane. The σ^E band is indicated with an arrowhead. A cross-reacting band, which is present in strains lacking the *rpoE* gene, runs directly above the σ^E band. A representative blot is shown and similar results were obtained in three separate experiments.

the growth phase-dependent increase in transcription by Eσ^E (data not shown).

Regulation of σ^E-dependent transcription by ppGpp

As ppGpp does not affect the level of σ^E, it must alter its activity. ppGpp binds to core RNAP and could directly affect transcription by Eσ^E with or without the assistance of DksA. Alternatively, ppGpp could indirectly affect σ^E-dependent transcription. Indirect regulation could be achieved by several mechanisms. For example, ppGpp could regulate the expression of a co-activator protein or molecule that is required specifically for transcription by Eσ^E. ppGpp could alter the competition among sigma factors, including σ^E, for core RNAP, as suggested by studies on the regulation of transcription by the alternative sigma factors σ^S and σ^N by ppGpp (Magnusson *et al.*, 2005). The model that ppGpp alters the competition among sigma factors for RNAP is supported by the observation that the *rpoD*P504L and *rpoD*S506F mutations, which lower the affinity of σ^{70} for core RNAP (Hernandez and Cashel, 1995; Sharp *et al.*, 1999; Gruber *et al.*, 2001), restore σ^N activity during entry into stationary phase in cells lacking ppGpp (Sze and Shingler, 1999; Laurie *et al.*, 2003). These mutations were originally isolated as suppressors of growth defects of ppGpp° strains on media lacking amino acids, suggesting that they also suppress defects in Eσ^{70} activity in ppGpp° strains (Hernandez and Cashel, 1995). To determine whether these σ^{70} variants could suppress the defect in σ^E activity in strains lacking ppGpp, transcription by Eσ^E was monitored in ppGpp° strains carrying these mutations. In the ppGpp° strains with either the *rpoD*S506F or *rpoD*P504L alleles, not only was transcription by Eσ^E restored during entry into stationary phase, but it was also nearly constitutive throughout the growth curve. σ^E activity increased by early exponential phase to a level comparable to that observed during entry into stationary phase in the wild-type strain (Fig. 4A, D and E). Similar results were obtained with the σ^E-dependent *fkpA* promoter in *rpoD*S506F ppGpp° and *rpoD*P504L ppGpp° strains (data not shown). These results indicate that ppGpp itself is not absolutely required for σ^E activity. The σ^{70} variants not only suppress the requirement of ppGpp for σ^E activity, but also nearly eliminate the growth phase-dependent regulation of transcription by Eσ^E throughout the growth curve.

The observation that mutations in σ^{70} that reduce its affinity for core RNAP render σ^E activity nearly constitutive suggests that σ^E activity is higher because it can compete better against σ^{70} for binding to core RNAP, and ppGpp is no longer needed. Therefore, other mechanisms to reduce the ability of σ^{70} to bind to RNAP should also restore σ^E activity in a strain lacking ppGpp. Rsd is a σ^{70}-specific anti-sigma factor that binds to σ^{70} and blocks its association with core RNAP (Jishage and Ishihama, 1998). To determine if overexpression of *rsd* could also restore σ^E activity in a strain lacking ppGpp, transcription by Eσ^E was monitored in a ppGpp° strain carrying a plasmid with the *rsd* gene placed under the control of an IPTG-inducible promoter. Overexpression of *rsd* restored the growth phase-dependent increase in σ^E activity during entry into stationary phase (Fig. 4B, D and E). These results provide further evidence that ppGpp itself is not absolutely required for this activation. Rsd overproduction will also restore σ^S and σ^N activity in a ppGpp° background, indicating that altering the competition among sigma factors for core RNAP by disabling σ^{70} is a general mechanism to restore alternative sigma factor activity in the absence of ppGpp (Jishage *et al.*, 2002; Laurie *et al.*, 2003).

We demonstrated that the growth phase-dependent regulation of transcription by Eσ^E is disrupted in a Δ*dksA* Δ*rpoZ* strain (Fig. 2A and C), presumably because RNAP cannot respond to ppGpp. As overexpression of *rsd* suppressed the requirement for ppGpp, it should also suppress the defects in σ^E activity in the Δ*dksA* Δ*rpoZ* strain, unless there are additional defects unrelated to ppGpp that affect σ^E activity in this strain. Overexpression of *rsd* did restore σ^E activity during entry into stationary phase (Fig. 2B and C), providing further evidence that at least

