The EMBO Journal (2004) 23, 4473–4483 | © 2004 European Molecular Biology Organization | All Rights Reserved 0261-4189/04
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THE EMBO JOURNAL

An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation

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As an approach to the study of rRNA synthesis in Gram-positive bacteria, we characterized the regulation of the *Bacillus subtilis rrnB* and *rrnO* rRNA promoters. We conclude that *B. subtilis* and *Escherichia coli* use different strategies to control rRNA synthesis. In contrast to *E. coli*, it appears that the initiating NTP for transcription from *B. subtilis* rRNA promoters is GTP, promoter strength is determined primarily by the core promoter (−10/−35 region), and changes in promoter activity always correlate with changes in the intracellular GTP concentration. rRNA promoters in *B. subtilis* appear to be regulated by changes in the initiating NTP pools, but in some growth transitions, changes in rRNA promoter activity are also dependent on *relA*, which codes for ppGpp synthetase. In contrast to the situation for *E. coli* where ppGpp decreases rRNA promoter activity by directly inhibiting RNA polymerase, it appears that ppGpp may not inhibit *B. subtilis* RNA polymerase directly. Rather, increases in the ppGpp concentration might reduce the available GTP pools, thereby modulating rRNA promoter activity indirectly.

The EMBO Journal (2004) **23,** 4473–4483. doi:10.1038/sj.emboj.7600423; Published online 21 October 2004
Subject Categories: chromatin & transcription; microbiology & pathogens
Keywords: *B. subtilis*; GTP concentrations; ppGpp; promoters; rRNA transcription

Introduction

Ribosomal RNA synthesis is the rate-limiting step in ribosome synthesis in both *Escherichia coli* and *Bacillus subtilis* (Henkin, 2002; Paul *et al*, 2004b). rRNA promoters are tightly regulated with nutritional conditions to accommodate the cell's changing translational requirements while preventing overinvestment of biosynthetic resources in energetically costly ribosome synthesis.

Each of the seven rRNA operons in *E. coli* contains two promoters, *rrn* P1 and *rrn* P2. The core (−10/−35) region in *rrn* P1 promoters is preceded by upstream (UP) elements that increase promoter activity ~20- to 50-fold by binding the C-terminal domains of the two α subunits of RNA polymerase (RNAP) (Ross *et al*, 1993; Hirvonen *et al*, 2001). This region is preceded by binding sites for the transcription factor Fis that increase promoter activity an additional three- to eight-fold, depending on the operon (Hirvonen *et al*, 2001). Fis does not activate *rrn* P2 promoters, and UP elements play a much smaller role in P2 than in P1 activity (Murray *et al*, 2003a).

Most regulation of *E. coli rrn* P1 and P2 promoter activity is attributable to the effects of small molecule effectors, ppGpp and NTPs, whose concentrations change at specific times in growth and alter rRNA transcription (Murray *et al*, 2003b; Murray and Gourse, 2004). (ppGpp refers collectively here to both ppGpp and its precursor pppGpp.)

Transcription initiation begins with the interaction of RNAP and the promoter to form an initial closed complex. This step is followed by the formation of several kinetic intermediates, culminating in open complex(es) in which the transcription initiation site is exposed. RNA synthesis starts with the incorporation of incoming NTPs and the transition to an elongation complex capable of processive transcription.

E. coli rRNA promoters form open complexes with extraordinarily short half-lives compared to most other promoters (Gourse, 1988; Murray and Gourse, 2004). We have proposed that this is the kinetic property that makes rRNA promoters sensitive to changing concentrations of their initiating NTP (iNTP); increasing NTP concentration could increase transcription simply by mass action (Gaal *et al*, 1997; Barker and Gourse, 2001). Alternatively, in theory the iNTP(s) could induce a conformational change in RNAP to facilitate transcription (Lew and Gralla, 2004). In any case, increasing concentrations of the iNTP directly stimulate *E. coli rrn* P1 and *rrn* P2 promoter activity *in vitro* and *in vivo* (Murray *et al*, 2003b; Murray and Gourse, 2004).

ppGpp is made in response to amino-acid starvation and some other nutritional stresses (Cashel *et al*, 1996). ppGpp binds directly to RNAP and increases the rate of open complex collapse at all promoters (Barker *et al*, 2001). We have proposed that rRNA transcription is specifically inhibited by ppGpp, at least in part, because this step is rate determining for rRNA promoters (Barker *et al*, 2001). In support of this model, studies on a large number of mutant *rrnB* P1 promoters indicate that there is a strict correlation between formation of a short-lived open complex and effects of ppGpp and the iNTP on transcription *in vitro* and *in vivo* (Barker, 2001; Barker *et al*, 2001; Barker and Gourse, 2001).

It has also been proposed that ppGpp competes directly with iNTP binding (Jores and Wagner, 2003). The recent X-ray structure of ppGpp in complex with RNAP suggests that ppGpp binds close to, but not overlapping, the catalytic center (Artsimovitch *et al*, 2004), but direct effects of ppGpp on NTP binding have not been ruled out. There may also be cases where ppGpp inhibits transcription by a mechanism

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Received: 20 July 2004; accepted: 25 August 2004; published online: 21 October 2004

different from effects on open complex collapse or competition for NTP addition (Potrykus *et al*, 2002).

Here we address whether bacteria evolutionarily distant from *E. coli*, such as the spore-forming Gram-positive bacterium *B. subtilis*, use strategies similar to *E. coli* to regulate rRNA synthesis. *B. subtilis* rRNA synthesis has been studied in the past (e.g. Testa and Rudner, 1975; Deneer and Spiegelman, 1987; Wellington and Spiegelman, 1993; Henkin, 2002), but the detailed molecular mechanisms responsible for regulation remain unclear. *B. subtilis* contains 10 *rrn* operons (Henkin, 2002). Six operons appear to contain tandem P1–P2 promoters, and four only a single promoter. When the *B. subtilis rrnB* P1 and P2 promoters were measured in *E. coli*, *rrn* P2 activity changed with growth rate more than *rrn* P1 activity (Deneer and Spiegelman, 1987).

