The Role of the Alarmone (p)ppGpp in σN Competition for Core RNA Polymerase*

Andrew D. Laurie‡‡, Lisandro M. D. Bernardo‡, Chun Chau Sze‡‡, Eleonore Skärfstad‡, Agnieszka Szalewska-Palasz‡, Thomas Nyström§, and Victoria Shingler**

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Some promoters, including the DmpR-controlled σ^N-dependent Po promoter, are effectively rendered silent in cells lacking the nutritional alarmone (p)ppGpp. Here we demonstrate that four mutations within the housekeeping σ^D-factor can restore σ^D-dependent Po transcription in the absence of (p)ppGpp. Using both in vitro and in vivo transcription competition assays, we show that all the four σ^D mutant proteins are defective in their ability to compete with σ^N for available core RNA polymerase and that the magnitude of the defect reflects the hierarchy of restoration of transcription from Po in (p)ppGpp-deficient cells. Consistently, underproduction of σ^D or overproduction of the anti-σ^D protein Rsd were also found to allow (p)ppGpp-independent transcription from the σ^D-Po promoter. Together with data from the direct effects of (p)ppGpp on σ^D-dependent Po transcription and σ-factor competition, the results support a model in which (p)ppGpp serves as a master global regulator of transcription by differentially modulating alternative σ-factor competition to adapt to changing cellular nutritional demands.

Escherichia coli holoenzyme RNA polymerase is composed of a core enzyme (E,1 subunit composition α₂ββ’ω) associated with one of seven sigma (σ)-factors that program the complex to engage and initiate transcription at different sets of promoters (1). Thus, the levels and binding properties of alternative σ-subunits together with factors that modulate their ability to associate with core RNA polymerase are critical for the relative composition of the multiple holoenzymes available for transcription of the distinct promoter classes within the prokaryotic genome. The seven different σ-factors of E. coli fall into two groups. The larger of these comprises six factors that share notable sequence and functional similarities to the major σ^D (σ^D)-factor that is responsible for transcribing “housekeeping” genes (2). Recent structural studies have shown that the σ^D-like proteins comprise three globular domains that encompass previously identified conserved regions (σD 1.2 to 2.4, σD 3.0 to 3.1, σD 4.1 to 4.2)) that are tethered by flexible linkers (3–6). In contrast, the alternative σ^D (σ^D)-factor is in a class on its own, bearing little sequence homology to other σ-factors and determining recognition of the well conserved but unusual −24, −12 (TGGCA-C-N_7-TGGC) promoter sequences (7). In addition, there are significant differences in the action of the cognate holoenzymes at promoters. Unlike Eσ^D, which can undergo transition from the initial closed complex to the transcriptionally competent open complex without any other regulatory factor, the Eσ^N closed complex is kinetically and thermodynamically stable and Eσ^N cannot melt promoter DNA on its own. Transition to the open complex is dependent on interaction with, and nucleotide hydrolysis by, a member of the bacterial family of enhancer binding proteins (reviewed in Ref. 8).

Promoters of the σ^N-dependent −24, −12 class direct transcription of genes involved in a variety of physiological processes responsive to nutrient limitation such as nitrogen assimilation and fixation, substrate-specific transport systems, and utilization of alternative carbon and energy sources. Appropriate environmental signals lead to activation of the cognate regulator by diverse mechanisms that result in a common active form of the regulator (9). For DmpR, which controls transcription of the σ^D-Po promoter of an operon encoding the enzymes for metabolism of (methyl)phenols in Pseudomonas CFD600, the activation mechanism is direct. Binding of aromatic phenolic pathway substrates to its N-terminal regulatory A-domain alleviates interdomain repression to give the active form of the protein (10, 11), and DmpR mediated aromatic effector- and ATP-dependent transcription from Po can be fully reconstituted in vitro (12, 13).

In addition to the specific DmpR-mediated control mechanism described above, transcription from the σ^N-Po promoter is dependent on the unusual nucleotides guanosine tetraphosphate (pppGpp) and guanosine pentaphosphate (ppppGpp), collectively referred to as (p)ppGpp. These molecules are heralds of metabolic stress and were originally identified as the mediators of the classical stringent response to down-regulate superfluous stable RNA synthesis upon amino acid starvation (reviewed in Refs. 14 and 15). Synthesis of these molecules by ribosome-associated RelA (p)ppGpp synthetase is activated by the arrival of an uncharged tRNA on the ribosome, whereas the dual function SpoT protein (which is primarily responsible for (p)ppGpp degradation) catalyzes (p)ppGpp synthesis in response to glucose starvation (16). Since their discovery, however, these signaling molecules have also been implicated in the up-regulation of transcription from many classes of promot-
ers including those dependent on $\sigma^D$ (e.g. Refs. 16 and 17), the stationary phase $\sigma$-factor $\sigma^B$ (e.g. Ref. 18–22), the heat shock $\sigma$-factor $\sigma^E$ (e.g. Refs. 22–24), as well as $\sigma^N$ (e.g. the Po and Pu promoters; Refs. 25–27).