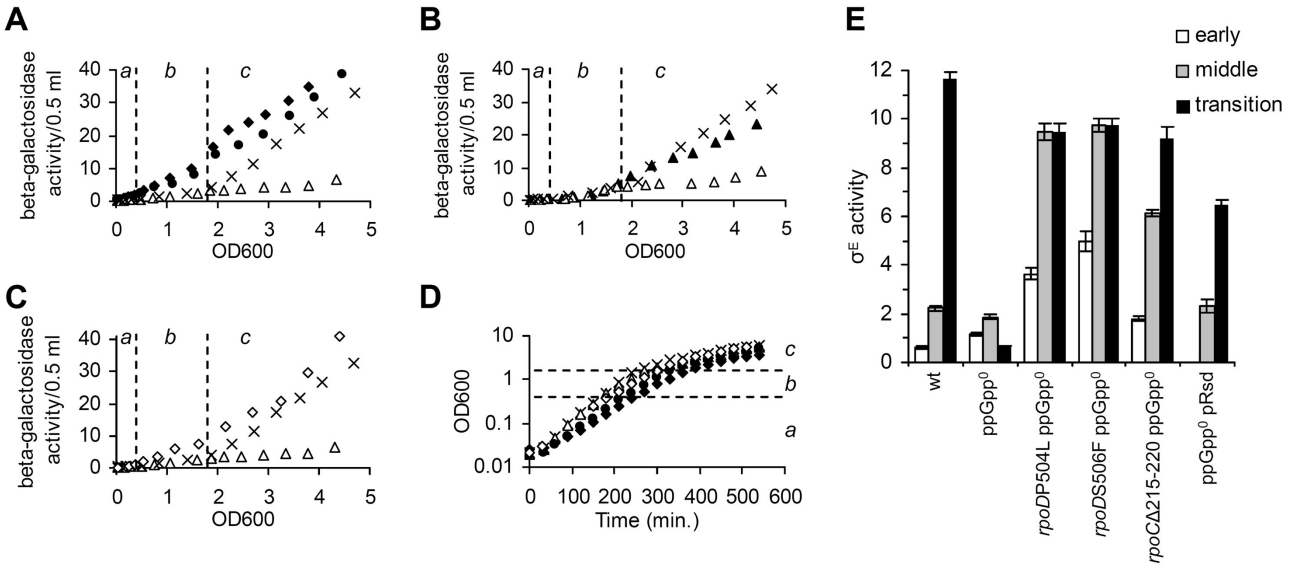

Fig. 4. Suppression of the requirement for ppGpp for the increase in σ^E activity during entry into stationary phase by (A) mutations in *rpoD*, (B) overexpression of *rsd* and (C) a mutation in *rpoC*. A–C are differential rate plots displaying the accumulation of β-galactosidase activity in 0.5 ml samples (*o*-nitrophenol min^{-1}) produced from the σ^E-dependent *rpoH* P3::*lacZ* reporter as a function of growth and D shows growth curves for the strains. Each panel displays data for the wild-type strain (crosses, SEA001), the ppGpp° (open triangles, SEA2010) strain, and the following strains: (A and D) ppGpp° strain with the σ^{70} variants *rpoD*P504L (filled circles, SEA2027) and *rpoD*S506F (filled diamonds, SEA2028), (B and D) ppGpp° with the pRsd plasmid (filled triangles, SEA2144), and (C and D) ppGpp° strain with the β′ variant *rpoC*(Δ215-233) (open diamonds, SEA2136). In (B) *rsd* overexpression was induced at an OD_{600} = 0.2. The portions of the plots corresponding to early exponential phase (a), mid-exponential phase (b), and the transition into stationary phase and early stationary phase (c) are indicated as described for Fig. 2. Representative data sets are shown in A–D and variation between data sets was less than 10%. (E) σ^E activity, determined from the slope of the line on the differential rate plot, for each part of the growth curve is shown. Slopes were determined using compiled data from at least three experiments, and error bars represent the standard error of the slope. For the *rpoD* mutant strains σ^E activity increased before mid-exponential phase and remained high (see text), as such σ^E activity in early exponential phase was calculated before this increase. No value is shown for σ^E activity in early exponential phase for the pRsd ppGpp° strain as *rsd* overexpression was not induced until the end of this phase of growth.

some aspects of the Δ*dksA* Δ*rpoZ* strain phenocopy a ppGpp° strain. The expression of *rsd* itself is regulated by ppGpp, and increases during entry into stationary phase (Jishage and Ishihama, 1999). This observation, along with the results that overexpression of *rsd* can restore the growth phase-dependent regulation of σ^E activity, suggested that ppGpp may indirectly affect σ^E activity by increasing the expression of *rsd*. If this were true, then the pattern of $E\sigma^E$ activity during the growth of cells lacking *rsd* should resemble that of cells lacking ppGpp, i.e. no activation during entry into stationary phase. However, no defects in the growth phase-dependent regulation of transcription by $E\sigma^E$ were observed in a Δ*rsd* strain (data not shown). Therefore, either *rsd* is not required for ppGpp to act, or the regulation is redundant and another regulator controls σ^E activity in the absence of *rsd*.

