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The guanosine tetraphosphate (ppGpp) alarmone, DksA and promoter affinity for RNA polymerase in regulation of σ^{54}-dependent transcription

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Summary

The RNA polymerase-binding protein DksA is a cofactor required for guanosine tetraphosphate (ppGpp)-responsive control of transcription from σ^{70} promoters. Here we present evidence: (i) that both DksA and ppGpp are required for *in vivo* σ^{54} transcription even though they do not have any major direct effects on σ^{54} transcription in reconstituted *in vitro* transcription and σ-factor competition assays, (ii) that previously defined mutations rendering the housekeeping σ^{70} less effective at competing with σ^{54} for limiting amounts of core RNA polymerase similarly suppress the requirement for DksA and ppGpp *in vivo* and (iii) that the extent to which ppGpp and DksA affect transcription from σ^{54} promoters *in vivo* reflects the innate affinity of the promoters for σ^{54}-RNA polymerase holoenzyme *in vitro*. Based on these findings, we propose a passive model for ppGpp/DksA regulation of σ^{54}-dependent transcription that depends on the potent negative effects of these regulatory molecules on transcription from powerful stringently regulated σ^{70} promoters.

Introduction

The σ^{54}-factor is involved in controlling many physiological processes that are responsive to environmental cues ranging from assembly of motility organs and chemotaxis transducers, through nitrogen assimilation and the utilization of different carbon sources, to alginate biosynthesis (reviewed in Valls *et al*., 2004). Hence, global regulatory factors that alter the activity or availability of σ^{54}-RNA polymerase (σ^{54}-RNAP) have the potential to mediate far-reaching effects on integrated bacterial responses. One such factor is the nucleotide alarmone guanosine tetraphosphate (ppGpp), which is synthesized in response to a variety of nutritional limitations and physicochemical stresses through the action of RelA (synthetase I) and the dual-function SpoT protein (synthetase II) (reviewed in Cashel *et al*., 1996). This nutritional/stress alarmone was first identified through its role in negative regulation of powerful stringent σ^{70}-dependent promoters (e.g. rRNA and tRNA promoters) to adjust translational capacity to growth demands. ppGpp is assisted in this process by the RNAP-binding protein DksA which acts synergistically with ppGpp to amplify its effects on σ^{70} transcription (Paul *et al*., 2004a,b; 2005). Structural modelling suggests that the coiled coil of DksA protrudes through the secondary channel of RNAP to stabilize ppGpp bound adjacent to the active site, thus providing a structural basis for the observed synergy of ppGpp and DksA on transcription (Artsimovitch *et al*., 2004; Perederina *et al*., 2004).

The molecular mechanism(s) by which ppGpp and DksA affect transcriptional initiation from σ^{70} promoters is not fully resolved. Both of these regulatory molecules reduce the lifetime of competitor-resistant open complexes at all σ^{70} promoters analysed so far. The lifetimes of competitor-resistant complexes at rRNA promoters are intrinsically very short, and further shortening by ppGpp/DksA has been proposed to underlie the direct negative effects of ppGpp and DksA on transcription from these promoters (Paul *et al*., 2004a, and references therein). Support for this mechanism also comes from suppressor mutants within the β- and β′-subunits of core RNAP, which likewise destabilize competitor-resistant complexes at σ^{70} promoters (Zhou and Jin, 1998). During rapid growth, transcription from the powerful stringent σ^{70}-rRNA promoters occupies approximately 60–70% of the transcriptional machinery (Bremer and Dennis, 1996). Thus, a likely consequence of ppGpp/DksA-mediated downregulation is an increase in the available pool of core RNAP for holoenzyme formation. In addition to having direct negative effects on transcription from stringent σ^{70} promoters, ppGpp and DksA also exert direct positive effects on transcription from σ^{70} promoters controlling amino acid biosynthetic operons (Paul *et al*., 2005). The lifetimes of competitor-resistant complexes at ppGpp/DksA-stimu-

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lated amino acid biosynthetic promoters are unusually long, and ppGpp/DksA-reduced half-life is not rate-limiting for transcription in these cases. Rather, ppGpp and DksA have been proposed to stimulate transcription by reducing the energy of a transition state intermediate(s) to accelerate rate-limiting formation of open complexes (Barker *et al*., 2001; Paul *et al*., 2005).

Previous work has also demonstrated that efficient *in vivo* transcription from many promoters that are dependent on alternative σ-factors also requires ppGpp (Jishage *et al*., 2002, and references therein). In this capacity, ppGpp has been proposed to modulate the outcome of σ-factor competition for limiting amounts of core RNAP resulting in increased association of alternative σ-factors such as stationary-phase σ^S, the heat-shock σ^H and the structurally distinct σ^{54}, over that of the housekeeping σ^{70}-factor (reviewed in Nystrom, 2004; Magnusson *et al*., 2005). Although yet to be tested, stabilization of ppGpp binding by DksA also implicates DksA in these global regulatory mechanisms. In the case of σ^{54}-dependent transcription, this proposed role of ppGpp is based on analysis of the DmpR-regulated σ^{54}-Po promoter that controls transcription of a (methyl)phenol catabolic operon of the pVI150 plasmid of *Pseudomonas* sp. strain CF600 (reviewed in Shingler, 2003). The σ^{54}-factor is structurally unrelated to the σ^{70} family of proteins, and programmes the RNAP to bind the unusual –24, –12 class of promoters (consensus TGGCAC N5 TTGCa/t; Barrios *et al*., 1999). Expression levels of σ^{54} are about 16–20% of the levels of σ^{70}, and the levels of both of these σ-factors are constant throughout the growth curves and under different growth conditions (Ishihama, 2000). In contrast to σ^{70} and σ^{70}-like alternative σ-factors, σ^{54} imposes kinetic constraints on open complex formation by the holoenzyme polymerase (reviewed in Zhang *et al*., 2002). Consequently, transcription from this class of promoters requires activators that utilize nucleotide hydrolysis to remodel the closed complex to allow initiation of transcription. For DmpR, binding of aromatic phenolic compounds to its N-terminal regulatory domain is required to alleviate interdomain repression to give the transcription-activating form of the protein (O'Neill *et al*., 1998; 2001; Wikström *et al*., 2001).

Superimposed on the aromatic-responsive regulation of σ^{54}-Po transcription mediated through DmpR, global regulation mediated by ppGpp links the output from Po to the physiological status of the cell. Transcription from Po is low during exponential growth on rich media, but swiftly increases at the transition from exponential to stationary phase where ppGpp rapidly accumulates, and in response to growth conditions or artificial manipulations that elicit high ppGpp levels (Sze *et al*., 1996; Sze and Shingler, 1999). Consistently, transcription from this σ^{54}-dependent promoter is severely reduced in *Escherichia coli* and *Pseudomonas putida* strains devoid of ppGpp (Sze and Shingler, 1999; Sze *et al*., 2002). Two main lines of evidence indicate that this dominant level of physiological regulation involves ppGpp-mediated modulation of the ability of σ^{54} to gain access to limiting core RNAP in intact cells: (i) that the requirement for ppGpp can be suppressed by underproduction and/or sequestering of σ^{70}, leading to transcription from the σ^{54}-Po promoter during exponential growth and to a dramatic increase (> 10-fold) in transcriptional output in the post-exponential phase, and (ii) that the *in vivo* requirement for ppGpp can also be suppressed by four mutant σ^{70} proteins that all exhibit defects in competing against σ^{54} for core RNAP *in vitro* (Laurie *et al*., 2003). Hence, ppGpp-mediated enhancement of the otherwise poor ability of σ^{54} to access limited amounts of available core RNAP has been proposed to lead to elevated levels of the σ^{54}-RNAP holoenzyme, thus allowing occupancy and transcription from the Po promoter (Laurie *et al*., 2003).

The identification of DksA as a critical protein in ppGpp-mediated positive and negative regulation of σ^{70} promoters prompted us to evaluate the role of DksA in ppGpp-mediated regulation of σ^{54}-dependent transcription. Based on the results from both *in vitro* and *in vivo* assays, we propose a passive model for ppGpp/DksA regulation of σ^{54}-dependent transcription that depends on their potent negative effects on transcription from powerful stringently regulated σ^{70} promoters.

Results

Both ppGpp and DksA are required for efficient σ^{54} transcription of Po in vivo

Transcription from the Po promoter of pVI150 is growth phase-regulated, and growth phase-dependent transcription from this promoter is maintained with a Po–*luxAB* transcriptional reporter when carried as a single copy on the host chromosome or in multiple copies on an RSF1010-based plasmid in *P. putida* (Sze *et al*., 1996; 2002; Sze and Shingler, 1999). For *in vivo* transcription analysis of promoter activity in wild-type and mutant *E. coli* strains, we used the RSF1010-based σ^{54}-Po promoter luciferase transcriptional reporter plasmid pVI466 (*dmpR*-Po–*luxAB*), which carries the *dmpR* gene in its native configuration with respect to Po, with the *luxAB* genes fused at +291 relative to the transcriptional start. Because of the low expression from the native promoter of *dmpR* in *E. coli*, this genetic system maintains regulator levels close to those of the native pVI150 plasmid of *Pseudomonas* CF600, and reproduces the transcriptional profile observed from the Po promoter in its native context in *P. putida* (Fig. 1A, squares; Sze *et al*., 1996; Sze and Shingler, 1999). The dependence of the Po promoter on