Down-regulation of transcription from stringent promoters is believed to occur through the effects of binding of (p)ppGpp at the interface of the $\beta$- and $\beta'$-subunit of RNA polymerase (28, 29). Binding of (p)ppGpp destabilizes $\sigma^D$-promoter complexes, and, because open complexes formed by $E\sigma^D$ at RNA P1 promoters are intrinsically very unstable, it has been proposed that these promoters are likely to be particularly sensitive to (p)ppGpp destabilization (30). Support for this mechanism comes from analysis of suppressor mutations within $\beta$ and $\beta'$ (isolated by rescue of the polyauxotrophic growth defect of (p)ppGpp-deficient strains) that also destabilize $E\sigma^D$-promoter open complexes in vitro (31–33). One possible consequence of down-regulation of transcription from the powerful stringent promoters is an increase in the pool of core RNA polymerase that is normally sequestered in producing these abundant transcripts. Thus, accumulation of (p)ppGpp and, by analogy, suppressor mutations has been proposed to increase the amount of $E\sigma^D$ available for (p)ppGpp-stimulated $\sigma^D$-promoters that are difficult to saturate and have $E\sigma^D$ recruitment as the rate-limiting step (33–35).

The seven $\sigma$-factors of E. coli exhibit quite different affinities for core RNA polymerase (reviewed in Ref. 36), and (p)ppGpp has the potential to play multiple roles in their function. In addition to the potential to directly alter the promoter recognition and kinetics of transcriptional initiation at promoters, (p)ppGpp could also plausibly modulate the $\sigma$-association properties of the core RNA polymerase. Models involving the influence of (p)ppGpp on $\sigma$-factor competition for limiting available core have been put forward to explain poor induction of $\sigma^D$-dependent promoters in (p)ppGpp-deficient cells (20, 21, 37). Most recently evidence for a direct effect of (p)ppGpp on the competitive abilities and levels of $\sigma^D$ and $\sigma^N$ associated with core RNA polymerase has been presented (22). Primarily based on the ability of two mutations in $\sigma^D$ to restore transcription to a $\sigma^D$-dependent promoter in the absence of (p)ppGpp, we have previously postulated that modulation of $\sigma$-factor competition may at least in part underlie the (p)ppGpp dependence of the Po promoter that requires the action of the structurally and functionally distinct $\sigma^N$ protein (25). Here, we dissect the potential of (p)ppGpp to directly affect $\sigma^D$-dependent Po transcription and to modulate $\sigma^N$ competition by (i) identifying new suppressor mutations that restore transcription from Po in (p)ppGpp-deficient cells and (ii) performing in vitro and in vitro $\sigma$-factor competition in the presence and absence of (p)ppGpp. We present evidence that (i) the levels of alternative $\sigma$-factors have a large impact on the output from the Po promoter and (ii) the requirement for (p)ppGpp can be suppressed by four mutations in $\sigma^D$ that exhibit defects in competition against $\sigma^3$, or by underproduction or sequestering of $\sigma^D$. These results strongly support a model in which (p)ppGpp serves as a master regulator of transcription through $\sigma$-factor competition for limiting core RNA polymerase and brings the performance of the structurally and functionally distinct $\sigma^N$ protein within the global control of the stringent response.

### EXPERIMENTAL PROCEDURES

**Strains and Culture Media**—Strains used are listed in Table I and were routinely cultured and assayed in Luria broth (LB; Ref. 38) supplemented with appropriate antibiotics for selection. Where indicated, assays were also performed using rich defined medium consisting of M9 minimal medium (38) supplemented with 22 amino acids (50 g/ml, Sigma kit), serine at 1 g/liter as the carbon source, all five nucleotides (20 g/ml), trace metals, and thiamine (0.05 mM). E. coli MG1655Δrac and CF1693Δrac strains carrying $P_{\text{rpoD}}$ were generated by transfecting (CAM1) $P_{\text{rpoD}}$ from E. coli MC14100 (CAM1) $P_{\text{rpoD}}$ into these strains by P1-mediated transduction using the tetracycline resistance aer-3075::Tn10 marker and testing for chloramphenicol resistance and/or indole-3-acrylic acid (IAA) dependence. Prior to analy-
sis, all the rpo alleles were introduced in clean genetic backgrounds by P1-mediated transduction exploiting the thi-39-Tn10 marker for rpoBC alleles, aer-3075-Tn10 for rpoD alleles, and dcm-61-Tn10 for the rpoN allele. DNA sequencing was used to monitor co-transduction of the mutant alleles. Suppressor phenotypes were assessed using a Po-luxAB reporter and the ability of the strains to grow on M9 minimal media plates supplemented with 10 mM glucose and 100 μg/ml thiamine.

Plasmids and DNA Manipulations—Plasmids and primers are listed in Tables II and III. Plasmids were constructed by standard recombinant techniques and the fidelity of all PCR-derived DNA confirmed by sequencing. Plasmids pV1664 and pVI684 are equivalent to pVI693 and pVI-luxAB lacI site encompassing the DmpR binding sites (upstream activating sequences –26 to 7 bp downstream of the termination codon). The PCR-amplified spectinomycin/streptomycin gene, rpoB, encompassing the DmpR binding sites (upstream activating sequences –26 to 7 bp downstream of the termination codon).

Selection of Second Site Suppressor Mutants—Independent cultures of E. coli CF1693Δlac harboring pVI687 (dmpR-Po-tet) were grown for 2 h with shaking at 30 °C in 2 ml of LB supplemented with carbenicillin (50 μg/ml) and 0.5 mM 2-methylphenol (the most potent aromatic effector of DmpR). Tetracycline was then added to 20 μg/ml and the culture incubated under the same conditions for another 2 h prior to dilution and plating on equivalent solid media and growth overnight at 30 °C. Equal numbers of large and small independent isolates were re-streaked and screened for DmpR dependence of the tetracycline resistance phenotype by testing for TCr in the presence and absence of 0.5 mM 2-methylphenol. Of the original 102 isolates, all exhibited DmpR-dependent tetracycline resistance. However, 33 were discarded as being unstable, P1 phage-resistant, and/or having suppressor phenotypes too poor to map by P1 transduction as described below.