The above results demonstrate that the requirement for ppGpp to increase σ^E activity can be complemented by conditions in the cell that decrease the ability of σ^{70} to compete for core RNAP. Many mutations that suppress the growth defects in ppGpp-deficient cells have also been mapped to the β and β′ subunits of RNAP (Bartlett *et al.*, 1998; Zhou and Jin, 1998). Several of these mutations will also complement defects in σ^N activity in ppGpp° strains (Sze and Shingler, 1999). We examined the ability of one such mutation, a deletion of amino acids 215–220 of the β′ subunit, to restore $E\sigma^E$ activity in a ppGpp deficient strain (Bartlett *et al.*, 1998). This deletion in β′ is not at the sigma/core interface and is unlikely to affect the affinity of σ^{70} for core RNAP. The increase in σ^E-dependent activity during entry into stationary phase was restored in the ppGpp° strain containing the *rpoC*Δ215-220 mutation, indicating that alterations in β′ can also complement the defects in σ^E activity observed when ppGpp is lacking (Fig. 4C, D and E). σ^E activity was also elevated during mid-exponential phase in this strain, approximately threefold compared with the wild-type strain, but not to the same extent as in the strains with mutations in *rpoD* (Fig. 4E). Again, similar results were obtained from the *fkpA* promoter in the ppGpp°*rpoC*Δ215-220 strain (data not shown). This mutation in β′ most likely restores σ^E activity indirectly via its destabilizing effects on ppGpp-sensitive σ^{70}-dependent rRNA promoter complexes, thereby increasing the availability of core RNAP for binding to σ^E (see *Discussion*). We have not formally ruled out that this mutation could also directly favour tran-

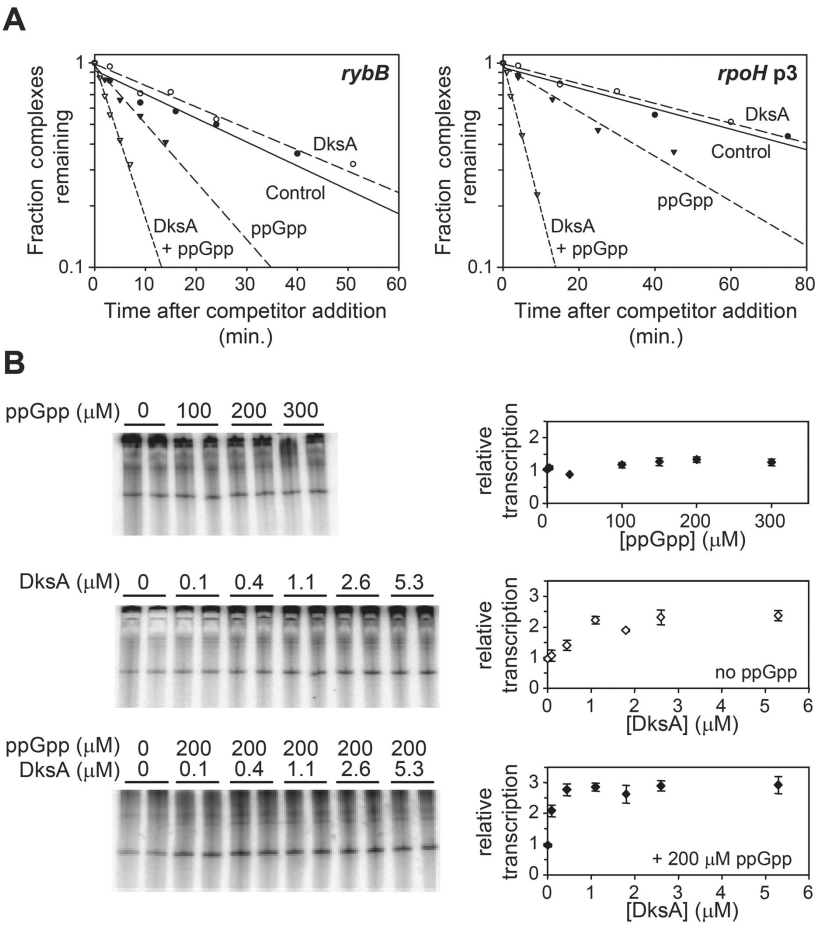

Fig. 5. Effects of ppGpp and DksA on Eσ^E-dependent transcription.
A. DksA and ppGpp together, as well as ppGpp alone, increase the RNAP–promoter open complex decay rate *in vitro* at the Eσ^E-specific *rpoH* P3 and *rybB* promoters. Lifetimes of the competitor-resistant open complex were measured using a transcription-based assay under the following conditions: 2 μM His-DksA, 100 μM ppGpp, 10 nM Eσ^E and 1 nM supercoiled plasmid template (pSEB014 or pSEB015) with 10 μg ml^{-1} heparin as a competitor (see *Experimental procedures* for additional details). Representative decay curves are shown; the absolute rates differed over three assays but the ratios with and without DksA and ppGpp were reproducible (≤ 15% variation).
B. DksA and ppGpp together, as well as DksA alone, increase Eσ^E-dependent transcription in multiround transcription assays from the *rpoH* P3 promoter. To initiate transcription Eσ^E was added to a final concentration of 20 nM to reaction mixes containing 2.5 nM supercoiled plasmid template carrying the *rpoH* P3 promoter (pSEB015), nucleotides and the indicated amounts of ppGpp and/or His-DksA (see *Experimental procedures* for additional details). Representative gels are shown on the left and quantitation of the data on the right. The fold increase in transcription represents the amount of transcript in reactions with ppGpp and/or DksA relative to that in a reaction without ppGpp and DksA. The data were compiled from a minimum of three experiments, average values are shown and error bars represent the standard deviation.

scription initiation by Eσ^E, thus compensating for the loss of activation in the absence of ppGpp.