We report here the properties and regulation of the *B. subtilis rrnB* and *rrnO* promoters in their natural host. We demonstrate that the *rrn* P1 promoters display more pronounced changes with growth rate and phase than the *rrn* P2 promoters, and that DNA sequences upstream of the core promoters contribute much less to promoter activity than in *E. coli* rRNA promoters. Transcription from these *B. subtilis* promoters initiates with GTP and is regulated by changes in GTP and ppGpp concentrations *in vivo*. However, in contrast to the situation in *E. coli*, ppGpp appears to regulate rRNA promoter activity indirectly by affecting GTP pools.

Results

B. subtilis rRNA promoters initiate with GTP

As a first step in studying *B. subtilis* rRNA transcription, we constructed *lacZ* fusions to the P1 and P2 promoters from two rRNA operons, *rrnB* and *rrnO*, and from a control promoter, P*veg*, that is expressed constitutively during vegetative growth (Fukushima *et al*, 2003). *rrnB* was chosen because it had been investigated extensively previously (Deneer and Spiegelman, 1987; Wellington and Spiegelman, 1993). *rrnO* was chosen as representative of the rRNA promoter class differing in sequence from *rrnB*. The fusions were integrated in single copy at the *B. subtilis amyE* locus (see Supplementary material).

The DNA sequences of *rrnB* and *rrnO* P1 and P2 and P*veg* are shown in Figure 1A. Primer extension from RNAs synthesized *in vivo* identified start sites 8 bp downstream of the presumptive *rrn* P1 −10 hexamers and 7 bp downstream of the presumptive *rrn* P2 −10 hexamers, either in constructs in which the two promoters were in their natural tandem configuration or in which they were separated from each other (Figure 1B–D and data not shown). All four *rrn* promoters initiated with GTP. The *veg* transcript initiated with ATP (data not shown), as reported previously (e.g. Fukushima *et al*, 2003). For experiments described below, *rrn* P1 and P*veg* promoters were created with A and G at position +1 (nontemplate strand), respectively, and their transcription start sites were verified by primer extension (data not shown). When appropriate, the identity of the +1 position is indicated in the promoter name (e.g. *rrnB* P1+1A).

Since the *rrnB* and *rrnO* P1 and P2 core promoter constructs made the exact same RNA, their activities could be compared directly by quantitative primer extension (Materials and methods). The four core promoters had similar activities in LB medium (Figure 1E). These results, in conjunction with the small effects of their upstream regions (see below), indicate that *rrnB* and *rrnO* promoter activities are similar in rich medium. This result does not support the conclusion of an earlier study employing *lacZ* fusions that the *rrnB* promoters are much weaker than the *rrnO* promoters (Okamoto and Vold, 1992; see below and Materials and methods).

Sequences upstream of the B. subtilis rrn P1 and P2 core promoters increase transcription much less than in E. coli

The RNAP αCTD(s) interact with sequences upstream of the core promoter in many *B. subtilis* operons, increasing transcription (e.g. Fredrick *et al*, 1995; Helmann, 1995; Meijer and Salas, 2004). We initially used promoter-*lacZ* fusions with different upstream end points, measuring β-galactosidase activities to estimate the effects of upstream sequences in rRNA operons. However, we found that β-galactosidase activities consistently underestimated rRNA promoter activities at the highest growth rates (data not shown; see Materials and methods). Therefore, we used quantitative primer extension to measure promoter activities directly from the same fusions (Figure 2A). In contrast to the situation with *E. coli rrn* P1 promoters, the native sequence upstream of the −35/−10 region of *rrnB* P1 increased expression only two- to three-fold (Figures 1A, 2A, and B) relative to constructs containing plasmid-derived sequences or the G+C-rich 'SUB' sequence (which does not bind the *E. coli* αCTD; Rao *et al*, 1994) fused to the core promoter. Only small increases were observed when the native *rrnB* P1 sequence was extended as far upstream as −352 (Figure 2B). Even smaller effects of upstream sequences (<2-fold) were observed for *rrnB* P2, *rrnO* P1, and *rrnO* P2 (Figure 2A–C). Consistent with the ~2-fold effect of the *B. subtilis rrnB* P1 upstream sequence on promoter activity *in vivo*, we detected only slight stimulation (<2-fold) of this promoter by its upstream sequence with *B. subtilis* RNAP *in vitro* (Figure 2D).

As controls to verify that these methods would likely have detected stimulation by *rrn* upstream sequences, we measured the effects of previously characterized UP elements using purified RNAP *in vitro* and by primer extension *in vivo*. The *E. coli rrnB* P1 UP element stimulated its core promoter ~30-fold using *E. coli* RNAP *in vitro* (Figure 2E) and ~45-fold by primer extension with RNA extracted from *E. coli* cells (Figure 2F), consistent with previous observations (Rao *et al*, 1994). Furthermore, our methods detected a 14-fold stimulatory effect of the *B. subtilis hag* UP element *in vivo* (Figure 2G), in good agreement with a previous report (Fredrick *et al*, 1995).

Taken together, these results suggest that classical transcription activators and UP elements do not make large contributions to the activities of *B. subtilis* rRNA promoters. At least under the conditions tested, *B. subtilis rrnB* and *rrnO* promoter strength derives primarily from the core promoter.

rrn P1 promoters display more pronounced changes in activity with changing nutritional conditions than rrn P2 promoters

E. coli rRNA promoter activity increases proportionally with growth rate (Paul *et al*, 2004b). Regulation of *B. subtilis* rRNA promoter activity was estimated by growing cells at different steady-state growth rates (determined by the nutritional