Localization and Identification of Mutations—Given the predominance of rpoBC mutants in a previous prototrophy rescue screen (40), isolates were first screened for alterations in rpoBC by using a non-isotopic RNA cleavage assay (NIRCA) kit (MutationScreener™) supplied by Ambion, Inc. and/or P1 transduction. For NIRCA analysis, each ~5-kb gene was amplified by colony PCR from each mutant strain using rpoB B1/B2 and rpoC C1/C2 primers and Expand High Fidelity Taq polymerase (Roche Molecular Biochemicals). Using these products as templates, five overlapping regions (of ~1000 bp) of each gene were amplified using primers that incorporated the T7 promoter region at the 5' end (BT7–1/AB to BT7–5A/B and CT7–1AB to CT7–5A/B; Table III). These products were then mixed with the corresponding section amplified from wild-type E. coli MG1655, transcribed to form RNA, and the hybridized products subjected to limited RNase cleavage. The sizes of RNase-cleaved bands resulting from mismatches were used to estimate the location of the mutation, which was subsequently confirmed by DNA sequencing. For the majority of isolates, assignment was made to the rpoBC region as soon as a mutation was detected during sequencing analysis from the 5’ region of rpoB through to the 3’ region of rpoC.

In this study, NIRCA analysis of the entire rpoBC region of each strain revealed the single genetic changes listed in Table I. However, even after introduction into a clean genetic background, because NIRCA analysis does not detect all mutations (see below), there is a small possibility that these derivatives may also contain additional closely linked but undetected mutations that contribute to their phenotype.

The NIRCA analysis assigned mutations in 53 isolates to rpoBC. Mutations in an additional 12 isolates were assigned to the rpoBC region by P1 linkage analysis. The assay system used to determine linkage frequencies utilized the thi-39-Tn10 marker and CF1693Δlac harboring the reporter plasmid pVI686. Plasmid pVI686 mediates DmpR-Po-dependent expression of the dmpB-encoded catechol-2,3-dioxygenase, which converts catechol to the bright yellow product 2-hydroxymuconic semialdehyde.

In the case of the three rpoB and three rpoC alleles further analysed in this study, NIRCA analysis of the entire rpoBC region of each strain revealed the single genetic changes listed in Table I. However, even after introduction into a clean genetic background, because NIRCA analysis does not detect all mutations (see below), there is a small possibility that these derivatives may also contain additional closely linked but undetected mutations that contribute to their phenotype.

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The remaining four isolates with mutations unlinked to rpoBC were subjected to NIRCAl analysis for mutations in both rpoD and rpoN alleles of this strain were only present in the absence of (p)ppGpp (Fig. 1A). This plasmid confers aromatic effector-dependent tetracycline resistance (pVI687, dmpR-controlled Po promoter driving transcription of a promoterless tetracycline resistance gene (pVI687, pVI695). In vitro transcription assays were performed at 37 °C in transcription buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.275 mg/ml bovine serum albumin. Core RNA polymerase (10 mM) was allowed to associate with variable amounts of wild type or mutant αD and/or αD to form a core polymerase (10 nM) that was used to initiate transcription with RpoN as described previously (44). Antibody decorated bands were revealed using chemiluminescence reagents (Amersham Biosciences) as directed by the supplier. Differences in expression levels were assessed by comparison of different dilutions of extracts from various strains. Specificity of antibodies was monitored using genetic control strains proficient and deficient in expression of the gene product and/or purified proteins.

The fourth isolate, with a mutation in rpoD, was selected for further study. This isolate was used to inoculate the media indicated. Luciferase activity of LuxAB within whole cells was assayed with a 1:2000 dilution of decanal as described previously (44).

In Vivo Luciferase Transcription Assays—Assays for (p)ppGpp—In vivo luciferase transcription assays were grown overnight in the test media, diluted and grown to early exponential phase, and then diluted once more prior to initiation of the experiment by the addition of 0.5 mM of the DmpR effector 2-methylphenol. For cultures of strains harboring the P_dmpR-rpoD system, cultures were grown as described above in LB supplemented with 0.2 mM IAA, concentrated by centrifugation, and then used to inoculate the media indicated. Luciferase activity of LuxAB was assayed with a 1:2000 dilution of decanal as described previously (44).
of DmpR. P1 transduction and/or a non-isotopic RNA cleavage assay were used to determine the location of the mutations in the independent isolates as described under “Experimental Procedures.” Of the 69 mutants that proved amenable to analysis, 65 harbored mutations in **rpoBC** (**β′**), 1 had a mutation in **rpoD** (**σ**), 1 had multiple mutations in **rpoN** (**σ**N), and 2 had mutations elsewhere in the genome. Thus, the majority of isolates harbor mutations in components of the RNA polymerase, and, of these, 97% had mutations in **rpoBC** and 3% had mutations in **rpoD**.

By screening a selection of the mutants using the dmpR-Po-luxAB luciferase reporter plasmid pV1466, we found that the degree of restoration of Po transcription in (p)ppGpp proficient and -deficient E. coli. Panel A, luciferase activity (------) and growth (——) of ppGpp + MG1655Δlac (closed squares) and its otherwise isogenic ppGppΔ counterpart CF1693Δlac (open circles) harboring the dmpR-Po-luxAB reporter pV1466. The results are the average of triplicate independent experiments with cells grown and assayed as described under “Experimental Procedures.” Minimal and maximal values are 3 ± 2 to 1069 ± 265 and 2 ± 0.5 to 1171 ± 330 LUXₐₙₖₕₛ for MG1655Δlac and CF1693Δlac, respectively. LUXₐₙₖₜ units. Panel B, luciferase activity of ppGppΔ CF1693Δlac suppressor derivatives harboring pV1466 grown and treated as in A. Results are the average of two independent experiments and represent the peak output values that occur at the same point as shown for MG1655Δlac pV1466. Upper and lower dashed lines indicate the peak values observed for the ppGpp-proficient and -deficient strains shown in panel A.