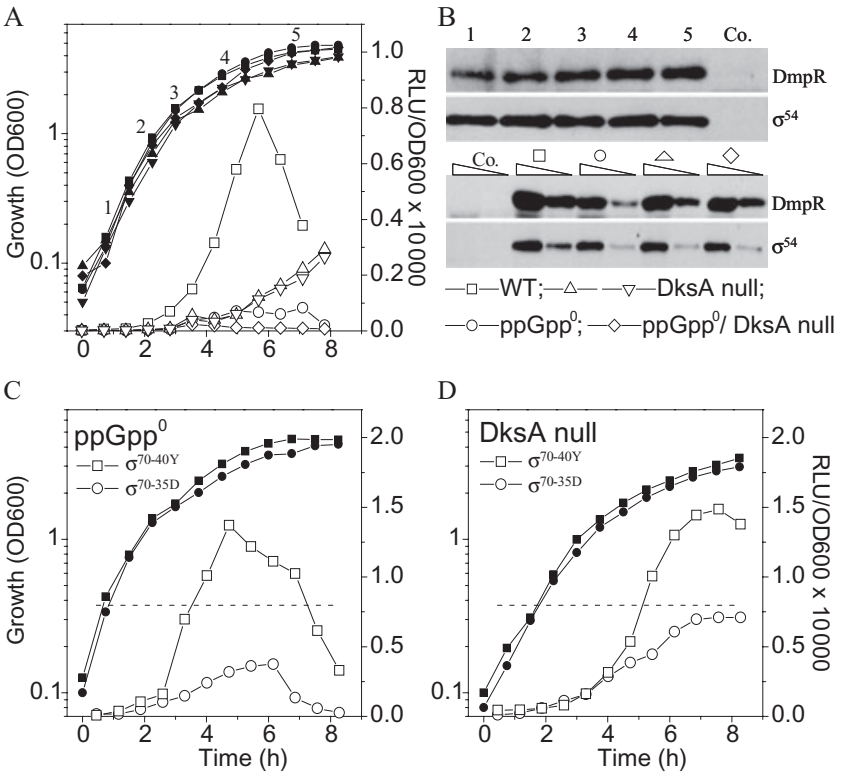

Fig. 1. Luciferase reporter assays of σ⁵⁴-Po promoter.
A. Growth (closed symbols) and luciferase activity (open symbols) in LB-grown MG1655-based *E. coli* strains harbouring pVI466 (*dmpR*-Po–*luxAB*). Strains: wild-type ppGpp+/DksA+ MG1655 (squares), ppGpp⁰ CF1693 (circles), DksA null RK201 (MG1655Δ*dksA*::Km) and MG1655-*dksA*::Tc (up- and down-triangles respectively), and ppGpp⁰/DksA null CF1693-*dksA*::Tc (diamonds).
B. Western analysis of DmpR and σ⁵⁴ in SDS-PAGE-separated crude extracts. Top: 20 μg crude extracts from MG1655 (pVI466) harvested at the indicated time points; bottom: 20 and 10 μg crude extracts from the cultures shown in (A) and harvested at an OD_{600} of 2.5–3.0. Co.: control, is a σ⁵⁴ null mutant of MG1655 lacking the DmpR encoding plasmid pVI466.
C and D. Luciferase reporter assays from the σ⁵⁴-Po promoter of pVI466 in strains harbouring the indicated σ⁷⁰ alleles, cultured as in (A). Derivatives of ppGpp⁰ CF1693 (C); derivatives of DksA null RK201 (D). Dashed lines indicate the maximal activity achieved in wild-type MG1655.

ppGpp leads to a 7- to 10-fold decrease in transcription in the ppGpp⁰ strain (Fig. 1A, circles; Laurie *et al*., 2003, and references therein). Using this genetic system, we also found a large decrease in transcription from the σ⁵⁴-Po promoter in two independent DksA null mutant strains (Fig. 1A, triangles). When combined, the lack of both ppGpp and DksA essentially abolishes detectable transcription from Po in *E. coli* (Fig. 1A, diamonds). These results demonstrate the potent effects of both of these regulatory molecules in DmpR-controlled σ⁵⁴ transcription. The additive negative effect upon loss of both of these regulatory molecules is consistent with the proposed role of DksA in stabilizing the binding of ppGpp to RNAP. However, as DksA can also modulate transcriptional properties in the absence of ppGpp (Paul *et al*., 2004a; 2005), the ppGpp-independent effects of DksA may also contribute to the net *in vivo* effect on σ⁵⁴ transcription.

Western blot analysis revealed that the temporal expression profiles of DmpR and σ⁵⁴ in wild-type and mutant strains are similar. As shown in Fig. 1B for the wild type, DmpR levels increase approximately twofold over the growth curve while σ⁵⁴ levels remain constant. We found a previously undetected small decrease in DmpR levels (approximately twofold; Sze and Shingler, 1999) in the strain lacking ppGpp, while the levels σ⁵⁴ are similar (Fig. 1B, lower). However, coexpression of additional *dmpR* from a plasmid (pVI899) to provide DmpR levels in the ppGpp⁰ strain slightly exceeding those in the wild type did not influence the level of dependence on ppGpp (data not shown). Thus, we conclude that ppGpp and DksA exert their action *in vivo* mainly through a mechanism that is independent of associated alterations in the levels of DmpR or σ⁵⁴.

Mutants of σ⁷⁰ suppress the need for ppGpp and DksA for efficient in vivo *σ⁵⁴ transcription*

The individual and combined *in vivo* effects of lack of ppGpp and DksA are consistent with the known synergistic and ppGpp-independent effects of DksA in regulation of σ⁷⁰ promoters (Paul *et al*., 2004a; 2005). The genetic system described in the preceding section has been used previously to demonstrate that mutants of σ⁷⁰ that are defective in competing against σ⁵⁴ for core RNAP *in vitro* by-pass the need for ppGpp for efficient σ⁵⁴-Po transcription *in vivo* (Laurie *et al*., 2003). Thus, it was of interest to determine whether σ⁷⁰ mutants could also suppress the need for DksA for efficient σ⁵⁴-dependent transcription. To this end, we monitored transcription in strains harbouring the $\sigma^{70\text{-}40Y}$ allele, which has a three-amino-acid insert [∇DSA(536–538)] and exhibits the most extensive defects in *in vitro* competition assays, and the $\sigma^{70\text{-}35D}$ allele, which harbours a single-amino-acid substitution (Y571H) and shows the least extensive defects in competition assays

(Laurie *et al.*, 2003). As shown in Fig. 1C and D, both σ^{70} mutant proteins result in increased σ^{54}-Po transcription in both ppGpp^0 and DksA null strains. The $\sigma^{70\text{-}40Y}$ allele is an efficient suppressor in both cases, restoring transcription in the absence of ppGpp or DksA to approximately 150% and 175% of the levels observed in the wild-type parent respectively. The $\sigma^{70\text{-}35D}$ allele is a comparatively poor suppressor, restoring transcription in the ppGpp^0 and DksA null strains to ~50% and 85% of the level observed in the wild-type parent respectively. These data clearly demonstrate that altered σ^{70} properties can efficiently compensate for the need for ppGpp or DksA *in vivo* to restore transcription from the σ^{54}-dependent Po promoter, and are consistent with both of these regulatory molecules mediating their effect through σ^{70}-dependent transcription.

DksA and ppGpp do not have any direct stimulatory effect on reconstituted in vitro *σ^{54} transcription*

To assess the potential direct effects of ppGpp and DksA on σ^{54} transcription *in vitro*, we used a previously developed multiple-round transcription assay that fully reconstitutes aromatic effector- and ATP-dependent transcription from Po with purified *E. coli* components (O'Neill *et al.*, 2001). To compare the effects of these two regulatory molecules, we used a template carrying σ^{54}-Po (pVI695) in parallel with assays using templates that carry σ^{70} promoters previously shown to be either positively regulated by ppGpp/DksA (P*thrABC*, pRLG5073), or negatively regulated by ppGpp/DksA (*rrnB* P1, pRLG6214) (Paul *et al.*, 2004a; 2005). As a first step in the analysis, we added increasing concentrations of either ppGpp (0–300 μM) or DksA (0–5 μM) alone into reaction mixtures. Neither ppGpp nor DksA had any major stimulatory effect on σ^{54}-Po transcription (Fig. 2A). With the exception of a previously observed stimulatory effect of DksA alone on transcription from the σ^{70}-P*thrABC* (Paul *et al.*, 2005), neither ppGpp nor DksA alone had any notable influence on transcription from either σ^{70}-P*thrABC* or σ^{70}-*rrnB* P1 when tested in isolation. In contrast, increasing concentrations of ppGpp in the presence of a constant level of DksA (2 μM), or increasing concentrations of DksA in the presence of a constant level of ppGpp (200 μM), both resulted in the anticipated synergistic effects on transcription from σ^{70} promoters (positive for P*thrABC* and negative for σ^{70}-*rrnB* P1; Fig. 2B). However, the simultaneous presence of these two regulatory molecules had no influence on the levels of transcription from the σ^{54}-Po promoter (Fig. 2B). Thus, neither ppGpp and/or DksA directly stimulates transcription from the σ^{54}-Po promoter under *in vitro* conditions that recapitulate known positive and negative effects on σ^{70} promoters.