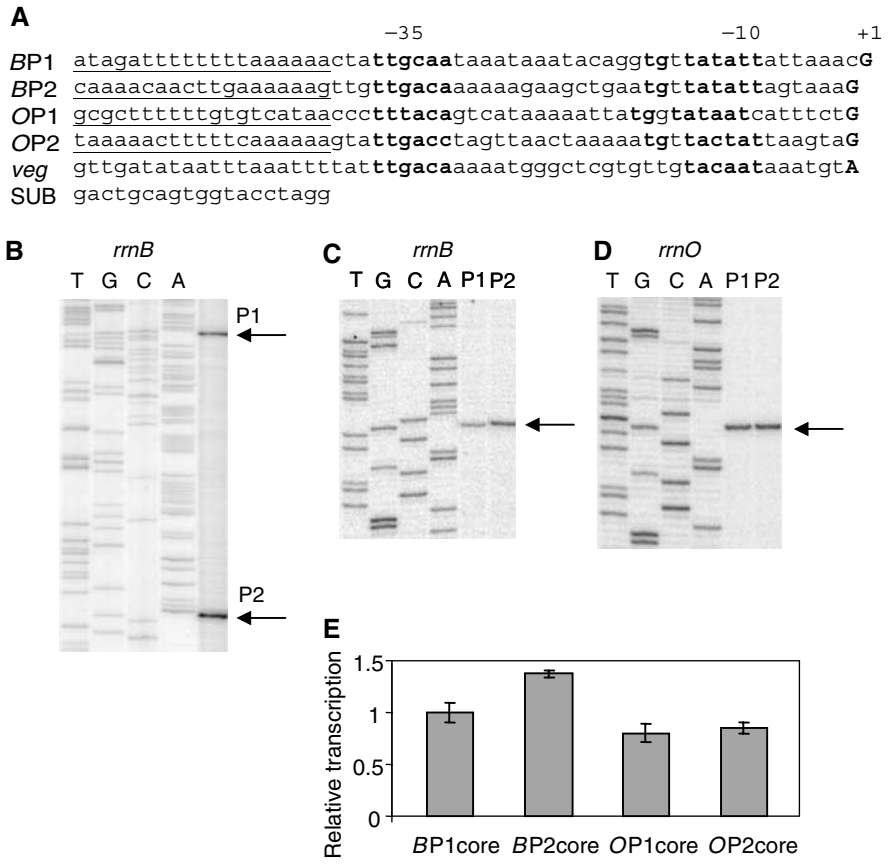


Figure 1 (**A**) Sequences of *B. subtilis* promoter constructs used in this study. Putative −10 and −35 hexamers and the +1 positions are in bold. Putative UP elements are underlined. Core promoter constructs contain native sequence from 3 bp upstream of the −35 element (−39 in *rrnB* P1) to +1. The arbitrary triplet TCT was inserted adjacent to the +1 position, followed by the *Hin*dIII site, to avoid positioning an A next to +1. In indicated core promoter constructs, the SUB sequence (Rao *et al*, 1994) was substituted for the same length of native sequence upstream of the −35 element. The *veg* promoter is described in the text. (**B**) Primer extension mapping of start sites from cells grown in rich medium containing a *B. subtilis rrnB* P1–P2 tandem promoter construct (RLG6930; contains *rrnB* sequence from −248 upstream of *B*P1 to +8 of *B*P2). (**C, D**) Primer extension mapping of start sites from isolated *B. subtilis rrnB* and *rrnO* P1 and P2 promoter constructs (−39 to +1 for *B*P1, −38 to +1 for *B*P2, *O*P1, and *O*P2 (RLG7554, RLG7553, RLG7369, and RLG7370, respectively). Since the isolated P1 and P2 promoters make the same RNA in these fusions, the primer extension products migrate to the same position in the gel. Arrows indicate start sites. Sequencing ladders are shown for P1 promoters only. (**E**) Relative activities of *rrnB* and *rrnO* core promoters. Cells containing the core promoter constructs used in (C, D) were grown concurrently in LB to OD_{600} ~0.3, and promoter activities were measured by primer extension, normalized to cell density and to the same RNA recovery marker (see Materials and methods). Activities are in arbitrary units, relative to the *rrnB* P1 core promoter.

composition of the medium) and measuring transcription from *rrnB* P1 and P2 promoter constructs by primer extension. Since the constructs made the exact same RNA transcript, differences in transcription reflected only differences in promoter activity and not some potential difference in RNA half-life (see also Supplementary material). Each promoter activity is plotted as a function of growth rate, normalized at the lowest growth rate, to facilitate visualization of differences in regulation (Figure 3). Contrary to the conclusion reached previously (from measurements of *B. subtilis rrnB* promoters in *E. coli*; Deneer and Spiegelman, 1987), *rrnB* P1 increased much more with growth rate than *rrnB* P2. Similar results were obtained for the *rrnO* P1 versus *rrnO* P2 promoters, and for the *rrnB* P1 versus *rrnB* P2 promoters in their natural tandem configuration (data not shown). Thus, regulation of *rrn* promoter activities in a heterologous host may not mimic the native situation.

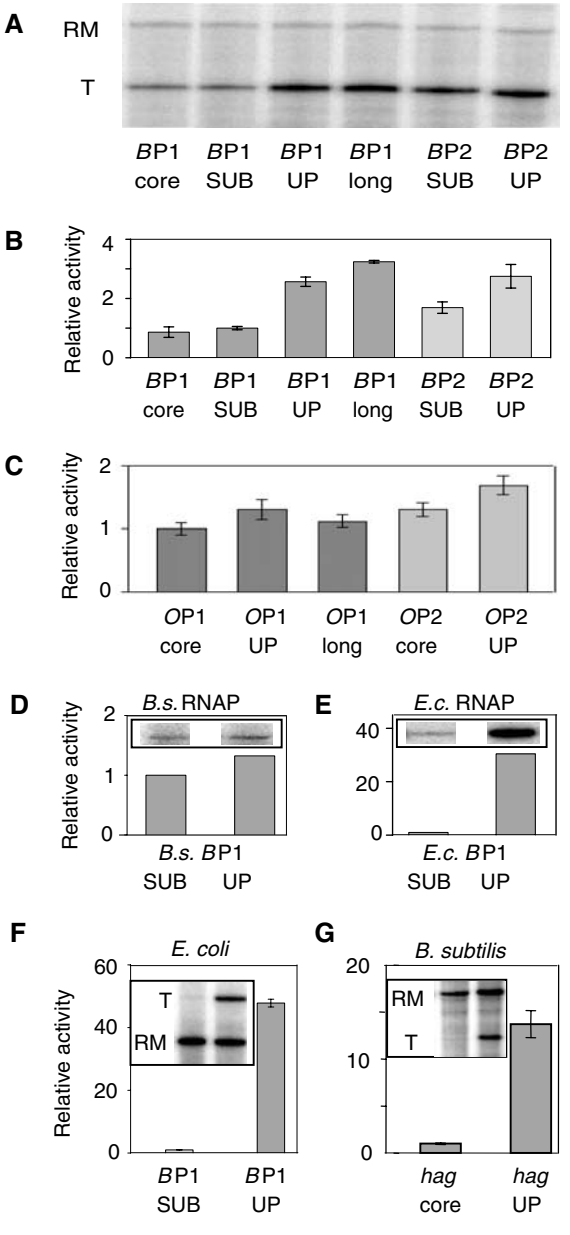
Although the full-length P1 promoters and full-length P2 promoters account for similar amounts of rRNA transcription in rich medium (Figure 2B), at slow growth rates the P2 promoters are less inhibited than the P1 promoters and thus account for the majority of rRNA synthesis (as in *E. coli*; Murray *et al*, 2003a; Murray and Gourse, 2004). The growth rate-dependence curves derived from measuring total RNA:protein ratios (data not shown) were very similar to those derived from the analysis of the individual promoters, suggesting that regulation of the *rrnB* and *rrnO* promoters is typical for *B. subtilis* rRNA operons. The *rrnB* P2 and *rrnO* P2 promoters also exhibited much less pronounced changes in activity than their respective P1 promoters during outgrowth from, and entry into, stationary phase (data not shown), further suggesting that *B. subtilis rrn* P1 promoters are regulated more than their respective P2 promoters.