To explore further the mechanism(s) responsible for activation of σ^E activity, direct effects of ppGpp and DksA were tested on promoter complexes formed by Eσ^E. ppGpp and DksA destabilize RNAP complexes formed on all promoters examined to date (Barker *et al*., 2001a,b; Paul *et al*., 2004a; 2005; Bernardo *et al*., 2006; Mallik *et al*., 2006), but their effects on Eσ^E promoter complexes have not been tested previously. Complexes were formed on two promoters, *rpoH* P3, the same Eσ^E-dependent promoter used in the *in vivo* experiments described above, and *rybB*, the promoter for a σ^E-dependent small RNA whose expression is also regulated by ppGpp/DksA (Thompson *et al*., 2007). The fraction of complexes in the presence of ppGpp, DksA, or the two together that remained at different times after addition of the competitor heparin was measured by *in vitro* transcription (Barker *et al*., 2001). Both promoters formed relatively stable complexes with Eσ^E under these solution conditions ($t_{1/2}$ = 72 min for *rpoH* P3 and $t_{1/2}$ = 26 min for *rybB*; Fig. 5A). DksA by itself had little effect on the lifetimes of competitor-resistant complexes at either promoter, while ppGpp by itself destabilized complex stability by approximately 2.5-fold (Fig. 5A). In contrast, DksA and ppGpp together greatly decreased the lifetimes of the promoter complexes, by sixfold for the *rybB* promoter and 15-fold for the *rpoH* P3 promoter (Fig. 5A). Future studies will be needed to address why DksA alone had little effect on the half-lives of these promoters under these conditions; nevertheless, these results show that ppGpp and DksA can together function on promoter complexes containing Eσ^E RNAP.

Although ppGpp and DksA destabilize open complexes on all promoters yet examined, their effect on overall transcription depends on the properties of the individual promoters (Barker *et al*., 2001a,b; Paul *et al*., 2004a; 2005; Bernardo *et al*., 2006; Mallik *et al*., 2006). Therefore, effects of ppGpp and DksA on transcriptional output were measured by multiround transcription from *rpoH* P3 (representative assays are shown at the left in Fig. 5B, and the results from multiple assays are quantified at the right in Fig. 5B). As observed previously with certain σ^{70}-dependent amino acid biosynthesis promoters (Paul *et al*., 2005), ppGpp by itself exerted little or no effect (Fig. 5B), and as also observed previ-

ously, DksA by itself activated transcription up to approximately twofold (Fig. 5B). When 200 μM ppGpp was also included in the reactions, transcription increased slightly more (up to approximately threefold) and was observed at lower concentrations of DksA (Fig. 5B). Similar effects were obtained on transcription from the *rybB* promoter, although the magnitude of the activation was smaller. In preliminary experiments DksA alone (2 μM) increased transcription from P*rybB* approximately 1.5-fold and DksA/ppGpp (2 μM/100 μM respectively) increased transcription from P*rybB* 1.8-fold, while ppGpp alone had no effect (data not shown). We conclude that ppGpp and DksA can directly activate transcription by Eσ^E, and, as with Eσ^{70}-dependent promoters (Paul *et al*., 2005), different promoters are activated to different extents.

Discussion

The data presented here are consistent with the model that ppGpp works as a general alarm system to redistribute RNAP from promoters for genes required for rapid growth to promoters for genes that are important for survival during stress, shifting the spectrum of genes transcribed by Eσ^{70} and activating genes transcribed by alternative sigma factors (Nystrom, 2004; Magnusson *et al*., 2005; Costanzo and Ades, 2006; Gourse *et al*., 2006). Our data also support the model that ppGpp, along with its cofactor DksA, changes the distribution of RNAP holoenzymes on promoters, both through direct effects on promoter complexes sensitive to ppGpp and DksA and also through indirect effects achieved primarily by reducing transcription of rRNA genes (Zhou and Jin, 1998; Barker *et al*., 2001a; Jishage *et al*., 2002; Paul *et al*., 2005; Szalewska-Palasz *et al*., 2007). In this manner, ppGpp and DksA provide a general mechanism to co-ordinately activate alternative sigma factors.