Transcription from σ^{54} promoters is strictly dependent on binding and hydrolysis of nucleotides by the obligatory

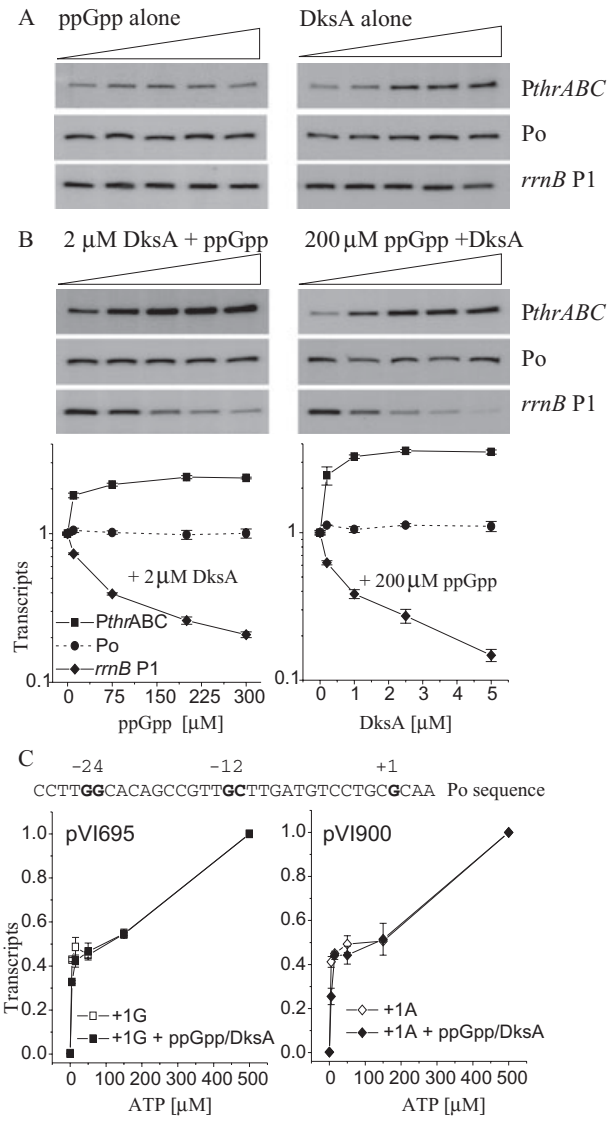

Fig. 2. Multiple-round *in vitro* transcription in the absence or presence of ppGpp and DksA.
A. Autoradiographs of independent ppGpp (0, 25, 75, 200, 300 μM) and DksA (0, 0.3, 1, 2.5, 5 μM) titrations performed at 30°C in T-buffer with 0.5 nM template and 5 nM σ^{70}-RNAP or 5 nM σ^{54}-RNAP as described in *Experimental procedures*. Templates: σ^{54}-Po (pVI695), σ^{70}-P*thrABC* (pRLG5073), σ^{70}-*rrnB* P1 promoter (pRLG6214).
B. Results of ppGpp and DksA titrations performed as in (A), but in the presence of constant concentrations of DksA (2 μM) or ppGpp (200 μM) respectively. Graphs show the normalized data from two independent experiments performed for each promoter with the zero ppGpp or DksA value set as one.
C. ATP titrations (0, 5, 15, 50, 150, 500 μM) with the indicated σ^{54}-Po templates under conditions as in (A), but with 4 mM dATP as the DmpR regulator nucleotide; the remaining nucleotides were at a constant concentration of 200 μM for GTP and CTP, and 80 μM UTP. Graphs are the normalized data from two independent experiments performed for each promoter, with the 500 μM ATP value set as one.

transcriptional activator. DmpR is a dedicated (d)ATPase that can use either ATP or dATP efficiently, but not other nucleotides, to activate transcription from the +1G start of the Po promoter (Wikström *et al.*, 2001). The levels of the

initiating nucleotide influences the observed effects of ppGpp and DksA at σ^{70}-rRNA promoters, and has been proposed to compete with ppGpp at the active site of RNAP (Jores and Wagner, 2003; Paul *et al.*, 2004a,b). Thus, we considered that the 4 mM ATP used in the *in vitro* transcription assays might mask potential effects of ppGpp and DksA on transcription from the σ^{54}-Po promoter. To test this possibility, we used multiple-round transcription assays as described under Fig. 2, but with dATP replacing ATP as the regulator nucleotide. The assays used pVI695, which has the native +1G of the σ^{54}-Po promoter, or pVI900, in which an A replaces the +1G. ATP titrations into reaction mixtures remained unchanged in the presence of ppGpp and DksA with both the +1G or +1A template (Fig. 2C). Similar results were obtained when GTP was titrated into reaction mixtures (data not shown). Thus, we conclude that the level of initiating nucleotide has no influence on the absence of effect of these two regulatory molecules on σ^{54}-dependent transcription from the Po promoter *in vitro*.

Lifetime of competitor-resistant complex at σ^{54}-Po is shortened by ppGpp

The molecular mechanism(s) by which ppGpp and DksA exert their direct effects on transcription from σ^{70}-dependent promoters are still ill-defined. Both these regulatory molecules reduce the lifetime of competitor-resistant open complexes at ppGpp-inhibited and ppGpp-stimulated σ^{70} promoters alike. However, reduced open complex stability will only have regulatory consequences on transcription from negatively regulated promoters such as rRNA operon promoters that have open complex stability as the rate-limiting step. The potential effects of ppGpp and DksA on competitor-resistant open complex stability at σ^{54} promoters are unknown. We therefore tested the lifetimes of competitor-resistant complexes at the σ^{54}-Po promoter in the presence and absence of ppGpp/DksA using an assay based on *in vitro* transcription. In these experiments, open complexes were first accumulated under the same conditions as described above, and then new open complex formation was blocked by addition of competitors. The level of transcript formation at subsequent time points measures the stabilities of the preformed competitor-resistant open complexes. As shown in Fig. 3A, the lifetime of competitor-resistant complexes at σ^{54}-Po lay between those of σ^{70}-*rrnB* P1 promoter (Fig. 3B) and σ^{70}-P*thrABC* (Fig. 3C). Addition of ppGpp (200 μM) reduced the lifetime (Fig. 3A). In contrast, addition of DksA (2 μM) had little effect on the lifetimes when added either alone or in combination with ppGpp (Fig. 3A). Thus, ppGpp appears to be more potent than DksA at reducing the lifetime of competitor-resistant complexes at the σ^{54}-Po promoter. However, this reduction in lifetime does not result in reduced transcription from the σ^{54}-Po promoter under the same conditions (Figs 2 and 3), suggesting that open complex stability is not the rate-limiting step for σ^{54}-dependent transcription from this promoter. Taken together with the data described in the preceding sections, these results suggest that potential ppGpp and DksA effects on transcription through open complex formation or stability do not account for the major stimulatory effect of these molecules on σ^{54}-Po transcription observed *in vivo* (Fig. 1).

DksA and ppGpp do not directly alter σ-factor competition in vitro

Holoenzyme RNAPs utilizing σ^{70} or σ^{54} do not show cross-recognition of the distinct promoter classes that they control. Thus, it is highly unlikely that the σ^{70} mutants compensate for lack of ppGpp or DksA (Fig. 1) by acting

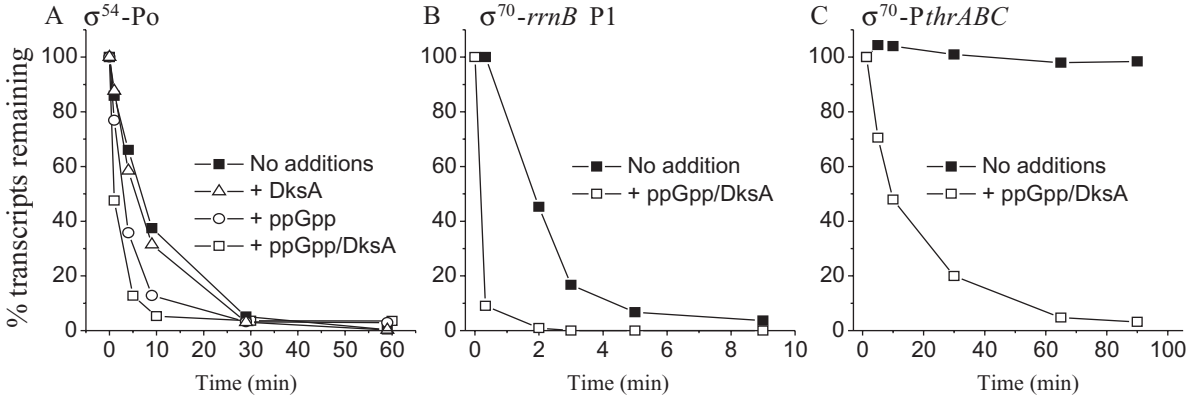

Fig. 3. Competitor-resistant open complex stability in the presence or absence of ppGpp and DksA. Complexes were preformed at 30°C in T-buffer in the absence or presence of DksA (2 μM) and/or ppGpp (200 μM) as described in *Experimental procedures*. Templates: (A) σ^{54}-Po (2 nM pVI695, 5 nM σ^{54}-RNAP), (B) σ^{70}-*rrnB* P1 promoter (4 nM pRLG6214, 10 nM σ^{70}-RNAP) and (C) σ^{70}-P*thrABC* (2 nM pRLG5073, 5 nM σ^{70}-RNAP). Data are presented with the value at time zero for each condition set as 100%.

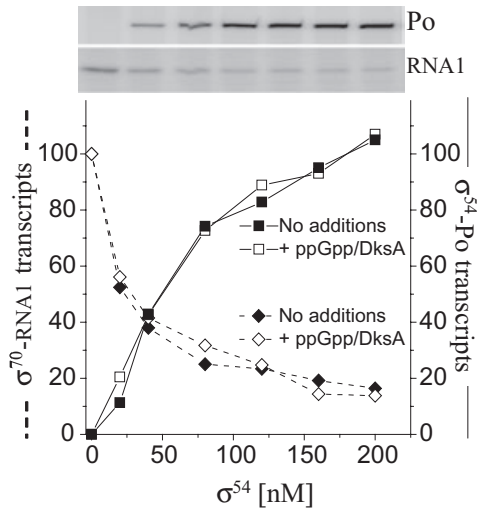

Fig. 4. Multiple-round *in vitro* competition between σ^{70} and σ^{54} in the absence or presence of ppGpp and DksA. Core RNAP (10 nM), σ^{70} (20 nM) and increasing concentrations of σ^{54} (0, 20, 40, 80, 120, 160, 200 nM) were pre-incubated in T-buffer at 30°C in the absence or presence of DksA (2 μM) and ppGpp (200 μM) for 2 h before addition of 5 nM pVI695. Transcript levels are given as a percentage of those achieved for σ^{70}-RNA1 in the presence of 20 nM σ^{70} and the absence of σ^{54} (-----), or for σ^{54}-Po in the presence of 160 nM σ^{54} and in the absence of σ^{70} (——), under each condition.