B. subtilis rrn P1 promoters require high concentrations of their iNTP but appear insensitive to ppGpp in vitro

We next investigated potential mechanisms for regulation of *B. subtilis* rRNA promoter activity. Changing concentrations of the iNTP and ppGpp account for much of the regulation of

rRNA transcription in *E. coli* (Murray *et al*, 2003b; Murray and Gourse, 2004). In support of this conclusion, *E. coli* rRNA promoters require higher concentrations of the iNTP for transcription than other promoters *in vitro*, and ppGpp moderately but specifically inhibits transcription from rRNA promoters *in vitro* (Barker *et al*, 2001). Therefore, we measured the effects of iNTP and ppGpp concentrations *in vitro* on the more regulated of the two *B. subtilis rrn* promoters, *rrn* P1, using purified *B. subtilis* RNAP (Figures 4 and 5).

Relative to the control, P*veg* + 1G (Figure 4C), *rrnB* P1 + 1G required high levels of GTP, but not ATP, for maximal transcription (Figure 4A), and relative to P*veg* + 1A (Figure 4C), *rrnB* P1 + 1A required high levels of ATP and not GTP (Figure 4B). *rrnO* P1 + 1G and + 1A displayed NTP dependences similar to those of *rrnB* P1 (data not shown). Thus, like *E. coli* rRNA promoters, *B. subtilis* rRNA promoters displayed iNTP dependences *in vitro* characteristic of regulation of promoter activity by changes in NTP concentrations *in vivo*. The identity of the + 1 position in *B. subtilis rrn* P1



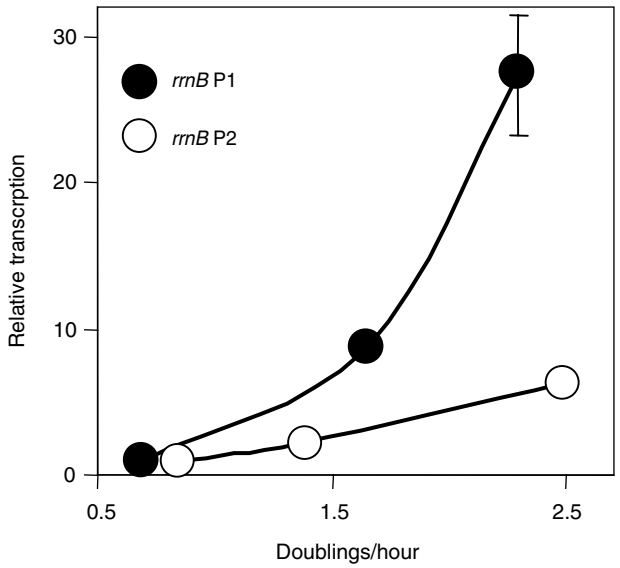


Figure 3 Growth rate-dependent control of *B. subtilis* rRNA promoters (*rrnB* P1: −39 to +1, RLG7554; *rrnB* P2: −38 to +1, RLG7553). Promoter activity (arbitrary units) was measured by primer extension from RNA extracted from cells grown in different media. Slowest to fastest growth rate: (i) MOPS, 1% glucose, phenylalanine and tryptophan; (ii) MOPS, 1% glucose, 20 amino acids; (iii) LB. To facilitate comparison of slopes, promoter activities were normalized to the activity of its own promoter at the lowest growth rate.