**Fig. 1. In vivo transcription from the σ**N**-dependent Po promoter in ppGpp-proficient and -deficient E. coli.** Panel A, luciferase activity (-----) and growth (——) of ppGpp + MG1655Δlac (closed squares) and its otherwise isogenic ppGppΔ counterpart CF1693Δlac (open circles) harboring the dmpR-Po-luxAB reporter pV1466. The results are the average of triplicate independent experiments with cells grown and assayed as described under “Experimental Procedures.” Minimal and maximal values are 3 ± 2 to 1069 ± 265 and 2 ± 0.5 to 1171 ± 330 LUXₐₙₖₛ for MG1655Δlac and CF1693Δlac, respectively. LUXₐₙₖₜ units. Panel B, luciferase activity of ppGppΔ CF1693Δlac suppressor derivatives harboring pV1466 grown and treated as in A. Results are the average of two independent experiments and represent the peak output values that occur at the same point as shown for MG1655Δlac pV1466. Upper and lower dashed lines indicate the peak values observed for the ppGpp-proficient and -deficient strains shown in panel A.

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By screening a selection of the mutants using the dmpR-Po-luxAB luciferase reporter plasmid pV1466, we found that the degree of restoration of Po transcription in (p)ppGppΔ CF1693Δlac varied considerably from barely detectable to 4-fold the level observed in the (p)ppGppΔ MG1655Δlac counterpart in the case of one rpoB allele (data not shown). This suggests that the selection procedure is very sensitive and allows isolation of suppressors that differ by >40-fold in their ability to restore transcription from Po. The two rpoD alleles, the single rpoN allele, and six rpoBC alleles (Table I) were chosen for direct comparison with two previously isolated prototrophy-restoring rpoD alleles (P504L and S506F; Ref. 40) that also restore transcription to Po in (p)ppGppΔ E. coli (25). The majority of the mutants shown in Fig. 1B restored Po **σ**N-dependent transcription to between 0.5- and 1.5-fold of the levels observed in (p)ppGppΔ MG1655Δlac. However, the rpoD-35 (Y571H) and rpoN-35 (E150D/I165M) mutants are markedly poorer in their ability to restore transcription.

**Mutant σ**N** Subunits Are Defective in Competition against σ**N** in Vitro**—The data above clearly demonstrate that mutations in **σ**N can efficiently compensate for the effect of (p)ppGpp in vivo to restore transcription to the **σ**N-dependent Po promoter. Given the distinct classes of promoters recognized by the two **σ**-factors, it is unlikely that the **σ**N derivatives directly affect Po output. The most plausible interpretation of this data is that the mutant **σ**N subunits mediate their effects through deficient competition for limiting core RNA polymerase. To directly test this idea, we set up a multiple-round in vitro transcription (IVT) assay to simultaneously monitor the output of the **σ**N-dependent Po promoter and the **σ**D-dependent RNA1 promoter present on the template pV1685. This assay system, employing either a constitutively active form of DmpR deleted of its regulatory A-domain or aromatic effector-activated DmpR, results in clearly distinguishable **σ**N- and **σ**D-dependent transcripts (Fig. 2A, compare lanes 1 and 2), which can be simultaneously monitored within the same reaction (Fig. 2A, lane 4). Inclusion of 0.5 mM aromatic effector 2-methylphenol used in assays with DmpR-His did not alter specificity or the output from the IVT assay.

As a first step in the analysis, we added increasing amounts of the individual **σ**N subunits (Fig. 2B) or **σ**N (Fig. 2C) to a set concentration of core (10 nM) and supercoiled template (0.5 μg). In addition, all assays contained effector-activated DmpR-His (50 nM) and ATP (4 mM) required for **σ**D-dependent transcription, and IHF (10 nM) required for optimal Po output (39). Consistent with previous data, comparison of the saturation curves for wild type **σ**D and **σ**N (closed symbols in Fig. 2, B and C) indicate that these two **σ**-factors have a similar high affinity for core (46–48). For the **σ**D mutants, the plateau saturation values are all lower than observed for wild type **σ**D (Fig. 2B).

Because formation of the holoenzyme is a prerequisite for promotor binding and transcriptional initiation by EσD, transcription by EσN can be considered as at least a second order co-operative binding event. Lower plateau values would thus be predicted for a defect in the initial binding step, which would lead to lower levels of the holoenzyme at equilibrium. However, it is possible or even probable that the mutations may also have some other defect in the complex series of events of transcriptional initiation that may also contribute to the lower net output.

To assess relative competitiveness of the different **σ**D-factors, we first performed a direct comparison of the ability of increasing concentrations of **σ**D to compete for 10 nM core polymerase in the presence of 20 nM **σ**D-wt or the most severely affected **σ**D-derivative (σDΔ-DSA-536–538). The results in Fig. 3A illustrate the severe competitive defect of **σ**DΔ-DSA-536–538 and show that, at this fixed **σ**D concentration, greater than 5-fold more **σ**N is required to achieve a 50% reduction of RNA1 transcript levels with **σ**D-wt than with **σ**DΔ-DSA-536–538.