directly at the σ^{54}-Po promoter. A more plausible interpretation of these *in vivo* results would be that the σ^{70} mutants, which are defective in competing against σ^{54} for limiting amounts of core RNAP, operate at the level of σ-factor association with core RNAP, by either an active or a passive mechanism (Laurie *et al*., 2003). Both σ^{54} and σ^{70} had similarly high affinity for core RNAP when assessed in isolation; however, σ^{54} was significantly poorer at out-competing σ^{70} than the converse in a previously developed multiple-round *in vitro* transcription competition assay that simultaneously monitors the transcriptional output from the σ^{54}-Po promoter and the σ^{70}-RNA1 promoter of pVI695 (Laurie *et al*., 2003). Addition of ppGpp alone does not influence competition between σ^{54} and σ^{70} in this assay system (Laurie *et al*., 2003). Here we used this competition assay to test for a possible active role of ppGpp in the presence of DksA in facilitating association of σ^{54} over that of σ^{70}. The experiment shown in Fig. 4 compares the ability of increasing concentrations of σ^{54} to compete with 20 nM σ^{70} for 10 nM core RNAP in the presence or absence of both ppGpp (200 μM) and DksA (2 μM). Although σ^{54} effectively competed with σ^{70} to reduce σ^{70}-RNA1 transcription to ~20% at the highest concentration tested, the addition of ppGpp and DksA did not alter the level of competition observed. Furthermore, addition of ppGpp and DksA did not enhance σ^{54} transcription over that of σ^{70} transcription in similar experiments in which components were allowed to associate for 5 min rather than for 2 h as in Fig. 4, nor when fixed concentrations of core RNAP (10 nM) and σ^{54} (40 nM) were challenged with increasing concentration of σ^{70} (data not shown). The lack of any direct effects of addition of ppGpp and DksA in σ^{54} versus σ^{70} *in vitro* competition assays (Fig. 4) contrasts with the direct effects of ppGpp on competition between σ^{H} and σ^{70} that are apparent in similar assays (Jishage *et al*., 2002). However, this finding is fully consistent with previous *in vivo* data on manipulation of the levels or availability of σ^{54}, σ^{S} and σ^{70} in wild-type and ppGpp^{0} strains, namely that *in vivo* σ-factor competition, although integral to the efficiency of σ^{54} transcription, is not directly influenced by the presence or absence of ppGpp *in vivo* (Laurie *et al*., 2003). The lack of any detectable direct effect of ppGpp and DksA on *in vitro* competition between σ^{70} and σ^{54} suggests that these two regulatory molecules do not collaborate to alter the binding properties of σ^{70} or σ^{54} for core RNAP to favour association of one σ over that of the other.

Hybrid promoters of Po have different affinities for σ54-RNAP

The Po promoter has relatively low affinity for σ^{54}-RNAP (Sze *et al*., 2001). The effects of ppGpp and DksA on transcription from other σ^{54}-dependent systems that depend on promoters that have different affinities for the holoenzyme have not been tested previously. All the results described above are consistent with a model in which ppGpp and DksA mediate their effects on σ^{54}-dependent transcription *in vivo* indirectly through the activity or availability of σ^{54}-RNAP holoenzyme. If this were indeed the case, then poorly occupied low-affinity promoters would be more susceptible to loss of these regulatory molecules than high-affinity σ^{54} promoters which are easy to saturate. These considerations prompted us to generate and test the activities of a series of hybrid σ^{54} promoters that differed in their innate affinity for σ^{54}-RNAP.

To generate σ^{54} promoters with different affinities for σ^{54}-RNAP as the test variable, we constructed a template that allows simple replacement of the Po –24, –12 promoter region by DNA linkers specifying the desired sequence as depicted in Fig. 5A. This strategy maintains the test promoters in the same context as the native Po promoter with respect to its integration host factor (IHF) binding site and the upstream activating sequence (UAS) binding sites for the divergently transcribed *dmpR* gene product, and places the promoters in control of the luciferase *luxAB* genes. In addition to reconstructing the Po promoter, designated Po/Po, hybrid promoters containing the –33 to +2 regions of σ^{54} promoters originating from different bacteria were generated. These included: (i) the *P. putida*-derived Pu promoter which, like Po, appears to have low affinity for σ^{54}-RNAP (Bertoni *et al*., 1998; Sze *et al*., 2001), (ii) the *Klebsiella pneumoniae*-derived *nifH* promoter and its

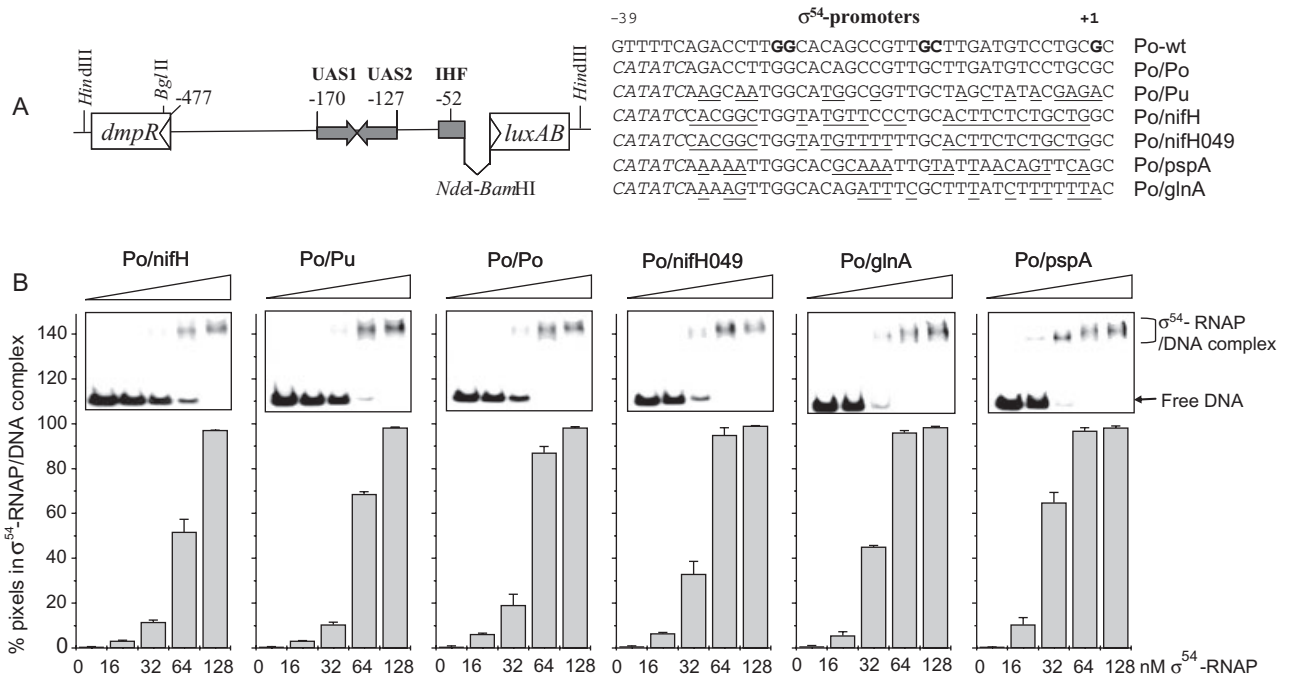

Fig. 5. Hybrid σ^{54}-Po/Px promoter regions.
A. The DNA configuration and restriction enzyme sites used in DNA manipulations are shown, along with the location of coding regions of *dmpR* and the luciferase *luxAB* reporter genes as open boxes, with arrowheads indicating the direction of transcription (not to scale). The locations of the DNA binding sites for DmpR (inverted shaded arrows; UAS1, UAS2) and the IHF recognition sequence (shaded box) are also indicated. The nucleotide sequences of the −39 to +2 regions of hybrid promoters generated upon insertion of linkers with compatible NdeI and BamHI ends into the template are shown to the right, with the consensus −24 GG and −12 GC sequences shown in bold. Nucleotides that differ from Po in the −33 to +2 region are underlined. The sequence of the NdeI site that is destroyed upon insertion of the linkers is shown in italics. Co-ordinates are given relative to the +1 transcriptional start of Po.
B. Band-shift assay of closed complexes between increasing concentrations of σ^{54}-RNAP and 2 nM end-labelled linear DNA fragments (−171 to +126) of the Po/Px hybrid promoters shown in (A). Bar graphs represent the average of two independent experiments.

mutant derivative *nifH049*, in which substitution of the −17 to −15 CCC by TTT increases both affinity and transcriptional output *in vitro* and *in vivo* (Morett and Buck, 1989; Buck and Cannon, 1992) and (iii) the strong *E. coli*-derived *pspA* promoter (Weiner *et al*., 1995) and *glnA* promoter that form stable closed complexes with σ^{54}-RNAP (Popham *et al*., 1989).

To directly compare the relative affinities of the hybrid promoters, EcoRI fragments spanning from −171 to +126 of the hybrid promoters were used in a mobility shift assay to assess closed complex formation in the presence of increasing concentrations of σ^{54}-RNAP (Fig. 5B). The relative affinities of the hybrids were found to lie in the order Po/nifH < Po/Pu < Po/Po < Po/nifH049 < Po/glnA < Po/pspA, which is consistent with limited comparative data available on these promoters in their native context (Morett and Buck, 1989; Buck and Cannon, 1992; Sze *et al*., 2001). *In vitro* occupancy of the hybrid promoters was also measured by following the formation of productive open transcriptional complexes over time. The data in Fig. 6 show that promoter occupancy kinetics differs such that the higher-affinity promoters rapidly approach plateau values, while the lower-affinity promoters show slower kinetics. Similar results were obtained when DmpR and its aromatic effector (which are required for open complex formation) were omitted until 8 min before each time point (data not shown). The relative order of the slower kinetics for the lower-affinity promoters (Po/nifH slower than Po/Pu slower than Po/Po) was the same as the comparative affinities for σ^{54}-RNAP (Fig. 5B). These results define the relative affinities and hierarchy of promoter occupancies of the hybrid promoters under *in vitro* conditions (namely, Po/nifH < Po/Pu < Po/Po < Po/nifH049 < Po/glnA and Po/pspA), and suggest that the promoters would be capable of recruiting σ^{54}-RNAP from the available *in vivo* pool with the same hierarchy.