Figure 2 Contribution of sequences upstream of the −35 element to *B. subtilis* rRNA promoter activity. (**A**) Representative primer extension bands from RNAs synthesized in cells grown in LB medium from *B. subtilis rrnB* P1 and *rrnB* P2 (promoter end points: *B*P1 core: −39 to +1, RLG7554; *B*P1 SUB: −39 to +1, RLG7372; *B*P1 UP: −58 to +1, RLG7373; *B*P1 long: −352 to +1, RLG7584; *B*P2 SUB: −38 to +1, RLG7374; *B*P2 UP: −57 to +1, RLG7375). The test (T) and recovery marker (RM) reverse transcripts are indicated (see also Materials and methods). (**B**) Effects of upstream sequences on transcription from *B*P1 and *B*P2 (constructs described in panel A, normalized to the activity of *B*P1 SUB). (**C**) Effects of upstream sequences on transcription from *rrnO* P1 and *rrnO* P2 (*O*P1 core: −38 to +10, RLG7027; *O*P1 UP: −77 to +10, RLG7028; *O*P1 long: −227 to +10, RLG7030; *O*P2 core: −38 to +10, RLG6937; *O*P2 UP: −77 to +10, RLG7029). Promoter activities are normalized to *O*P1 core. (**D, E**) Effect of upstream sequences on *in vitro* transcription from (D) *B. subtilis rrnB* P1 using *B. subtilis* (*B.s.*) RNAP and supercoiled templates containing *B.s. B*P1 SUB (−39 to +1, pRLG7599) or *B.s. B*P1 UP (−58 to +1, pRLG7598) or (E) *E. coli rrnB* P1 using *E. coli* (*E.c.*) RNAP and supercoiled templates containing *E.c. B*P1 SUB (−41 to +50, pRLG2230) or *E.c. B*P1 UP (−66 to +50, pRLG6214). Promoter activities are from quantitation (phosphorimager units) of the *in vitro* transcripts shown at the top of the panels. The fold effect of the *B. subtilis rrn* P1 upstream sequence varied from 1.3- to 1.6-fold, while the fold effect of the *E. coli rrn* P1 upstream sequence varied from 15- to 30-fold. (**F**) Effect of *E. coli rrnB* P1 UP element on transcription *in vivo* in *E. coli*. Transcripts were measured by primer extension from RNAs transcribed from *E. coli rrnB* P1 SUB (−39 to +50, RLG3097) and *E. coli rrnB* P1 UP (−66 to +50, RLG3074) constructs in single copy in the *E. coli* chromosome. Activities are normalized to the core promoter construct. (**G**) Effect of a *B. subtilis hag* promoter UP element on transcription in *B. subtilis* using the same methods as in (A–C). *B. subtilis* promoters (*hag* core, −43 to +4, RLG7391; *hag* promoter with upstream sequences = *hag* UP, −96 to +4, RLG7392) were integrated in the *B. subtilis* chromosome. The activity of *hag* UP is normalized to *hag* core. For the experiments in panels A–C and F, total RNA was extracted in early exponential phase (OD_{600} ~0.3) from cells grown in LB for at least four doublings. In (G), RNA was extracted from cells in late exponential phase (OD_{600} ~2.0) when the *hag* promoter is most active.

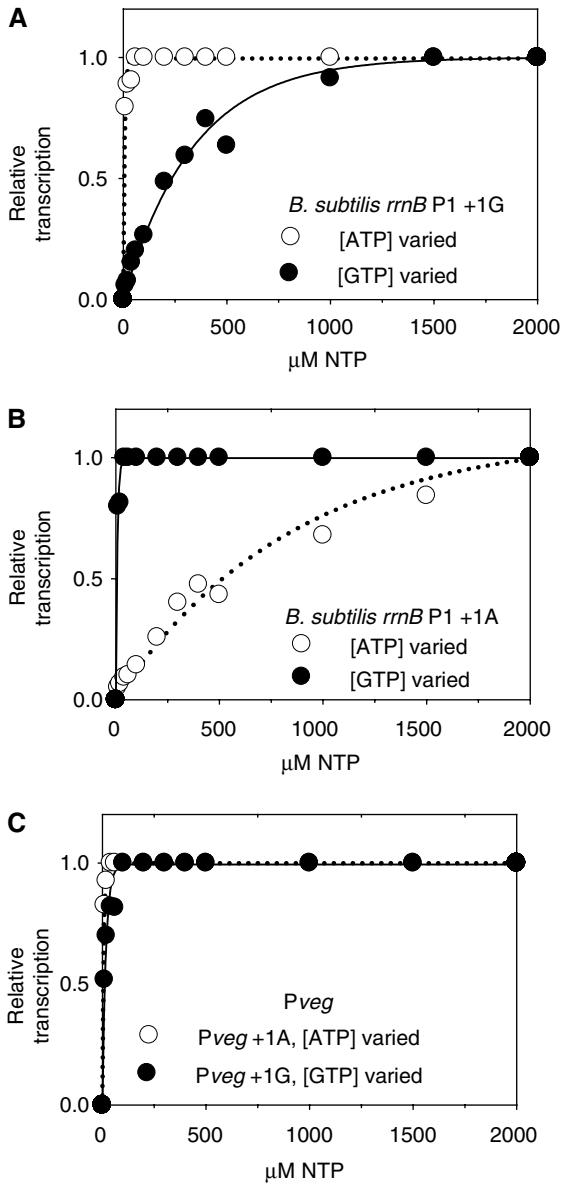


Figure 4 Effects of changing NTP concentration on *rrnB* P1 promoter activity *in vitro*. *B. subtilis* promoters on supercoiled templates were transcribed with *B. subtilis* RNAP and normalized to transcription at the highest NTP concentration (2000 μM). Transcription (arbitrary units) from (**A**) *rrnB* P1 +1G (pRLG7596), (**B**) *rrnB* P1 +1A (pRLG7597), and (**C**) P*veg* + 1A (pRLG7595) or +1G (pRLG7558) at varying ATP or GTP concentration.

promoters was important for specifying the NTP to which the promoter responded, but it was not responsible for the high iNTP concentration requirement (see Discussion).

Complexes containing *B. subtilis* rRNA promoters and *E. coli* RNAP are inhibited by ppGpp *in vitro* (Wellington and Spiegelman, 1993). To our surprise, *rrnB* P1 +1G (or *rrnB* P1 +1A, data not shown) with *B. subtilis* RNAP was not inhibited by ppGpp *in vitro* using single round or multiple round transcription assays at any of several salt concentrations (Figure 5A and data not shown). ppGpp also failed to inhibit *B. subtilis* RNAP transcribing a tandem *B. subtilis rrnB* P1 and P2 construct (data not shown). The same ppGpp preparation inhibited *E. coli rrnB* P1 ~2-fold using *E. coli* RNAP (Figure 5A and data not shown).