Based on the results above, we determined the relative competitiveness of all the mutant **σ**D subunits by measuring the ability of 100 nM **σ**N to compete for 10 nM core polymerase in the presence of 40 nM (Fig. 3B) or 20 nM (Fig. 3C) of the **σ**D mutants. The results show that the mutants differ substantially in their ability to compete, ranging from minimally effected (σDΔ- Y571H) to severely affected (σDΔ-DSA-536–538). Consistent with a critical role in **σ**-factor competition in vivo, the relative order of the defects of the these mutants follows the order of their ability to restore Po transcription in the absence of (p)ppGpp (Fig. 1B), namely σDΔ-DSA-536–538 > σDΔ-P504L > σDΔ-S506F > σDΔ-Y571H.

**ppGpp Has No Major Influence on EσN Transcription from Po in Vitro**—To assess the potential direct effects of ppGpp on
Po promoter output, we measured Po transcription in the presence of 200 nM to 800 μM ppGpp in a multiple-round IVT assay. As shown in Fig. 4A, no major stimulatory effect of ppGpp was observed, and the highest concentration resulted in moderate inhibition. The minor stimulatory effect observed in the low micromolar ppGpp range was also obtained when the assay was performed with a constitutively active form of DmpR, ΔA2-His-DmpR, deleted of its regulatory A-domain (data not shown). No further stimulatory effects were observed by changing assay parameters likely to modulate promoter kinetics, namely (i) reduction of DmpR concentrations from the saturating 50 nM concentration to as low as 2.5 nM, (ii) reducing temperature from 37 °C to 30 or 20 °C, or (iii) shortening open complex formation time from 20 min to 8 or 3 min (data not shown). Hence we conclude that ppGpp has no major stimulatory effects on DmpR-mediated Po transcriptional output under the in vitro conditions used.

To test whether ppGpp has any direct effect on σ^N-dependent competition in vitro, we added ppGpp to a competition assay for limiting core (10 nM). In these experiments σ^N was held at a constant concentration of 40 nM and challenged with increasing amounts of σ^D from 0 to 100 nM. As shown in Fig. 4B, although σ^D effectively competes with σ^N, leading to a decrease in σ^N-dependent Po transcription to ~20% at the highest concentration tested, addition of either 20 or 180 μM ppGpp had no discernible effect. Using a similar experimental set-up, addition of 180 μM ppGpp to an in vitro competition assay has recently been shown to result in an ~2-fold difference in inhibition of transcription from the σ^D-dependent dnaK promoter caused by competing σ^N (22). Thus, in contrast to the case of the
These considerations prompted the series of experiments performed with overlapping concentrations of ppGpp. The data show that increasing concentrations of ppGpp results in a 2–2.5-fold enhancement of Po transcription in both (p)ppGpp-deficient and (p)ppGpp-proficient strains. However, the absolute output levels in the parental (p)ppGpp− MG1655Δlac derivatives are 25–45% lower than in the cognate (p)ppGpp+ CF1693Δlac derivatives. Because both the level and the competitive ability of σ2N for available core are dramatically increased in (p)ppGpp-proficient strains (Ref. 22 and references therein), additional competition by σ2N is likely to occur in the (p)ppGpp− MG1655Δlac derivatives. As shown in Fig. 5C, the presence or absence of σ2N has the anticipated effects on the levels of Po transcription in vivo. As previously observed (44), lack of σ2N in the (p)ppGpp-proficient RH90 strain results in a 2-fold increase in Po transcription, whereas overexpression of σ2N in the absence of σ2N results in a 3.5-fold net increase over the maximal levels of σ2N-dependent Po transcription observed in the parental counterpart (Fig. 5C).

Hence, we conclude that σ2N is under significant competition with both σ2N and σ3D in (p)ppGpp− cells.

Modulating σ2N Levels Restores Output from Po in the Absence of (p)ppGpp—Both σ2N and σ3D levels in E. coli are constant throughout the different growth phases (49). The finding that σ3D more readily out-competes σ2N than the converse in vitro permitted us to assess the effect of decreased levels of σ3D on transcription from Po in vivo. To achieve underproduction of σ3D, we utilized a genetic system in which expression of σ3D is under control of the P rpoD promoter, transcription from which can be regulated by varying levels of the IAA that counteracts the action of the Trp repressor (50). Culturing of both (p)ppGpp− and (p)ppGpp+ strains carrying the P rpoD system in the presence of 0.2 mM IAA results in σ3D levels comparable with those present in the wild-type (p)ppGpp+ counterpart (Fig. 6A, compare lanes 1–4). Reduced IAA (0.002 mM) causes a 2–3-fold underproduction of σ3D in both P rpoD strains, as compared with those cultured with 0.2 mM IAA (see Fig. 6A, lanes 5–8). These culturing conditions did not alter the levels of the RNA polymerase α subunit (Fig. 6A, lower panels) or β' subunits (data not shown).