Hybrid promoters show different dependency on ppGpp and DksA

For *in vivo* transcription analysis of hybrid promoters, transcriptional reporter plasmids carrying the *dmpR*-Po/Px–*luxAB* cassettes were constructed. These plasmids are analogous to pVI466 (*dmpR*-Po–*luxAB*) used in Fig. 1, except that the *luxAB* genes are fused downstream of +2 relative to the transcriptional start. In the reconstructed

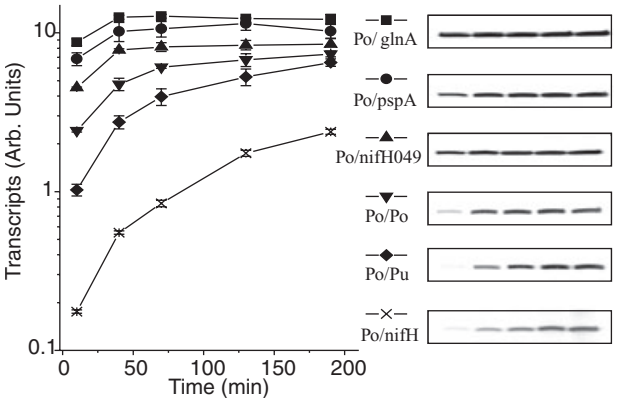

Fig. 6. Time-dependent *in vitro* σ^{54}-RNAP promoter occupancy of hybrid σ^{54}-Po/Px promoters. Transcription assays were performed at 20°C in G-buffer in the presence of 10 nM σ^{54}-RNAP, 25 nM IHF, 50 nM DmpR and 11 nM supercoiled templates, with increasing incubation times as indicated. Data given are the average of two normalized independent experiments. The autoradiographs show differential exposures of the transcripts from each hybrid promoter to illustrate the different kinetics.

Po/Po promoter, the only nucleotide changes from the wild-type sequence are limited to –39 to –36 relative to the transcriptional start, which are outside the critical binding sites for DmpR, IHF and σ^{54}-RNAP (Fig. 5A). As shown in Fig. 7A, the Po/Po promoter reproduced the induction profile and dependency on ppGpp and DksA for efficient promoter output, but with a previously noted approximately fivefold higher luciferase activity relative to the +291 fusion transcriptional reporter pVI466 used in Fig. 1 (Sze and Shingler, 1999; Sze *et al*., 2002).

The temporal expression profiles of all the hybrid promoters showed growth phase-dependent profiles similar to those shown for Po/Po and Po/glnA (Fig. 7A and B), with absolute output levels that differed by 18-fold (Fig. 7C). The powerful high-affinity promoters such as the Po/pspA and Po/glnA hybrids also allowed some level of transcription even in the exponential phase of growth (Fig. 7B, and data not shown). Most importantly, all the Po/Px hybrids were found to require ppGpp and DksA for full transcriptional output to an extent that reflects their affinity for σ^{54}-RNAP *in vitro* (Fig. 7C). The ratio between peak transcriptional output in the presence or absence of ppGpp or DksA from the low-affinity Po/nifH, Po/Po and Po/Pu promoters was reduced 6- to 10-fold while that from the higher-affinity Po/nifH049, Po/pspA and Po/glnA promoters was only reduced two- to threefold. Thus, while all the promoters require both ppGpp and DksA for maximal output, low-affinity σ^{54} promoters are markedly more sensitive to the loss of these two regulatory molecules *in vivo*. As is the case for the native Po promoter, addition of ppGpp and DksA had no significant stimulatory effect on *in vitro* transcription from these hybrid promoters (Fig. 7D). With the exception of the Po/Pu hybrid, the

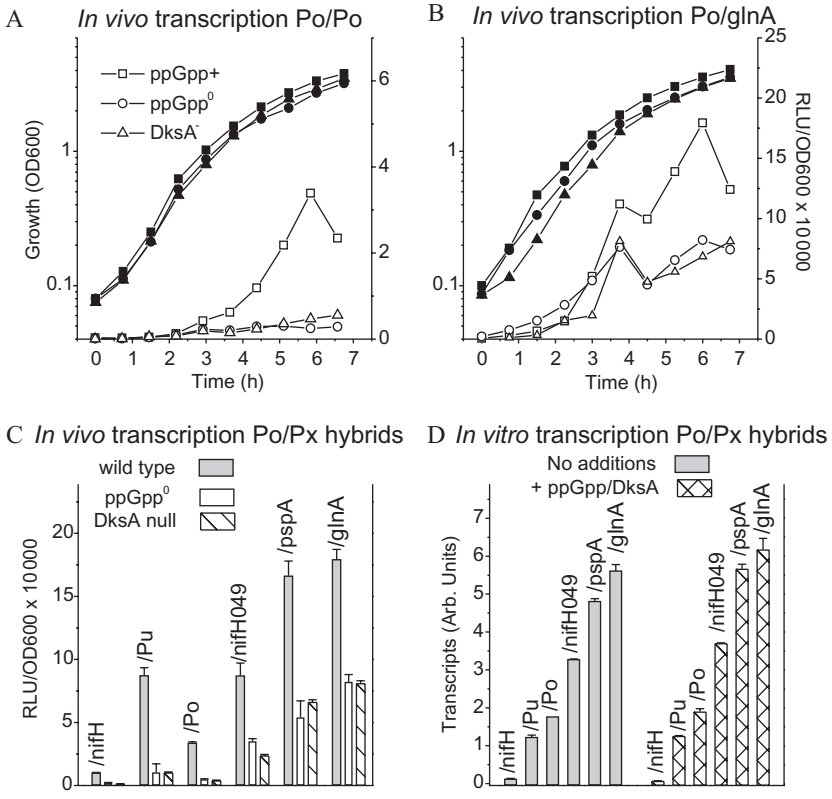

Fig. 7. Transcription of Po/Px hybrid promoters in the absence or presence of ppGpp and DksA.
A and B. Growth (closed symbols) and luciferase activity (open symbols) of LB-cultured *E. coli* MG1655Δ*lac* (squares) and its ppGpp^0 and DksA null counterparts (CF1693Δ*lac*, circles and RK201, triangles respectively) harbouring *dmpR*-Po/Px–*luxAB* reporter plasmids were monitored over time.
C. Peak luciferase activity at the 6 h time point in *E. coli* strains harbouring plasmids pVI704 to pVI727, carrying the hybrid promoters indicated.
D. Multiple-round *in vitro* transcription of pTE103-based supercoiled plasmids harbouring Po/Px hybrid promoters (–578 to +2; 0.5 nM pVI736 to pVI741) in the absence or presence of DksA (2 μM) and ppGpp (200 μM) at 30°C in T-buffer containing 5 nM σ^{54}-RNAP.

hierarchy of promoter strengths observed *in vivo* was the same as that observed *in vitro* (compare Fig. 7C and D).

Dependence on ppGpp and DksA is observed in the absence of IHF binding capacity

A simple interpretation of the data described above is that the greater sensitivity of the low-affinity promoters to loss of ppGpp and DksA is due to poor occupancy by the σ^{54}-RNAP available. However, optimal localization of the activator via IHF-mediated DNA bending is particularly important for transcriptional initiation from low-affinity σ^{54} promoters that are rarely occupied by σ^{54}-RNAP (Hoover *et al*., 1990; Claverie-Martin and Magasanik, 1992; Santero *et al*., 1992; Carmona *et al*., 1997). As IHF levels are partially under the control of ppGpp and show an abrupt increase at the exponential-to-stationary phase transition (Aviv *et al*., 1994; Ditto *et al*., 1994; Valls *et al*., 2002), we considered that the influence of ppGpp and DksA on IHF levels might contribute to the differences in dependence of the hybrid promoters on ppGpp/DksA *in vivo*. To test this possibility, we generated a series of promoter derivatives analogous to Po/Pu, Po/Po, Po/pspA and Po/glnA hybrids that lacked the IHF consensus binding site of Po. This approach, as opposed to the use of an IHF-deficient strain, avoids pleiotropic effects of the loss of IHF, which has global regulatory consequences *in vivo* (Arfin *et al*., 2000). Construction of these plasmids involved introduction of a non-native XhoI site at −122 to −117 relative to the transcriptional start, and alterations that disrupt the IHF binding site consensus but maintain the base composition of Po (Fig. 8A). These hybrids were designated xh-Po/Px and xh-Po(-IHF)/Px to indicate these changes.

In vitro transcription assays in the presence and absence of IHF showed the high IHF dependence of the low-affinity xh-Po/Pu hybrid (32- to 35-fold), the intermediate IHF dependence of xh-Po/Po (10- to 12-fold), and the relatively IHF-independent behaviour of high-affinity xh-Po/pspA and xh-Po/glnA hybrids (only approximately threefold stimulation) *in vitro* (Fig. 8B). As could be anticipated, transcription from cognate derivatives with a disrupted IHF consensus binding site was unaffected by addition of IHF (Fig. 8B). For *in vivo* transcriptional analysis, we used the same genetic systems as described under Fig. 7 to compare transcriptional output from promoters with or without the IHF binding capacity (Fig. 8C). IHF dependence was assessed as the fold decrease in transcriptional output of xh-Po(-IHF)/Px compared with cognate IHF binding-proficient xh-Po/Px derivatives (Fig. 8C, hatched bars). The results show an approximately fivefold decrease in transcription from the Po/Po promoter and an approximately 2.5- to 3.5-fold decrease from the Po/pspA and Po/glnA promoters in the absence of IHF binding *in vivo*. It was initially surprising that the activity of the xh-Po/Pu hybrid, which is extremely IHF-dependent *in vitro*, was unaffected by loss of IHF binding capacity in *E. coli* (Fig. 8C). However, this phenomenon has also been observed with the native Pu promoter at which the *E. coli* HU protein, which binds DNA with little or no site specificity, can functionally replace IHF – both *in vitro* and *in vivo* (Pérez-Martín and De Lorenzo, 1997; Valls *et al*., 2002). The action of HU on the Po/Pu promoter probably also underlies the somewhat different transcriptional profile observed *in vivo* (see below).