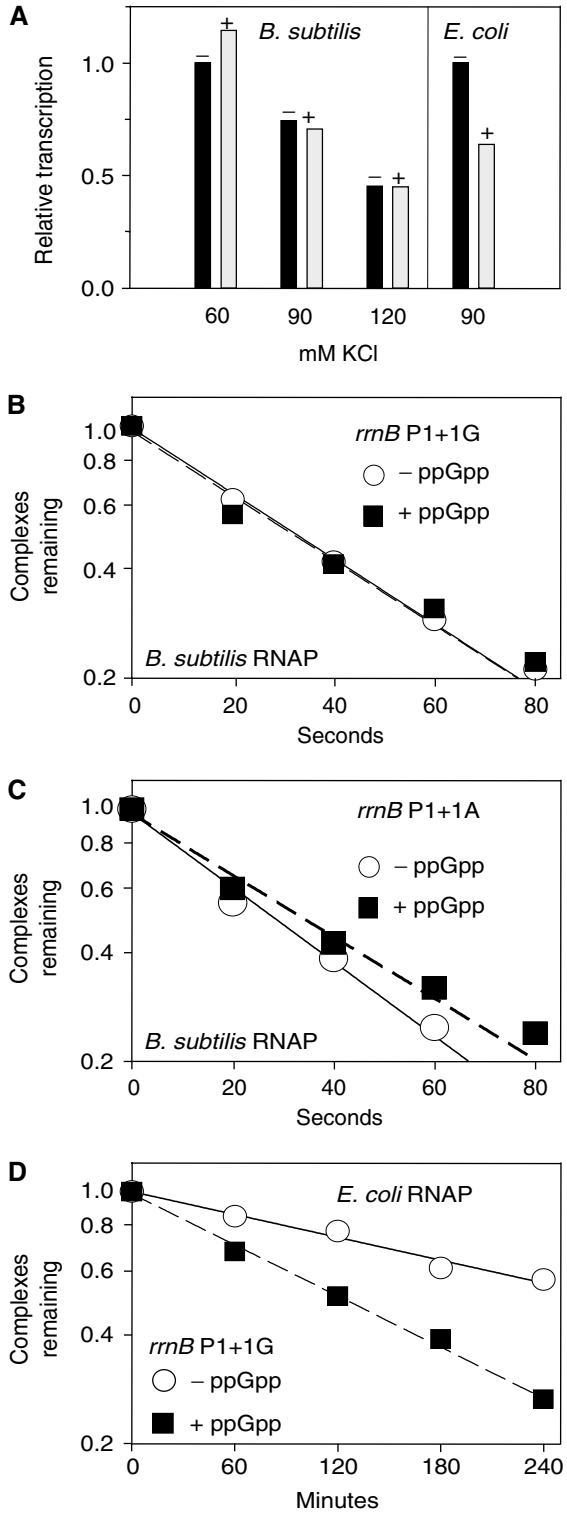


Figure 5 Effect of ppGpp on transcription by *B. subtilis* and *E. coli* RNAP *in vitro*. (**A**) Single round transcription from *B. subtilis rrnB* P1 +1G (pRLG7596) with *B. subtilis* RNAP and from *E. coli rrnB* P1 (pRLG6555) with *E. coli* RNAP. (+), ppGpp added at 0.5 mM. (**B–D**) Effect of ppGpp on open complex lifetime. *Y*-axis, fraction of competitor-resistant complexes. Representative experiments are shown; absolute values of the half-lives varied by only ~5% in different experiments. (B) *B. subtilis rrnB* P1 +1G (pRLG7596) with *B. subtilis* RNAP. (C) *B. subtilis rrnB* P1 +1A (pRLG7597) with *B. subtilis* RNAP. (D) *B. subtilis rrnB* P1 +1G (pRLG7596) with *E. coli* RNAP.

Inhibition of *E. coli* rRNA promoter activity by ppGpp *in vitro* requires using solution conditions in which the half-life of the promoter complex is rate determining. However, ppGpp binds to *E. coli* RNAP and decreases the half-lives of all open complexes, even at promoters where this lifetime is not rate determining for transcription (Barker *et al*, 2001). Hence, the ability of ppGpp to interact with RNAP can be detected from the effects on this lifetime, even under solution conditions where inhibition of transcription is not observed.

ppGpp did not decrease the half-lives of open complexes formed on either *B. subtilis rrnB* P1 +1G or *rrnB* P1 +1A (Figure 5B and C), consistent with its lack of an effect on transcription from these promoters. However, ppGpp did reduce the lifetimes of complexes containing *E. coli* RNAP, either using *E. coli* rRNA promoters (Barker *et al*, 2001) or using *B. subtilis rrnB* P1 +1G and the same solution conditions (Figure 5D; see also Wellington and Spiegelman, 1993). We tentatively conclude that purified *B. subtilis* RNAP is either insensitive, or at least not as sensitive, to ppGpp as *E. coli* RNAP. Interestingly, the open complexes formed using *B. subtilis rrnB* P1 +1G (or *rrnB* P1 +1A, data not shown) with *E. coli* RNAP were longer-lived than those formed with *B. subtilis* RNAP.

rrnB P1 promoter can 'sense' changes in NTP concentrations in vivo

We next examined rRNA promoter responses to three different kinds of downshifts (Figure 6) and an upshift (Figure 7). Similar approaches were instrumental in elucidating the differential roles of changing concentrations of NTPs and ppGpp in regulation of rRNA transcription in *E. coli* (Murray *et al*, 2003b; Murray and Gourse, 2004).

In order to determine whether regulation by the concentration of the iNTP *in vivo* is direct, we used the drug decoyinine to uncouple cellular GTP and ATP concentrations. Decoyinine inhibits GMP synthetase, thereby decreasing GTP but not ATP (Mitani *et al*, 1977). After decoyinine treatment, the activity of *rrnB* P1 +1G (Figure 6A), but not P*veg* + 1G (Figure 6B), decreased ~3-fold within the first 5 min, correlating with the decrease in GTP concentration (Figure 6A). The observed rate of decrease in *rrnB* P1 +1G activity was close to the maximal possible rate, since the half-life of the reporter RNA was ~4 min when measured by primer extension following rifampicin treatment (data not shown; see Materials and methods).