ppGpp and the regulation of σ^E expression

In addition to co-ordinately regulating alternative sigma factors by altering the distribution of RNAP holoenzymes, ppGpp and DksA exert specific effects on individual alternative sigma factors. ppGpp is thought to increase the production of σ^S, but not of σ^N (Gentry *et al*., 1993; Sze and Shingler, 1999; Kvint *et al*., 2000). We found that the production of σ^E is not dependent on ppGpp. Although one of the two promoters that directs transcription of the *rpoE* operon, *rpoE* P2, is dependent on σ^E (Rouviere *et al*., 1995), not all promoters transcribed by Eσ^E are necessarily subject to control by ppGpp/DksA. Only specific σ^{70}-dependent promoters have the kinetic characteristics that make them sensitive to ppGpp/DksA, and this is likely to be true of σ^E-dependent promoters as well (see below). Alternatively, as the *rpoE* gene is transcribed from two major promoters, one dependent on σ^{70} and the other dependent on σ^E, increased expression from the σ^{70}-dependent *rpoE* P1 promoter might compensate for decreased expression from *rpoE* P2 in the absence of ppGpp. Conversely, if transcription from *rpoE* P2 increases in the presence of ppGpp, transcription from *rpoE* P1 may decrease, thereby maintaining a constant amount of σ^E in the cell.

Direct regulation of σ^E-dependent transcription by ppGpp

ppGpp and DksA can have positive, negative, or no effects on overall transcription, even though these factors reduce the stability of open complexes on all promoters tested to date, including the two σ^E-dependent promoters tested here. This observation can be explained by the fact that ppGpp/DksA affect transcription from only a subset of promoters, those with specific kinetic characteristics. Studies with *rrnB* P1 promoter variants strongly suggest that ppGpp/DksA inhibit transcription by further destabilizing promoter complexes that are intrinsically short-lived. Decay of the open complex formed on these promoters is rate-limiting for transcription initiation (Haugen *et al*., 2006). In contrast, promoters that form stable open complexes are not inhibited by ppGpp/DksA, even though the open complexes are destabilized, because dissociation of the open complex is not rate-limiting for transcription initiation (Haugen *et al*., 2006). Consistent with this model, the open complexes formed by Eσ^E are relatively long-lived and ppGpp/DksA do not inhibit transcription from these promoters. It was somewhat surprising that effects of ppGpp alone but not DksA alone were observed on the lifetime of the open complex, while effects of DksA alone but not ppGpp alone were observed in multiround transcription assays. However, we emphasize that ppGpp/DksA together had parallel effects in the two assays, and this is the condition most relevant to cells.

The mechanism of positive control of transcription by ppGpp/DksA is much less well understood than negative control. Experiments with the σ^{70}-dependent P*argI* promoter suggest that ppGpp/DksA activate transcription by increasing the isomerization rate from the closed to open complex (Paul *et al*., 2005). A model based on the results with P*argI* and other amino acid biosynthetic promoters has been proposed explaining how DksA/ppGpp could reduce the free energy of a transition state on the pathway to open complex formation, and this could result in positive effects on transcription (Paul *et al*., 2005). The kinetics of transcription initiation for Eσ^E have not yet been studied in detail. In fact these studies present the first measurements of open complex stability for Eσ^E on any promoter. Studies on the mechanics of transcription initia-

tion at $E\sigma^E$-dependent promoters will be needed to elucidate how pGpp/DksA acts on these complexes.

Indirect regulation of alternative sigma factor transcription by ppGpp

$E\sigma^E$ activity increased fivefold during entry into stationary phase in the *in vivo* reporter assays with the *rpoH* P3 promoter. This increase was greater than that observed by ppGpp and DksA *in vitro*. Although the solution conditions may not adequately mimic the conditions *in vivo*, a likely explanation for the disparity between the magnitude of the observed effects *in vivo* and *in vitro* is that a component of the activation of σ^E-dependent promoters results from indirect effects of ppGpp and DksA. Previous models proposed to explain how ppGpp positively regulated transcription by $E\sigma^S$ and $E\sigma^N$ invoked alteration of the competition among sigma factors for RNAP (Hernandez and Cashel, 1995; Zhou and Jin, 1998; Jishage *et al*., 2002; Magnusson *et al*., 2005; Szalewska-Palasz *et al*., 2007).

Several hypotheses can explain how ppGpp alters the competition among sigma factors for RNAP. The observation that both mutations in *rpoD*, which reduce the affinity for core RNAP, and overexpression of the σ^{70}-inhibitor *rsd* bypass the requirement of ppGpp for $E\sigma^E$, $E\sigma^S$ and $E\sigma^N$ activity *in vivo* suggests that ppGpp could affect the association of sigma factors with RNAP favouring alternative sigma factors (Hernandez and Cashel, 1995; Sharp *et al*., 1999; Sze and Shingler, 1999; Jishage *et al*., 2002; Laurie *et al*., 2003). However, to our knowledge it has not been demonstrated that ppGpp and/or DksA directly alters the affinity of any sigma factor for core RNAP. The model also does not explain suppression of the defects in σ^E activity by the *rpoC* variant. The *rpoC* mutation, a deletion of amino acids 215–220, is not at the sigma/core interface and therefore is less likely than the *rpoD* mutants to alter the affinity of σ^{70} for core RNAP.