To determine the effect of loss of ppGpp and DksA in the absence of any potential influence through modulation of IHF levels, we measured the relative transcriptional output from the hybrids carrying the inactive shuffled IHF binding site [xh-Po(-IHF)/Px derivatives] in $ppGpp^0$ and DksA null strains. Figure 8D shows transcriptional profiles from the test σ^{54} promoters in the absence of ppGpp and/or DksA relative to that in wild-type *E. coli*. In the absence of any potential effect through IHF, ppGpp and DksA were still both required for maximal promoter output in all cases, with simultaneous loss of both molecules essentially abolishing detectable transcription (Fig. 8D), as was the case with the IHF binding-proficient Po promoter (Fig. 1A). Lack of ppGpp had somewhat less effect on σ^{54}-dependent transcription using these IHF-independent derivatives as compared with IHF binding-proficient derivatives, while loss of DksA had a similar effect (compare Figs 7A and B and 8D). Importantly, the low-affinity hybrid promoters, Po/Po and Po/Pu, still showed greater dependency on these two regulatory molecules *in vivo* than high-affinity Po/pspA and Po/glnA counterparts (Fig. 8D). Thus, we conclude that although IHF is required for optimal σ^{54} transcription in most cases, the major role of ppGpp and DksA in *in vivo* σ^{54} transcription is not mediated through their effects on IHF levels.

Discussion

In this study we have investigated the effects of ppGpp and DksA on σ^{54}-dependent transcription and the consequences of the loss of these two regulatory molecules with respect to regulation of σ^{54}-dependent promoters in *E. coli*. Both DksA and ppGpp are required for efficient *in vivo* transcription from the σ^{54}-dependent Po promoter and variants thereof that differ in their innate affinities for σ^{54}-RNAP (Figs 1A and 7). The effects of these regulatory molecules on σ^{54}-dependent transcription are major, with the simultaneous absence of both ppGpp and DksA essentially abolishing detectable transcription from the σ^{54}-Po promoter *in vivo* (Figs 1 and 8D). However, neither of these regulatory molecules, either alone or in combination, directly stimulated reconstituted *in vitro* σ^{54} transcription from six σ^{54} promoters tested (Figs 2 and 7D). These

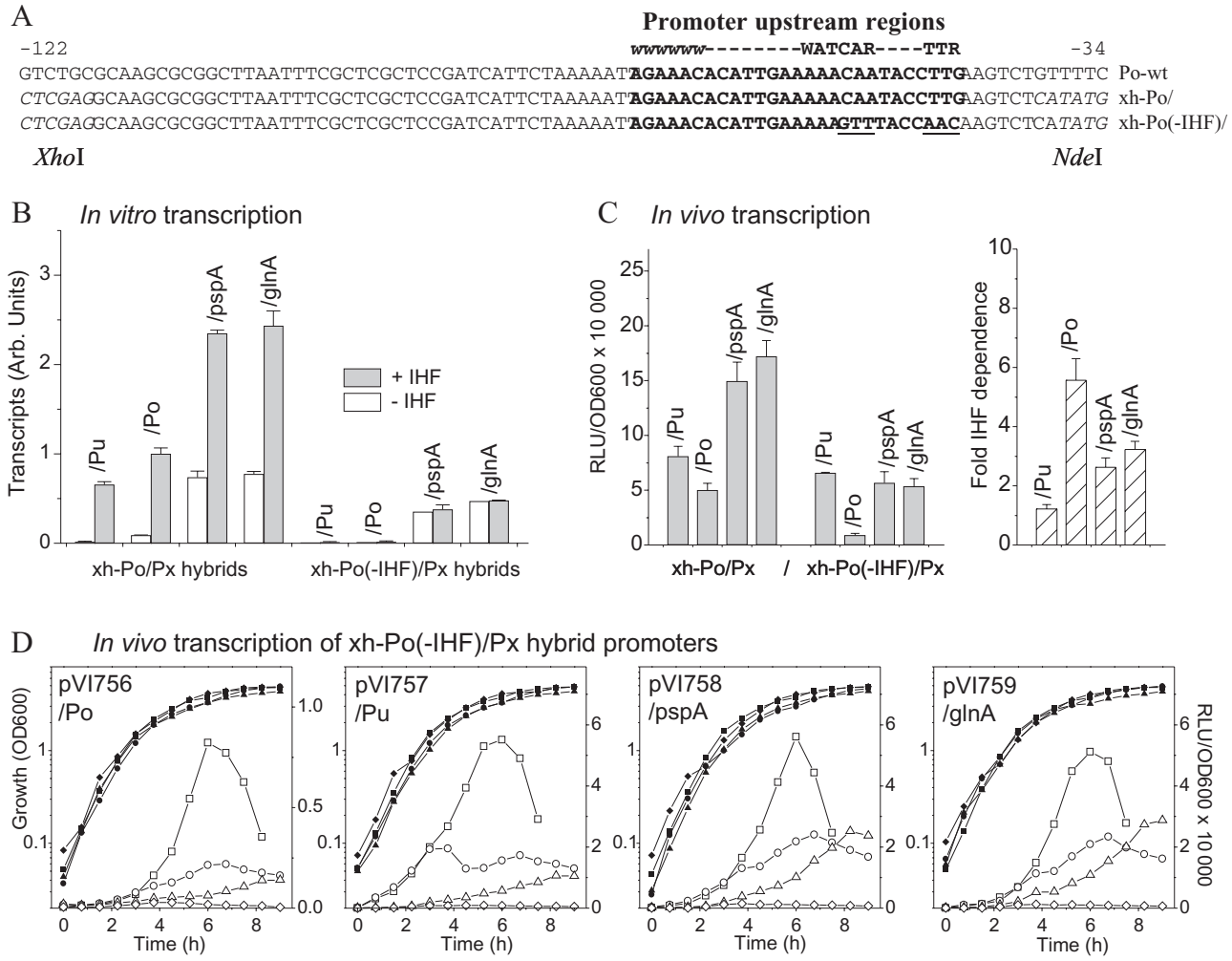

Fig. 8. Comparative transcription of IHF-binding competent and incompetent hybrid σ^{54} promoters.
A. Comparison of the nucleotide sequences of the upstream regions that vary in the plasmid used with that of the wild-type upstream sequence of Po (restriction sites that have been introduced are italicized). Note that the NdeI site is destroyed upon introduction of the linkers to generate the hybrid promoters, as shown in Fig. 5A and described in *Experimental procedures*. The IHF binding site of Po (Sze *et al*., 2001) is shown in bold and aligned with the consensus sequence, which includes the core 5′-WATCAR----TTR-3′ motif (where W is A or T, and R is A or G) separated from a less conserved A/T-rich tract of 4–6 bp (lower-case letters) (Goodrich *et al*., 1990). The residues shuffled to disrupt the core IHF consensus in xh-Po(-IHF) are underlined.
B. Single-round *in vitro* transcription of 11 nM templates pVI770 to pVI777 carrying the hybrid promoters indicated. Reactions were performed in G-buffer at 20°C as under Fig. 6, in the presence of 25 nM IHF (shaded bars) and the absence of IHF (open bars).
C. Luciferase reporter gene transcription from hybrid σ^{54} promoters in LB-grown cultures of MG1655Δ*lac* harbouring reporter plasmids pVI752 to pVI759. The data show the peak transcriptional output at the 6 h time point (shaded bars). Fold IHF dependence is calculated as the transcriptional output from the xh-Po/Px hybrids divided by that of the cognate xh-Po(-IHF)/Px hybrid (hatched bars).
D. Growth (closed symbols) and luciferase activity (open symbols) of LB-cultured *E. coli* strains harbouring *dmpR*-xh-Po(-IHF)/Px–*luxAB* transcriptional reporter plasmids pVI756 to pVI759. Strains: wild-type ppGpp+/DksA+ MG1655 (squares); $ppGpp^0$ CF1693 (circles), DksA null RK201 (MG1655Δ*dksA*::Km, triangles) and $ppGpp^0$/DksA null CF1693-*dksA*::Tc (diamonds).

data clearly suggest that DksA and ppGpp are not required for σ^{54}-dependent transcription *per se*, but rather that they mainly act in collaboration to mediate their effects *in vivo* indirectly. Our finding that the *in vivo* requirement for either ppGpp or DksA for efficient transcription from the σ^{54}-Po promoter can be by-passed in strains expressing mutant σ^{70} proteins (Fig. 1) provides independent support for this conclusion, and strongly suggests that ppGpp and DksA both affect σ^{54} transcription via their effects on σ^{70}-dependent transcription. We were unable to detect any alteration in the ability of σ^{54} and σ^{70} to compete for core RNAP upon addition of ppGpp and DksA to *in vitro* competition assays (Fig. 4). The lack of any direct effect of addition of ppGpp and DksA in σ^{54} versus σ^{70}*in vitro* competition assays is fully consistent with previous *in vivo* data that the outcome of manipulation of the levels or availability of σ^{54}, σ^{S} or σ^{70} is not directly influenced by the presence or absence of ppGpp

(Laurie *et al*., 2003). The lack of any evidence that ppGpp and/or DksA play an active role in competition between σ^{54} and σ^{70} leads us to propose a model in which these regulatory molecules affect σ^{54}-dependent transcription by a purely passive (indirect) mechanism, as depicted in Fig. 9.