The ATP concentration did not decrease following decoyinine treatment (in fact, it increased slightly; see also Lopez *et al*, 1979). *rrnB* P1 +1A (and P*veg* + 1A) were not inhibited by decoyinine, a qualitatively different response from that of *rrnB* P1 +1G (Figure 6A and B). Thus, a G at the + 1 position, as well as some property specific to rRNA promoters, is required for the response of *rrn* P1 promoters to the decrease in GTP concentration. This result is consistent with the model that *B. subtilis* rRNA promoters can be controlled directly by the cellular concentration of their iNTP, GTP.

ppGpp is required for stringent control of B. subtilis rrn P1 promoter activity, but only when the promoter initiates with GTP

The *in vitro* experiments described above suggested that the mechanism of inhibition of transcription by ppGpp might be different in *B. subtilis* than in *E. coli*. However, necessary components might have been missing from the *in vitro* reactions, and/or the solution conditions might have been inappropriate for detecting effects of ppGpp on *B. subtilis* RNAP. Therefore, we investigated inhibition of rRNA promoter activity following amino-acid starvation *in vivo* (stringent control), when the regulator responsible for controlling rRNA promoter activity in *E. coli* is ppGpp and not the iNTP (Cashel

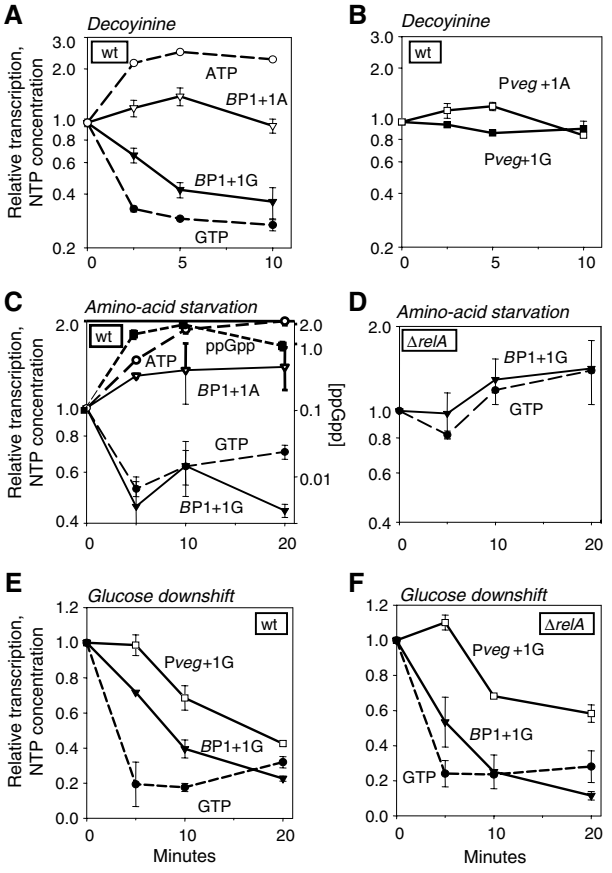


Figure 6 Correlation between GTP concentration and *B. subtilis rrnB* P1 promoter activity following three kinds of downshifts. NTP concentrations (dashed lines) and promoter activities (solid lines) are normalized to 1 at time 0. The GTP concentration was 45, 18, and 46% of the ATP concentration at time 0 in panels A, C, and E, respectively. Promoter activities were measured by primer extension from a wild-type strain: *rrnB* P1 +1G (RLG7554), *rrnB* P1 +1A (RLG7585), P*veg* + 1G (RLG7555), P*veg* + 1A (RLG7376), or from a Δ*relA* strain: *rrnB* P1 +1G (RLG7580), P*veg* + 1G (RLG7581). (**A, B**) Changes in promoter activity and NTP concentration after decoyinine addition. Cells were grown in a medium containing MOPS, 1% glucose, and 20 amino acids (50 μg/ml each). Decoyinine (final concentration 0.5 mg/ml) was added to exponentially growing cells at time 0 (OD_{600} ~ 0.3). (**C**) Effect of amino-acid starvation on *B. subtilis rrnB* P1 promoter activity. Cells were grown in a medium containing MOPS, 0.4% glucose, and six amino acids (FILMVW). Serine hydroxamate (1.5 mg/ml final concentration) was added to exponentially growing cells at time 0 (OD_{600} ~ 0.25). The ppGpp concentration is presented relative to the GTP concentration. Note the different scale for ppGpp. (**D**) Effect of amino-acid starvation on *B. subtilis rrnB* P1 promoter activity in a Δ*relA* strain. Conditions are as in (C). (**E**) Effect of glucose deprivation on *B. subtilis rrnB* P1 promoter activity. Cells were grown in a medium containing MOPS, 0.2% glucose, and 20 amino acids. α-Methyl glucoside (final concentration 2%) was added to exponentially growing cells at time 0 (OD_{600} ~ 0.3). The decrease in ATP concentration was ~2-fold by 5 min (data not shown). (**F**) Effect of glucose deprivation on *B. subtilis rrnB* P1 promoter activity in a Δ*relA* strain. Conditions are as in (E).

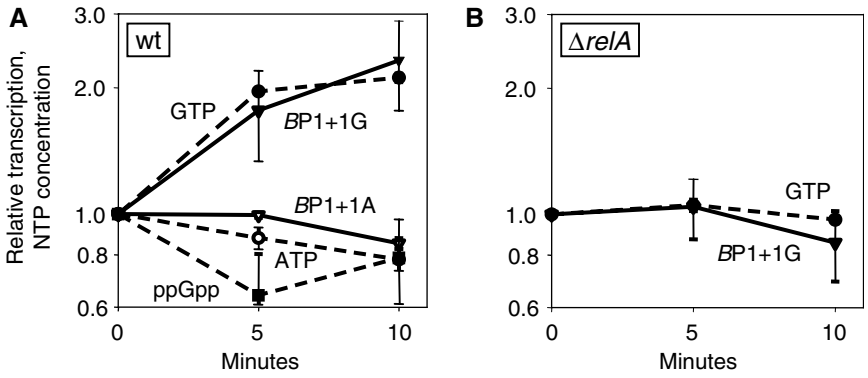


Figure 7 Effect of amino-acid upshift on *B. subtilis rrnB* P1 promoter activity. Cells were grown in MOPS, 0.2% glucose, and six amino acids (FILMVW, 50 μg/ml each). The remaining 14 amino acids were added to exponentially growing cells at time 0 ($OD_{600} \sim 0.25$) to a final concentration of 50 μg/ml each. Promoter activity was measured by primer extension from *rrnB* P1 +1G (RLG7554) and *rrnB* P1 +1A (RLG7585) in (**A**) and from *rrnB* P1 +1G (RLG7580) in (**B**) (Δ*relA*). NTP concentrations are normalized to 1.0 at time zero. The GTP concentration was ~16% of the ATP concentration and ppGpp was ~10% of the GTP concentration at time 0.