When rpoD is under control of its native promoter, −2–fold higher σ3D levels are observed in the LB-grown (p)ppGpp+ strain than in the (p)ppGpp+ strain (data not shown, and Ref. 40) and transcription from Po in (p)ppGpp-deficient strains is −10% of that in (p)ppGpp-proficient strains (see Fig. 1). Culturing of P rpoD strains in LB supplemented with 0.2 mM IAA to obtain σ3D levels in the (p)ppGpp+ CF1693Δlac P rpoD derivative equivalent to those in the (p)ppGpp− counterpart results in a comparative increase in σ2N-dependent Po transcription to ∼75% of that in the presence of (p)ppGpp (Fig. 6B). Further 2–3-fold down-regulation of σ3D levels by culturing with 0.002 mM IAA results in an additional increase in Po transcription in both strains (4–5-fold), with transcription in the (p)ppGpp+ strain now exceeding that in the presence of native σ2N levels and (p)ppGpp by ∼3-fold (Fig. 6B). Thus, consistent with a major role of σ-factor competition on Po transcription, down-regulation of σ3D levels can fully restore σ2N-dependent Po transcription in the absence of (p)ppGpp. Down-regulation of σ3D levels to below those in wild-type cells also allows transcription from Po during exponential growth where (p)ppGpp levels are low and the Po promoter is normally silent (compare Figs. 6C and 6D).
and thereby reducing access to core RNA polymerase (51, 52). Given the large effect of reduced levels of $\sigma^D$ on $\sigma^N$-dependent Po transcription, we also determined the effect of increased Rsd levels on Po transcription in vivo. This was achieved by introducing this gene on a high copy number plasmid into the P$_{rpoD}$ strains. As shown in Fig. 6B, Rsd overproduction alone increases Po transcription $\sim 3$-fold in both strains and is sufficient to restore Po transcription in the absence of (p)ppGpp to above that observed in the presence of (p)ppGpp and native levels of Rsd. Similar -fold increases in Po transcription by the presence of the Rsd plasmid were also observed in (p)ppGpp-proficient and deficient strains with rpoD in its native context (data not shown). Thus, like underproduction of $\sigma^N$, overproduction of Rsd has a major impact on $\sigma^D$-dependent Po transcription in vivo. The effects of these manipulations are more than additive, with simultaneous underproduction of $\sigma^N$ and overproduction of Rsd resulting in a 10–12-fold increase over the maximum levels of Po transcription in cells with wild-type levels of these proteins (Fig. 6B).

**DISCUSSION**

Here we report on the function of (p)ppGpp as a master determinant of the outcome of $\sigma^N$ competition for limiting core RNA polymerase. This role for (p)ppGpp is based on data using transcription of the $\sigma^N$-dependent Po promoter as a functional probe for ErN activity in both in vivo and in vitro assays. Four $\sigma^D$ suppressor mutations that functionally mimic (p)ppGpp share the common property of being defective in their ability to compete with $\sigma^N$ for limiting core RNA polymerase in vitro (Figs. 2 and 3). These mutations were isolated by different genetic strategies, two (P504L and S506F) on the basis of their ability to restore prototrophy (40) and two (Y571H) on the basis of restoration of Po transcription, in (p)ppGpp-deficient E. coli (Fig. 1). Consistent with the common property of defects in $\sigma$-factor competition underlying the mechanism of suppression in both cases, the magnitude of the defect of these four $\sigma^D$ mutations follows the same hierarchy as their ability to restore Po transcription in (p)ppGpp cells. This idea is further supported by the finding that underproduction of $\sigma^N$ and/or sequestering of $\sigma^D$ by Rsd in vivo can restore Po transcription in the absence of (p)ppGpp (Fig. 6). Moreover, underproduction of $\sigma^N$ allows ErN-dependent Po transcription in (p)ppGpp cells during exponential growth where (p)ppGpp levels are low and the Po promoter is normally silent. Thus, we

**Fig. 5. In vivo effects of modulation of $\sigma^N$ and $\sigma^D$ levels on transcription from the Po promoter.** A, CF1693Δlac (open bars) and MG1655Δlac (shaded bars) harboring pVI466 and either the P$_{rpoD}$-pwo expression plasmid pVI688 ( ) or a vector control plasmid pEXT21 ( ) were cultured in the presence of 0.5 mM IPTG. The results are represented as -fold increase over transcription in CF1693lac (pVI466, pEXT21). Inset shows a Western blot of the $\sigma^D$ levels in 25 μg of soluble proteins from the assayed cultures harvested 1 h into stationary phase. B, CF1693Δlac and MG1655Δlac derivatives with the indicated mutant rpoD alleles and harboring pVI466 and either pVI688 or a vector control plasmid pEXT21 were cultured as described under A. The results are represented as -fold increase of maximal transcription in strains overexpressing $\sigma^N$ from pVI688 versus vector control. Absolute values of luciferase activity from MG1655Δlac derivatives harboring pVI466/pEXT21 were 25–45% lower than their CF1693Δlac counterparts. C, MC1400 (open bars) and its isogenic rpoS-null mutant RH90 (hatched bars) harboring the dmpR-Po-luxAB reporter pVI466 and either the P$_{rpoD}$-pwo expression plasmid pVI688 ( ) or a vector control plasmid pEXT21 ( ) were grown and assayed as described under A. Results are the average of the maximal luciferase output from the Po promoter obtained from two or three cultures in independent experiments.

**Fig. 6. Effect of modulation of in vivo $\sigma^N$ levels on transcription from the Po promoter.** A, Western blot analysis of 25 μg of crude extract from (p)ppGpp-proficient (+) and (p)ppGpp-deficient (0) strains grown in rich media supplemented with 0.2 mM IPTG ( ) or 0.002 mM IPTG ( ) or 0.002 mM IPTG ( ). Expression of rpoD from chromosome in its wild type context (native-rpoD) or from the IAA-dependent P$_{rpoD}$ promoter (P$_{rpoD}$-rpoD) is indicated at the top of the panel. B shows the transcriptional output from Po of the luciferase reporter plasmid pVI684 in (p)ppGpp- CF1693Δlac P$_{rpoD}$-rpoD (open bars) or in (p)ppGpp- MG1655Δlac P$_{rpoD}$-rpoD (shaded bars) that harbor either the plasmid pBR-Rsd (indicated as Rsd ) or the pBR322 vector control (indicated as Rsd ). Results are the average of the data from two independent experiments with cultures inoculated into LB supplemented with 0.2 mM IPTG (indicated as $\sigma^D$ ) or 0.002 mM IPTC (indicated as $\sigma^D$ ). Qualitative similar results were obtained when strains were cultured in rich defined minimal media (data not shown). C, immediate Po transcription response (open symbols) upon down-regulation of $\sigma^N$ levels in CF1693lac P$_{rpoD}$-rpoD (pVI684, pBR322 circles) and its (p)ppGpp proficient counterpart (squares) MG1655Δlac P$_{rpoD}$-rpoD (pVI684, pBR322). Growth as measured by A$_{600}$ is indicated with closed symbols.