This proposed passive mechanism operates through predicted global regulatory consequences of the negative action of ppGpp and DksA at the seven powerful stringent σ^{70}-rRNA operon promoters of *E. coli*. While the levels of ppGpp change dramatically, DksA is produced at constant levels during all growth phases and under different growth conditions, and has been proposed to sensitize the transcriptional apparatus to physiological levels of ppGpp (Brown *et al*., 2002; Paul *et al*., 2004a). Thus, this passive model primarily operates through changes in ppGpp levels. The seven σ^{70}-rRNA operon promoters sequester approximately 60–70% of the transcriptional machinery during rapid growth of *E. coli* on rich media (Bremer and Dennis, 1996). Thus, under these conditions in which ppGpp levels are low (Fig. 9A), sequestering of the majority of the transcriptional machinery by the rRNA operons would leave little free core RNAP available for association with the constant levels of σ^{54} and σ^{70}, leading to low levels of σ^{54} holenzyme and cognate σ^{54} promoter occupancy and output. However, under conditions that elicit high levels of ppGpp (Fig. 9B), the dramatic downregulation of transcription from the σ^{70}-rRNA operon promoters in response to the synergistic action of ppGpp and DksA would lead to increased levels of available free core for association with σ-factors. Consequently, σ^{54} holenzyme levels would increase, leading to enhanced σ^{54} promoter occupancy. This passive model for the action of ppGpp and DksA would predict that low-affinity promoters that have σ^{54} holenzyme binding as a rate-limiting step would be more susceptible to loss of these regulatory molecules than their high-affinity counterparts. Here, we have shown that this indeed appears to be the case, as among six σ^{54} promoters tested, those with lower affinity for σ^{54} holenzyme were found to exhibit greater sensitivity to loss of ppGpp and DksA *in vivo* than high-affinity counterparts (Figs 7 and 8). Within this model, the σ^{70} suppressor mutants that have defects in their ability to compete against σ^{54} for core RNAP would by-pass the need for a ppGpp/DksA-mediated increase in the pool of core RNAP by simply allowing σ^{54} to associate with a greater fraction of that available. Consistent with this interpretation, the $\sigma^{70\text{-}40Y}$ mutant that is most severely defective in competing with σ^{54} is a better suppressor of the lack of both ppGpp and DksA than the $\sigma^{70\text{-}35D}$ mutant that is less severely defective in competing with σ^{54} (Fig. 1C and D; Laurie *et al*., 2003).

In vitro σ^{54} transcription can be reconstituted using just a few basic components, namely σ^{54} promoter DNA, an active form of the regulator, holoenzyme σ^{54}-RNAP and nucleotides. Lack of ppGpp and/or DksA does not alter the *in vivo* levels of σ^{54}, and has only a minor effect on the level of DmpR that does not account for their action *in vivo* (Fig. 1). Although not necessarily essential *in vitro*, IHF assists promoter output through architectural changes to allow productive contact between the regulator and the σ^{54} holoenzyme. IHF levels are partially under the control of ppGpp and increase at the transition from exponential to stationary phase of growth (Aviv *et al*., 1994; Ditto *et al*., 1994; Valls *et al*., 2002). Nevertheless, the *in vivo* requirement for ppGpp and DksA, although slightly different in magnitude, is still clearly manifested at σ^{54} promoters that lack the capacity to bind IHF (Fig. 8D). Thus, it seems reasonable to propose that passive regulation accounts for a large proportion of the affects of ppGpp and DksA on σ^{54}-dependent transcription. We can-

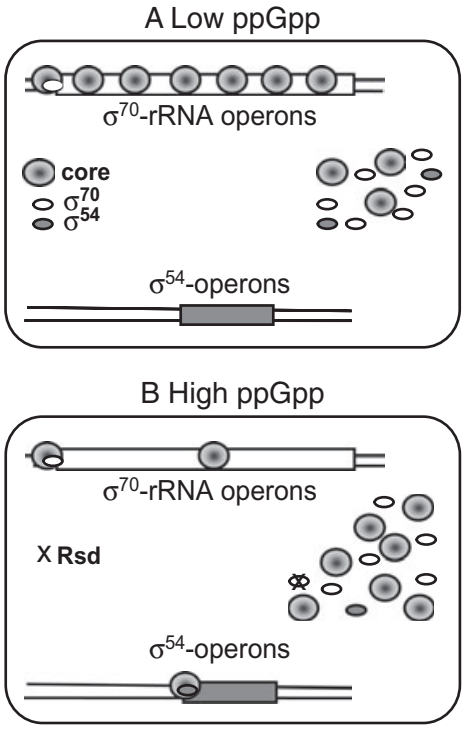

Fig. 9. Passive model for ppGpp/DksA control of σ^{54}-dependent transcription.
A. Growth conditions that elicit low levels of ppGpp. Availability of key transcriptional components is illustrated schematically. As detailed in the text, approximately 60–70% of the transcriptional machinery is sequestered in expressing the abundant transcripts from the multiple powerful rRNA operon promoters under these conditions. Thus, only low levels of core RNAP would be available for association with different σ-factors, leading to low levels of σ^{54} holoenzyme.
B. Conditions that elicit high levels of ppGpp. Reduced transcription from the rRNA operon promoters would increase the quantity of available core RNAP for holoenzyme formation, leading to higher levels of σ^{54}-RNAP holoenzyme. As depicted, the σ^{70}-binding Rsd protein may favour σ^{54} holoenzyme formation by binding and thus sequestering some of the competing σ^{70}.

not, however, exclude the possibility that a currently unknown regulator(s) could also contribute to the requirement for ppGpp and DksA for efficient σ^{54}-dependent transcription *in vivo*. Akin to the discovery of the role of DksA in ppGpp-mediated regulation of σ^{70} transcription, it is possible that some yet unknown factor could also act in conjunction with ppGpp and DksA to directly control σ^{54}-dependent transcription, or alternatively, could directly affect σ-factor competition for limiting core RNAP. However, only in the latter case could the existence of such an unknown player account for the σ^{54} promoter affinity-dependent differences in the requirement for ppGpp and DksA.

As depicted in Fig. 9B, the σ^{70}-binding Rsd protein, the levels of which are themselves partially under ppGpp control, may also aid access of σ^{54} to the available core RNAP by sequestering σ^{70} from association with core and by actively removing σ^{70} from the σ^{70} holoenzyme (Jishage and Ishihama, 1998; Jishage *et al*., 2001; Ilag *et al*., 2004; Westblade *et al*., 2004). While further work is required to directly investigate the participation of Rsd in passive regulation of σ^{54}-dependent transcription, support for its involvement comes from the observation that like underproduction of σ^{70}, overexpression of Rsd both restores and enhances σ^{54}-Po promoter output in ppGpp^0 cells to above that found in wild-type *E. coli* (Laurie *et al*., 2003). Future analysis of the *in vivo* occupancy of σ^{54} promoters under different growth conditions in strains lacking Rsd should help to directly test the comparative importance of the effects of both ppGpp/DksA effects on core RNAP pools and the potential effects of these regulatory molecules through their control of Rsd levels. Irrespective of the relative contributions of different factors that potentially contribute to passive regulation, transcription from even the strongest high-affinity σ^{54} promoters is still diminished in ppGpp- and DksA-deficient strains (Figs 7C and 8D). This suggests that passive regulation will affect the performance of many σ^{54} systems in response to nutritional stress and other cues that stimulate ppGpp production. However, as transcription from σ^{54} promoters requires co-occupancy of the regulator and the holoenzyme, the degree to which ppGpp/DksA-mediated passive regulation of σ^{54} transcription would affect different σ^{54} systems will depend on both the affinity of the promoter, and the levels and binding properties of the cognate regulator in each case.

Experimental procedures

General procedures

Escherichia coli strains (Table 1) were grown in Luria–Bertani medium (LB; Sambrook *et al*., 1989) supplemented with the following antibiotics as appropriate for the strain and resident plasmid selection: carbenicillin (Cb, 100 μg ml^{-1}), kanamycin (Km, 50 μg ml^{-1}), spectinomycin (Sp, 50 μg ml^{-1}), tetracycline (Tc, 5 μg ml^{-1}). *E. coli* DH5 was used for construction and maintenance of plasmids. *E. coli* strains carrying *rpoD* mutations linked to *aer-3075*::Tn*10* or the *dksA::Tc* mutation of TE8114 were generated by P1 transduction using the associated Tc resistance marker in each case (Table 1). DNA sequencing confirmed co-transduction of the *rpoD* alleles, as previously described (Laurie *et al*., 2003).

Plasmid construction

The *dmpR*-Po–*luxAB* luciferase transcriptional reporter plasmid pVI466 carries the *dmpR* gene and σ^{54}-Po promoter in their native configuration fused at +291 to the *luxAB* genes on an RSF1010-based vector (IncQ; 16–20 copies per cell) (Sze *et al*., 1996). To provide a compatible plasmid expressing additional controllable levels of DmpR, the KpnI site of the polylinker of the Sp^R pEXT21 vector (IncW; three copies per cell; Dykxhoorn *et al*., 1996) was converted to an NdeI site via a linker to give pVI898. Subsequent cloning of the *dmpR* gene as an NdeI to HindIII fragment from pVI399 (Shingler and Moore, 1994) between these sites of pVI898

Table 1. Bacterial strains.

Escherichia coli strain	Relevant properties	Reference
DH5	Prototrophic, Res$^-$	Hanahan (1985)
MG1655	Prototroph F$^-$, λ$^-$, K12	Xiao *et al*. (1991)
MG1655Δ*lac*	MG1655 Δ*lacX74*	Sze *et al*. (2002)
CF1693	Auxotrophic ppGpp^0, MG1655 Δ*relA251::Km* Δ*spoT207::Cm*	Xiao *et al*. (1991)
CF1693-dksA::Tc	ppGpp^0, DksA null; Km^R, Cm^R, Tc^R	This study
CF1693-*rpoD*^{40Y}	ppGpp^0, *rpoD*^{40Y} linked to *aer-3075*::Tn*10*, Km^R, Cm^R, Tc^R	This study
CF1693-*rpoD*^{35D}	ppGpp^0, *rpoD*^{35D} linked to *aer-3075*::Tn*10*, Km^R, Cm^R, Tc^R	This study
CF1693Δ*lac*	ppGpp^0, Δ*lacX74* Km^R, Cm^R	Sze *et al*. (2002)
CF1693Δlac -dksA::Tc	ppGpp^0, DksA null; ΔlacX74 Km^R, Cm^R, Tc^R	This study
CF1693Δ*lac* -*rpoD*^{40Y}	ppGpp^0, *rpoD*^{40Y} linked to *aer-3075*::Tn*10*, Km^R, Cm^R, Tc^R	Laurie *et al*. (2003)
CF1693Δ*lac* -*rpoD*^{35D}	ppGpp^0, *rpoD*^{35D} linked to *aer-3075*::Tn*10*, Km^R, Cm^R, Tc^R	Laurie *et al*. (2003)
RK201	Auxotrophic DksA null; MG1655 Δ*dksA::Km*, Km^R	Kang and Craig (1990)
RK201-*rpoD*^{40Y}	DksA null; *rpoD*^{40Y} linked to *aer-3075*::Tn*10*, Km^R, Tc^R	This study
RK201-*rpoD*^{35D}	DksA null; *rpoD*^{35D} linked to *aer-3075*::Tn*10*, Km^R, Tc^R	This study
TE8114	DksA null; MG1655-*dksA::*Tc, Tc^R	Brown *et al*. (2002)

Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline.

to give pVI899 places the *dmpR* gene cloned under the control of the *lacI*/P*tac* promoter of the vector.