An alternative explanation is that ppGpp alters the competition by increasing the amount of free RNAP available to bind to all sigma factors via its effects on transcription of ribosomal RNAs. In rapidly growing cells, up to 70% of the RNAs transcribed in the cell are stable rRNAs encoded in long operons (Bremer and Dennis, 1996). When ppGpp levels increase, transcription of these operons decreases and the core RNAP that was actively engaged in transcription will be released upon transcription termination (Cashel *et al*., 1996; Barker *et al*., 2001b). This release of core RNAP will increase the size of the free pool of the enzyme available to bind all sigma factors. This model is consistent with phenotypes of the *rpoC* mutation and with additional properties of the *rpoD* mutations, which appear to functionally mimic the effect of ppGpp/DksA on transcription initiation from the rRNA promoters (Bartlett *et al*., 1998; 2000; Barker *et al*., 2001a; Laurie *et al*., 2003; Szalewska-Palasz *et al*., 2007). RNAP containing the $\beta'\Delta$215-220 deletion reduced transcription from the *rrnB* P1 promoters, and the open complexes formed by the variant holoenzyme on this promoter are extremely unstable (Bartlett *et al*., 1998; 2000; Barker *et al*., 2001a). The σ^{70} variants, in addition to having a lower affinity for core RNAP than WT σ^{70}, also further destabilize competitor-resistant open complexes formed on the *rrnB* P1 promoter *in vitro* (Laurie *et al*., 2003; Szalewska-Palasz *et al*., 2007).

The combination of direct and indirect effects of ppGpp on gene expression provide a powerful means of transcriptional regulation. On a global level, ppGpp co-ordinately alters the activity of individual sigma factors, redistributing RNA polymerase among the sigma factors in the cell. This general regulatory mechanism works in conjunction with the ppGpp-independent signalling pathways that are specific for each alternative sigma factor and determine the overall amounts of the different alternative holoenzymes available to respond to changing ppGpp levels. For example, the overall amount of free σ^E available to interact with core RNAP is determined primarily by the amount and proteolytic stability of the anti-sigma factor RseA (Ades, 2004; Costanzo and Ades, 2006). Finally, the unique thermodynamic and kinetic properties of individual promoters ultimately determine the extent to which the direct effects of ppGpp on transcription and the changing amounts of alternative holoenzymes modulate gene expression.

Experimental procedures

Strains and growth conditions

Strains used in this work are listed in Table 1. Mutant alleles were moved into the appropriate strains using P1 transductions according to standard techniques (Miller, 1972). The *rpoD* and *rpoC* mutant alleles with tightly linked *zgh*-3075::Tn*10* (*rpoD* alleles) or *thi39*::Tn*10* (*rpoC* allele) insertions (Hernandez and Cashel, 1995; Bartlett *et al*., 1998) were transferred into strain SEA2010 by P1 transduction. Transductants were selected on tetracycline and the presence of the correct mutation was verified by sequencing. Experiments with the *rpoD*, *rpoC* and *dksA rpoZ* mutant strains were performed with at least two independent transductants to ensure that the results were not affected by spontaneous suppressor mutations. All ppGpp° strains were verified as being unable to grow on minimal media lacking amino acids, and the *rpoD* and *rpoC* suppressor strains were verified to have reverted this auxotrophy. Strain SEA2023 was made by a targeted disruption of the *lacYA* genes in the Φλ*rpoH*P3::*lacZ* reporter of SEA001. The genes were deleted according to the procedure of Datsenko and Wanner (2000) and the drug marker removed by FLP recombinase. Strain SEA2043 was made by transformation of SEA001 with pLC245 expressing the *rpoE* gene (Rhodius *et al*., 2005)

Table 1. Strains and plasmids.