and 1A). The finding that a 2–3-fold reduction in $\sigma^D$ causes a $\sim 5$-fold increase in $\sigma^N$ transcription (Fig. 6B), whereas a $> 10$-fold increase in $\sigma^N$ over native levels causes only a $\sim 2.5$-fold increase in Po output (Fig. 5), mirrors the in vitro finding that increased levels of $\sigma^D$ are poorer at out-competing $\sigma^N$ than the converse.

The E. coli Rsd protein has been proposed to act as an anti-$\sigma^D$-factor during stationary phase by binding to free $\sigma^D$ and thereby reducing access to core RNA polymerase (51, 52). Given the large effect of reduced levels of $\sigma^N$ on $\sigma^D$-dependent Po transcription, we also determined the effect of increased Rsd levels on Po transcription in vivo. This was achieved by introducing this gene on a high copy number plasmid into the P$_{rpoD}$ strains. As shown in Fig. 6B, Rsd overproduction alone increases Po transcription $\sim 3$-fold in both strains and is sufficient to restore Po transcription in the absence of (p)ppGpp to above that observed in the presence of (p)ppGpp and native levels of Rsd. Similar -fold increases in Po transcription by the presence of the Rsd plasmid were also observed in (p)ppGpp-proficient and deficient strains with rpoD in its native context (data not shown). Thus, like underproduction of $\sigma^N$, overproduction of Rsd has a major impact on $\sigma^D$-dependent Po transcription in vivo. The effects of these manipulations are more than additive, with simultaneous underproduction of $\sigma^N$ and overproduction of Rsd resulting in a 10–12-fold increase over the maximum levels of Po transcription in cells with wild-type levels of these proteins (Fig. 6B).

**DISCUSSION**

Here we report on the function of (p)ppGpp as a master determinant of the outcome of $\sigma^N$ competition for limiting core RNA polymerase. This role for (p)ppGpp is based on data using transcription of the $\sigma^N$-dependent Po promoter as a functional probe for ErN activity in both in vivo and in vitro assays. Four $\sigma^D$ suppressor mutations that functionally mimic (p)ppGpp share the common property of being defective in their ability to compete with $\sigma^N$ for limiting core RNA polymerase in vitro (Figs. 2 and 3). These mutations were isolated by different genetic strategies, two (P504L and S506F) on the basis of their ability to restore prototrophy (40) and two (Y571H) on the basis of restoration of Po transcription, in (p)ppGpp-deficient E. coli (Fig. 1). Consistent with the common property of defects in $\sigma$-factor competition underlying the mechanism of suppression in both cases, the magnitude of the defect of these four $\sigma^D$ mutations follows the same hierarchy as their ability to restore Po transcription in (p)ppGpp cells. This idea is further supported by the finding that underproduction of $\sigma^N$ and/or sequestering of $\sigma^D$ by Rsd in vivo can restore Po transcription in the absence of (p)ppGpp (Fig. 6). Moreover, underproduction of $\sigma^N$ allows ErN-dependent Po transcription in (p)ppGpp cells during exponential growth where (p)ppGpp levels are low and the Po promoter is normally silent. Thus, we
conclude that (p)ppGpp per se is not an absolute requirement for α^N-dependent Po transcription. Rather, elevated synthesis of (p)ppGpp at the onset of stationary phase allows successful competition of α^N for the available core, resulting in elevated levels of EαD_N sufficient to occupy and initiate transcription from the Po promoter. These findings do not preclude that sufficient EαD_N exists during rapid exponential growth to allow transcription from certain high affinity, easy to saturate α^N promoters. Thus, akin to the model put forward to explain (p)ppGpp stimulation of specific α^S-promoters (33), the extent to which (p)ppGpp modulation of EαD_N levels is manifested at different promoters will depend on their innate ability to recruit limiting EαD_N.

The data outlined above clearly support a recently proposed model for (p)ppGpp as a master regulator of alternative σ-factor competition (22), and extend the model to include the structurally and functionally distinct σ^N. Within this model (p)ppGpp directly modulates interaction of alternative σ-factors with the increased pool of core RNA polymerase generated by (p)ppGpp-mediated down-regulation of stringent promoters. Facilitation of association of alternative σ-factor by (p)ppGpp has been postulated to explain both decreases in the levels of α^N-holoenzyme and increases in the levels of α^S- and α^D-holoenzymes in extracts from (p)pGppN as compared with (p)pGpp^0 cells (22, 40). Most directly, addition of ppGpp to an in vitro competition assay has been shown to have a direct stimulatory effect on the outcome of competition between the low affinity α^D-factor and α^D for core. It is as yet unclear whether (p)ppGpp has an inhibitory effect on α^D binding, a stimulatory effect on α^S and α^D binding to core, or both (22). We could not document any effect of ppGpp on α^S competition against α^D (Fig. 4) under assay conditions that gave the predicted 2-fold effect on α^H competition (data not shown). These results suggest that detection of direct effects of (p)ppGpp on α-factor competition may be limited to low affinity α^D-like proteins, rendering systems dependent on α^D and α^S more sensitive to α-factor competition. This idea is supported by the data from the α^S suppressor mutants (YDSA-(536–538) and Y571H), where the effect of the poor suppressor α^D-Y571H that barely has detectable effects on α^S-competition (Figs. 1 and 3), has markedly greater effects on in vivo and in vitro competition assays involving α^D and α^S (22).