Construction of plasmids carrying different promoters was by a common step-wise procedure using standard DNA techniques, and the fidelity of all polymerase chain reaction (PCR)-generated fragments and linker sequences was confirmed by DNA sequence analysis. First, as depicted in Fig. 5A, templates that allowed introduction of the desired promoter sequence by insertion of linkers between a unique NdeI and BamHI sites were assembled on pBluescript-SK (Stratagene). Plasmid pVI769 harbours the native Po upstream region with an NdeI site at −39 to −34 relative to the transcriptional start of Po. Plasmid pVI733 carries the same DNA but with an XhoI site at −122 to −117. Plasmid pVI734, which harbours the Po upstream region with a disrupted IHF binding sequence, was generated by replacement of the XhoI to NdeI fragment of pVI733 with custom-designed linkers. In the next step, linkers with the desired promoter sequences, and designed to regenerate the BamHI site but not the NdeI site (Fig. 5A, right) were introduced into these three templates to generate the pBluescript-based plasmids listed in Table 2. To generate the *dmpR*–Px/Px–*luxAB* transcriptional reporter plasmids listed in Table 2, the RSF1010-based broad-host-range vector pVI432 was constructed by introducing HindIII–XhoI–HpaI sites via a linker into the SalI site of pVI397 (Pavel *et al*., 1994) to provide a PstI–HindIII–XhoI–HpaI–BamHI–SmaI–EcoRI polycloning cassette. The pVI432 vector was used to clone the *dmpR*–Px/Px–*luxAB* fusions as HindIII fragments.

Templates for *in vitro* transcription studies were generated using pTE103, which contains a strong T7 termination signal downstream of a polycloning site (Elliott and Geiduschek, 1984). Construction of pVI695, which carries DNA spanning the binding sites for DmpR and the Po promoter (−480 to +26) as an EcoRI–BamHI fragment, has been described previously (Laurie *et al*., 2003). An analogous plasmid, pVI900, with the +1G exchanged for an A, was constructed by first mutating the fragment on pBluescript-SK using the QuickChange™ from Stratagene, and subsequent cloning into pTE103. To generate pTE103-based plasmids carrying hybrid promoters, the Px/Px–*luxAB* fusions were first cloned as BglII to HindIII fragments between the BamHI and HindIII sites of pVI432 (Table 2). The EcoRI to BamHI fragments spanning the −578 to +2 hybrid promoter regions of the resulting plasmids were then cloned between these sites of pTE103 (Table 2).

Luciferase reporter gene assays

Luciferase assays of the *luxAB* gene product were performed on cultures grown and assayed at 30°C. To ensure balanced growth, overnight cultures were diluted and grown into exponential phase before a second dilution to an OD_{600} of 0.05–0.08, and initiation of the experiment by addition of the DmpR effector 2-methylphenol to a final concentration of 0.5 mM. Light emission from 100 μl of whole cells using a 1:2000 dilution of decanal was measured using a PhL Luminometer (Aureon Biosystems). Data points are the average of duplicate determinations from each of two or more independent experiments in which output values varied less than 20%.

Western blot analysis

Crude extracts of cytosolic proteins, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransfer and Western blot analysis were essentially as described previously (Shingler and Pavel, 1995). Monoclonal antibodies against *E. coli* σ^{54} were obtained from Neoclone (W0005). Polyclonal rabbit antibodies were raised against the N-terminal 232 residues of DmpR and further affinity purified against the same peptide with an SHHHHHH C-terminal fusion by Agrisera, Sweden. Antibody-decorated bands were revealed using Amersham Biosciences PVDF membrane and ECL-Plus reagents as directed by the supplier.

Gel retardation assay

Reactions were carried out at 20°C in 20 μl of glutamate buffer (G-buffer) comprising 20 mM Tris-glutamate, 10 mM

Table 2. Reporter and *in vitro* transcription plasmids carrying hybrid promoter regions.

Promoter Px/Px	*dmpR*–Px/Px–*luxAB* on pBluescript-SK	*dmpR*–Px/Px–*luxAB* on pVI432	Px/Px–*luxAB* on pVI432	Px/Px on pTE103
Po/Po	pVI700	pVI704	pVI708	pVI736
Po/Pu	pVI718	pVI723	pVI728	pVI737
Po/pspA	pVI719	pVI724	pVI729	pVI738
Po/nifH	pVI720	pVI725	pVI730	pVI739
Po/nifH049	pVI721	pVI726	pVI731	pVI740
Po/glnA	pVI722	pVI727	pVI732	pVI741
xh-Po/Po	pVI743	pVI752	pVI761	pVI770
xh-Po/Pu	pVI744	pVI753	pVI762	pVI771
xh-Po/pspA	pVI745	pVI754	pVI763	pVI772
xh-Po/glnA	pVI746	pVI755	pVI764	pVI773
xh-Po(-IHF)/Po	pVI747	pVI756	pVI765	pVI774
xh-Po(-IHF)/Pu	pVI748	pVI757	pVI766	pVI775
xh-Po(-IHF)/pspA	pVI749	pVI758	pVI767	pVI776
xh-Po(-IHF)/glnA	pVI750	pVI759	pVI768	pVI777

Promoters (Px/Px) of the different plasmids contain the indicated combinations of promoter upstream regions (−122 to −40) and promoter regions (−39 to +2) in the context of the *dmpR-dmpK* intervening region with co-ordinates given relative to the transcriptional start of Po. The prefix xh- denotes the presence of a non-native XhoI site, while (-IHF) indicates disruption of the IHF DNA binding sequence as shown in Fig. 8.

Mg-glutamate, 400 mM K-glutamate, 0.1 mg ml^{-1} BSA and 5% glycerol. EcoRI fragments generated by digestion of DNA amplified using primers 5′-GCGCCGAATTCATTTGCT CAAGCGGCC-3′ and 5′- CCACAGAATTCAGACGCTTTGC CCAG-3′ were end-labelled with [α-^{32}P]-dATP and the Klenow fragment of DNA polymerase as previously described (Sze *et al*., 2001). Labelled DNA (2 nM) was incubated for 30 min with the indicated concentration of σ^{54}-RNAP reconstituted at a 1:1 molar ratio of core to σ^{54} for 10 min before addition to the reaction mix. The entire reaction volume was then loaded onto a 4% non-denaturing polyacrylamide gel, electrophoresed, dried. The relative band intensities were quantified using a Molecular Dynamics Phosphorimager.

In vitro *transcription assays*

Reactions (20 μl) were performed at 20°C in G-buffer (see above), or at 30°C in T-buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA and 0.275 mg of BSA per ml), as indicated. *E. coli* core RNAP and σ^{70}-RNAP holoenzyme were obtained from Epicentre. Synthesis and purification of *E. coli* IHF, σ^{70}, σ^{54}, DmpR-His and ppGpp were as previously described (O'Neill *et al*., 1998; 2001; Laurie *et al*., 2003). N-terminally His-tagged *E. coli* DksA, which is functionally indistinguishable from native DksA in *in vivo* and *in vitro* assays (Paul *et al*., 2004a), was a generous gift from A. Åberg (Umeå University, Sweden).

For σ^{54}-RNAP holoenzyme formation, core RNAP was pre-incubated with an eightfold molar excess of σ^{54} for 5 min, unless otherwise stated. Holoenzyme RNAPs were incubated with ppGpp and/or DksA (or appropriate storage buffer) for 10 min, or as indicated, before initiation of experiments. Open complex formation (20 min) was initiated by the addition of supercoiled plasmid DNA. For σ^{54}-dependent transcription, reactions were also supplemented at the same time with IHF (10 nM unless otherwise indicated), DmpR-His (50 nM), 4 mM ATP (or dATP required for DmpR activity) and the DmpR aromatic effector 2-methylphenol (0.5 mM). Transcription was initiated by adding 2.5 μl of a mixture of ATP (final concentration, 0.4 mM), GTP and CTP (final concentration 0.2 mM each), UTP (final concentration 0.08 mM) and [α-^{32}P]-UTP (5 μCi at $>$ 3000 Ci mmol^{-1}; Amersham Biosciences). In multiple-round assays, re-initiation was prevented after 7 min by the addition of heparin (0.125 mg ml^{-1} final concentration) for σ^{54}-dependent transcription assays, or by addition of a 200-fold molar excess of a 60 bp double-stranded DNA fragment containing the *full con* promoter (Gaal *et al*., 2001) for σ^{70}-dependent transcription assays. Reactions were further incubated for 3–5 min to allow completion of initiated transcripts. For single-round assays, heparin or competitor DNA was added simultaneously with the nucleotide mix, reactions were incubated for 10 min then terminated by addition of formamide loading buffer, and transcripts were analysed on a 7 M urea-4% acrylamide gel as previously described (Laurie *et al*., 2003).

In competitor-resistant open complex stability assays, reaction components were incubated in T-buffer at 30°C in the presence or absence of ppGpp (200 μM) and DksA (2 μM) as described for transcription assays, to allow open complex formation. At time zero, heparin or a 200-fold molar excess of competitor *full con* promoter DNA was used to compete the formation of new complexes. At the indicated times, 20 μl aliquots were taken and single-round transcription was performed as described above.

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