Strain/plasmid	Genotype	Source, reference, P1 donor strain
Strains		
RLG7505	BL21(DE3) p RLG7067	Paul *et al.* (2004a)
SEA001	MG1655 Φλ*rpoH*P3::*lacZ* Δ*lacX74*	Costanzo and Ades (2006)
SEA2010	SEA001 Δ*relA*251::*kan*, Δ*spoT*207::*cam*	Costanzo and Ades (2006)
SEA2023	SEA001 Φλ*rpoH*P3::*lacZ* Δ*lacYA*	This work
SEA2025	SEA2023 pALS13	This work
SEA2026	SEA2023 pALS14	This work
SEA2027	SEA2010 *rpoD*(P504L) *zgh-3075*::Tn*10*	This work, P1 donor *rpoD*5 (Hernandez and Cashel, 1995)
SEA2028	SEA2010 *rpoD*(S506F) *zgh-3075*::Tn*10*	This work, P1 donor *rpoD*11 (Hernandez and Cashel, 1995)
SEA2043	SEA001 Δ*rpoE*::*kan* pLC245	This work
SEA2051	SEA001 Δ*dksA*::*tet*	Costanzo and Ades (2006)
SEA2103	SEA001 Δ*rsd*::*tet*	This work
SEA2136	SEA2010 *rpoC*(Δ215-220) *thi39*::Tn*10*	This work, P1 donor RLG3381 (Bartlett *et al.*, 1998)
SEA2144	SEA001 pRsd	This work
SEA5036	BL21(DE3) *slyD*::*kan* p*LysS* pPER76	This work, P1 donor BB101 (Chivers and Sauer, 1999)
SEA6017	SEA001 *rpoZ*::*cam* (Δ*spoS3*::*cam*)	This work, P1 donor CF2790 (Gentry *et al.*, 1991)
SEA6028	SEA001 *dksA*::*tet rpoZ*::*cam* (Δ*spoS3*::*cam*)	This work, P1 donors SEA2027 and CF2790 (Gentry *et al.*, 1991)
SEA6145	SEA6028 pRsd	This work
SEA6142	SEA6028 pTrc99a	This work
Plasmids		
pALS13	P*tac* truncated *relA*, active protein, Ap^R	Svitil *et al.* (1993)
pALS14	P*tac* truncated *relA*, inactive protein, Ap^R	Svitil *et al.* (1993)
pRLG7067	pET28a-His-*dksA*, Kan^R	Paul *et al.* (2004a)
pLC245	*rpoE* in pTrc99a, Ap^R	Rhodius *et al.* (2005)
pPER76	*rpoE* in T7 expression vector pET15b, Kan^R	Rouviere *et al.* (1995)
pRLG770	General transcription vector, Ap^R	Ross *et al.* (1990)
pRsd	*rsd* in pTrc99a, Ap^R	This work
pSEB014	*rybB* promoter and gene in pRLG770, Ap^R	This work
pSEB015	isolated *rpoH*P3 promoter in pRLG770, Ap^R	This work
pTrc99a	Vector, pBR322 ori, Ap^R	Pharmacia

followed by P1 transduction of the Δ*rpoE-rseC::kan* allele. Strains were grown in LB broth at 30°C with aeration unless otherwise noted.

Plasmid constructions

Plasmids used in this study are listed in Table 1. The plasmid pRsd was constructed by PCR amplifying the *rsd* gene from the chromosome and cloning it into the expression plasmid pTrc99a. Plasmids used as transcription templates are derivatives of pRLG770 (Ross *et al.*, 1990), containing the σ^E-dependent *rpoH* P3 (pSEB015) or *rybB* (pSEB014) promoters. The *rpoH* P3 promoter was amplified with the primers 5′-GGGCCGGAATTCGCCTTGATGTTACCCGAGAG-3′ and 5′-GGGCCAGGTGGAGACCCCCAGTCACGACGTTGTAAA-3′ from plasmid p2*rpoH*P3, which has the isolated σ^E-dependent *rpoH* P3 promoter cloned into the PstI-HindIII sites of pUC19. The *rpoH* P3 promoter sequence, from the −35 region to the +1 for transcription, is identical to that in the chromosomal *lacZ* fusion (λ*rpoH*P3::*lacZ*) used to study σ^E activity (Mecsas *et al.*, 1993). The resulting PCR product was digested with EcoRI and BsaI then cloned into the EcoRI and HindIII of pRLG770 to make pSEB015. The *rybB* promoter region and *rybB* gene, including the native transcription terminator, were amplified from genomic DNA by PCR with the primers: 5′-GGGCGGGAATTCGTTGTTCGGCGCAATGAT-3′ and 5′-GGGCCAAGCTTGTTGAGAGGGTTGCAGGGTA-3′ and cloned into the EcoRI and HindIII of pRLG770 to make pSEB014.

Western blotting

Whole-cell extracts were prepared as described (Gentry *et al.*, 1993). Briefly, cells were lysed in protein sample buffer, proteins were precipitated with acetone, and re-suspended in 2% SDS. Protein concentrations were determined using the BCA Protein Assay (Pierce). Ten micrograms of total protein from each sample was loaded onto 12% polyacrylamide-SDS gels and transferred to Hybond™-P, PVDF membrane (GE Healthcare). Bands containing σ^E were detected by probing the blots with rabbit polyclonal antibodies raised against σ^E (gift from CA Gross), then with alkaline phosphatase conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). The secondary antibody was visualized with the ECF reagent from GE Healthcare according to the manufacturer's instructions. Blots were scanned using Typhoon 8600 Imager in fluorescence mode.

β-Galactosidase assays

Overnight cultures were diluted to an OD_{600} of 0.02 and grown with shaking in a gyratory water bath at 30°C. Samples (0.5 ml) were collected throughout the growth curve. The β-galactosidase activity of each sample was measured by the standard assay (Miller, 1972) and is expressed as the OD_{420} of the reaction mixture divided by the reaction time (*o*-nitrophenol min^{-1}). The data are presented as differential rate plots in which β-galactosidase activity in each 0.5 ml sample is plotted versus the optical density (OD_{600}) of the

sample. β-galactosidase activity (*o*-nitrophenol min^{-1}) per 0.5 ml sample is plotted, rather than standard Miller Units (*o*-nitrophenol min^{-1} OD_{600}^{-1}), therefore the slope of the curve at each time point indicates the change in β-galactosidase activity with increased cell number. The plots illustrate how σ^E activity changes throughout the growth curve, in recovery from stationary phase, exponential phase and re-entry into stationary phase. A complete explanation of differential rate plots is presented in Costanzo and Ades (2006). Experiments were repeated a minimum of three times with independent cultures.