The role of (p)ppGpp in α-factor competition as described above does not exclude the possibility that transcription of some promoters are directly controlled by (p)ppGpp. Both positive and negative in vitro effects of adding (p)ppGpp to reconstituted transcription systems have been found; however, they are frequently notably lower than when assessed in vivo. More extensive positive stimulatory effects have been observed using coupled in vitro transcription-translation systems (53, 54). We have only observed minor direct stimulatory effects of low micromolar concentrations of (p)ppGpp on EαD_N transcription from the Po promoter in vitro (Fig. 4A). More substantial stimulation has been observed in a similar in vitro transcription assay employing the Po promoter and a truncated constitutively active form of XylR (27). We cannot as yet explain the differences in the stimulation levels observed in the two assays; however, it is possible that it is attributable to one of a number of differing properties between the two regulators (13). Nevertheless, the role of (p)ppGpp in determining the level of the pools of alternative holoenzyme RNA polymerases provides an in vivo mechanism to amplify even minor direct effects of (p)ppGpp at specific promoters.

The in vivo impact of α-factor competition significantly influences the levels of α^D-dependent Po transcription, with over-expression of α^D, or lack of α^S, causing a ~2.5-fold increase, and underproduction and sequestering of α^D causing a >10-fold increase over the maximum level of Po transcription achieved in wild-type cells. Given the great impact of α-factor competition on Po output, it may appear surprising that only one rpoN suppressor allele was isolated during the genetic selection. This mutant possesses two conservative substitutions (E150D and H165M) that both lie within a subportion of region II (alpha acids 120–215) of αD that is intimately associated with core (reviewed in Ref. 8). These mutations only mediate a low level suppressor phenotype (Fig. 1); however, we were unable to overproduce and purify αD-E150D/H165M using the αD-dependent temperature-sensitive λ P55 P55 expression system that has been successfully employed for overexpression and purification of native αD. As with two other overexpression systems that are ultimately dependent on αD (lacP4/P lac and the phage T7 system P7 promoter RNAP/P77), induction conditions were found to result in rapid growth arrest. Although anecdotal, these findings suggest that αD-E150D/H165M possesses an enhanced ability to compete with α^S, and that more pronounced increases in competitiveness might be lethal. In this respect it is interesting to note that, although both α^D and α^S exhibit similar affinities for core when assessed in isolation (Fig. 2), α^D is poorer at competing α^D than the converse in both in vivo and in vitro assays. The levels of these proteins within the cell are also disproportionate as compared with the number of genes requiring their activity. For an estimated ~30 potential α^D-dependent promoters, ~110 copies/cell of α^D are available, whereas only 600–700 copies/cell of α^S are available for >1000 actively transcribed α^D-dependent promoters (36, 55). The properties of constant comparatively high levels and high affinity of α^S, together with (p)ppGpp stimulation of its otherwise poor competitive ability against α^D, would provide a system for rapid alteration in occupancy and transcription of α^D-dependent promoters in response to nutritional changes in the environment without de novo synthesis of prerequisite α. Thus, for the Po promoter controlling the enzymes for methylphenol metabolism, lack of (p)ppGpp and concomitant high α-factor competition fulfill the same function as catabolite repression, namely causing silencing of energetically less favorable specialized catabolic functions until needed.

In the holoenzyme, α lies spread out across the upstream face of the enzyme with each of the σ^N4 domains and the linkers connecting them making extensive contacts with the core enzyme. The P504L and S506F substitutions lie just within the N-terminal end of the conserved 3.2-linker that joins the β1-associated σ^N and σ^N that interacts with the β-flap. The 3-amino acid insertion mutation YDSA-(536–538), respectively) substitution is directly adjacent to the σ^N4.2 conserved region (6). Thus, consistent with their defects in competition against α^N, all these mutants could directly or indirectly decrease the overall affinity of the extensive α^D-core interaction.

The four mutant rpoD alleles employed in this study provided an important mechanistic tool to dissect the role of α-factor competition without specifically affecting the kinetics of EαD_N at α^D-dependent promoters. The least and most severely affected proteins (Y571H and YDSA-(536–538), respectively) do not restore prototrophy, whereas the immediately affected proteins (P504L and S506F) do (Table I). These results suggest that restoration of α^D-Po transcription may provide a broader activity window than prototrophy for isolating suppressor mutations within α^D. The genetic selection strategy, as anticipated, also identified many mutations in the β (rpoB) and β′ (rpoC) subunits of the transcriptional apparatus that restore transcription to Po in the absence of (p)ppGpp. These mutations include some (e.g. R454H; Fig. 1) that have previously
been isolated on the basis of restoration of prototrophy (32). RpoB-R454H and some of the other newly isolated rpoBC suppressors also restore the ability to grow on minimal media, whereas others do not (Table I and data not shown). Similarly, only 7 of 15 rpoBC alleles isolated on the basis of restoration of prototrophy also exhibited the phenotype of restoring activity to the Po promoter in (p)pGpp-deficient strains (25). Thus, although some mutations in rpoBC make both phenotypes, others are specific to one phenotype. In addition to the potential to alter binding of αδ to core, mutations located within the β and β' subunits have the potential to directly and differentially affect transcription kinetics at different classes of promoters. Our future analysis of these mutations is aimed at clarifying the degree to which such modulation and effects on α-factor competition contribute to their different suppressor phenotypes.

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