For measurements of σ^E activity as a function of growth rate, cultures were grown in MOPS minimal media (Teknova) with 0.4% glycerol, MOPS minimal media (Teknova) with 0.2% glucose, EZ rich media (Teknova) and LB at 37°C. σ^E activity was determined in early exponential phase to avoid interference from any additional regulation owing to changing ppGpp levels during entry into stationary phase. Activity was determined by the slope of the line on a differential rate plot of β-galactosidase activity in 0.5 ml of culture (as described above) versus OD_{600}. Experiments were repeated a minimum of three times to ensure reproducibility. σ^E activity in early exponential phase reflects growth in fresh media at a low optical density. σ^E activity is the same whether it is measured in cultures directly following dilution of a saturated overnight or following repeated dilution of cultures that had reached exponential phase (OD_{600} ~0.3) to ensure that the cells are in so-called steady-state growth (Costanzo and Ades, 2006; and data not shown).

Protein purification

N-terminally His-tagged σ^E was purified from strain BL21(DE3) *slyD*::*kan* p*LysS* pPER76 as previously described (Campbell *et al*., 2003). Briefly, cells were grown at 25°C to an OD_{600} of 0.5 at which point IPTG was added to induce protein production. Following 1.5 h of induction, cells were harvested by centrifugation, re-suspended in lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole, 2.5 mM β-mercaptoethanol, 1 mM PMSF). Cells were lysed by sonication and the lysate was cleared by centrifugation. The supernatant containing soluble His-σ^E was loaded onto a NiNTA column. Bound proteins were eluted with a stepwise gradient of 20, 60, 100 and 200 mM imidazole in column buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2.5 mM β-mercaptoethanol). Fractions containing σ^E were pooled and dialysed into 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 2.5 mM β-mercaptoethanol.

His-tagged DksA was purified as described in Paul *et al*., 2004a or with minor modifications. Briefly, cells were grown at 37°C to an OD_{600} of 0.4 at which point IPTG was added to induce protein production. Following 3 h of induction, cells were harvested by centrifugation, re-suspended in buffer 1 (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol, 0.1 mM PMSF) and lysed by sonication. The lysate was cleared by centrifugation and the supernatant loaded onto a NiNTA column. Bound proteins were eluted with a stepwise gradient of buffer 1 with 75, 150 and 300 mM imidazole. Fractions containing His-DksA were combined and dialysed into buffer 2 (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1 mM DTT) then loaded onto a Hi-Trap™ Q FF column (GE Healthcare). Bound proteins were eluted with a stepwise gradient of buffer 2 with 200, 300 and 400 mM NaCl. Fractions containing His-DksA were pooled and dialysed into Buffer 2 with 20% glycerol.

RNAP–promoter complex decay assays

Lifetime of the competitor-resistant open complex was measured by single-round *in vitro* transcriptional assays as described in Barker *et al*. (2001b) with the exception of the use of $E\sigma^E$ (reconstituted at 30°C from 1:2 ratio of native core RNAP: His-σ^E). Briefly, 10 nM $E\sigma^E$ and 1 nM supercoiled plasmid DNA in transcription buffer (40 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 0.1 mg ml^{-1} BSA), and ppGpp and DksA (as indicated in Fig. 5) were incubated for 10 min at 30°C. Heparin was added to a final concentration of 10 μg ml^{-1}, and aliquots were removed to tubes containing NTPs (500 μM ATP, 200 μM GTP, 200 μM CTP, 10 μM UTP and 1.0 μCi [α-^{32}P]-UTP) at various times after heparin addition. Transcription reactions were stopped after 10 min with an equal volume of urea-based gel loading buffer. Transcripts were separated on a 6% polyacrylamide gel containing 7 M urea and then visualized and quantitated by phosphorimaging using ImageQuant software (Molecular Dynamics).

Multiround transcription assays

$E\sigma^E$ holoenzyme was formed by incubating 200 nM core RNAP (Epicentre) with 800 nM His-σ^E for 10 min at 30°C. Multiround transcription reactions were initiated by addition of $E\sigma^E$, to a final concentration of 80 nM σ^E and 20 nM core RNAP, to prewarmed (30°C) transcription mix containing 2.5 nM supercoiled plasmid template, 5% glycerol, transcription buffer, 500 μM ATP, 200 μM CTP, 200 μM GTP, 10 μM UTP, 2.5 μCi [α-^{32}P]-UTP, and the appropriate concentrations of ppGpp and/or His-DksA. After 10 min at 30°C, reactions were stopped by the addition of stop solution (80% formamide, 20 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). Samples were electrophoresed on 6% polyacrylamide gels containing 7.5 M urea, visualized by phosphorimaging and quantified using ImageQuant software (Molecular Dynamics).

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