et al, 1996; Murray *et al*, 2003b; Paul *et al*, 2004a). Serine hydroxamate (SHX), a competitive inhibitor of aminoacylation of serine tRNA, was used to induce a stringent response. SHX addition resulted in a ⩾10-fold increase in ppGpp, an ~2-fold decrease in GTP, and a corresponding decrease in *rrnB* P1 +1G activity (Figure 6C) (for technical reasons, the magnitude of this decrease in rRNA promoter activity following SHX treatment was somewhat smaller than reported for *E. coli*; Paul *et al*, 2004a). RelA is the sole ppGpp synthetase in *B. subtilis* (Wendrich and Marahiel, 1997). There was no decrease in GTP levels or *rrnB* P1 +1G activity in a Δ*relA* mutant (Figure 6D). Thus, ppGpp was required for the observed inhibition of *rrnB* P1 + G.

Although ppGpp was responsible for the decrease in *rrnB* P1 +1G activity after amino-acid starvation, *rrnB* P1 +1A activity was not inhibited and even increased slightly, correlating with a slight increase in ATP concentration. Thus, unlike the situation in *E. coli*, where rRNA promoters are inhibited by ppGpp irrespective of the identity of the iNTP (Murray *et al*, 2003b), *B. subtilis* rRNA promoters appear to require + 1G to respond to ppGpp, suggesting that ppGpp's effect might be indirect.

The concentration of the iNTP and not ppGpp controls rrn P1 promoter activity during a carbon source downshift in B. subtilis

B. subtilis RNAP was not affected by ppGpp *in vitro*, and *B. subtilis rrnB* P1 +1A activity was not inhibited by ppGpp following amino-acid starvation *in vivo*. Furthermore, GTP is consumed in the biosynthesis of ppGpp (Cashel *et al*, 1996), and it was reported previously that ppGpp decreases GTP levels in *B. subtilis* by inhibiting IMP dehydrogenase (Lopez *et al*, 1981). Together, these results suggested that ppGpp might elicit its effect on *B. subtilis* rRNA transcription indirectly by reducing GTP concentrations. To investigate this model further, we studied the effect of glucose deprivation in *B. subtilis*, another condition where increases in ppGpp concentrations rather than decreases in NTP concentrations directly regulated rRNA promoter activity in *E. coli* (Murray *et al*, 2003b).

Addition of α-methyl glucoside, a competitive inhibitor of glucose uptake, induces synthesis of ppGpp in *E. coli*, while NTPs remain relatively constant (Murray *et al*, 2003b). In *B. subtilis*, however, we did not detect an increase in ppGpp concentrations under the conditions tested (minimal medium supplemented with glucose and 20 amino acids); ppGpp concentrations remained below detection throughout the time course of the experiment (data not shown). In contrast to the situation in *E. coli*, GTP concentrations dropped ~5-fold (Figure 6E). At the same time, *rrnB* P1 +1G activity decreased by about the same magnitude, whereas the activity of the control promoter, P*veg* + 1G, decreased less, and this decrease was delayed (Figure 6E). *rrnB* P1 +1A and P*veg* + 1A decreased with kinetics similar to their respective + 1G versions, correlating with a two-fold decrease in ATP concentration (data not shown).

Since we were not able to detect ppGpp under these conditions, we repeated the experiment in a Δ*relA* strain to confirm that a potential increase in ppGpp levels was not responsible for the observed decrease in rRNA promoter activity. The absence of *relA* did not affect *rrnB* P1 +1G promoter behavior following α-methyl glucoside treatment (Figure 6F). We conclude that *B. subtilis* rRNA promoter activity correlates with changes in GTP concentration during mild carbon deprivation, and that under these conditions ppGpp has no direct or indirect effect on *rrnB* P1 +1G promoter activity.

Decreasing the ppGpp concentration during an upshift appears to increase rRNA promoter activity indirectly by increasing the GTP concentration

We also addressed whether ppGpp's effects on *B. subtilis* rRNA promoters might be indirect during a nutritional upshift. In *E. coli*, decreases in ppGpp concentration account for increases in rRNA promoter activity under these conditions, independent of the identity of the + 1 position (Murray *et al*, 2003b; data not shown). In *B. subtilis*, ppGpp concentration decreased following amino-acid upshift, and GTP, but not ATP, increased two-fold (Figure 7A; see also Lopez *et al*, 1981). Corresponding with the concentrations of GTP and ATP, *rrnB* P1 +1G activity increased two-fold, but *rrnB* P1 +1A did not (Figure 7A). The increase in GTP concentration and the corresponding increase in *rrnB* P1 +1G promoter activity were dependent on the decrease in ppGpp concentra-

tion, since the effects were abolished in a Δ*relA* strain (Figure 7B). These results further support a model in which increasing GTP concentrations directly stimulate *rrnB* P1 +1G promoter activity, and the effect of ppGpp on rRNA promoter activity is indirect.

Discussion

B. subtilis and E. coli appear to use different strategies to control rRNA synthesis

We systematically examined the properties and regulation of promoters from two of the 10 *B. subtilis* rRNA operons. Our primary conclusions are as follows: (i) *B. subtilis rrn* core promoters are intrinsically strong; that is, upstream sequences contribute much less to rRNA promoter activity than in *E. coli* rRNA promoters. (ii) Regulation of *B. subtilis* rRNA transcription can occur from direct effects of changes in the concentration of GTP, the initiating nucleotide, on rRNA promoter activity. (iii) When ppGpp inhibits *B. subtilis* rRNA promoter activity, it may do so indirectly by reducing GTP pools. Thus, although *B. subtilis* and *E. coli* rRNA promoters employ the same small regulatory molecules, they appear to use different strategies to control rRNA synthesis. A schematic diagram (undoubtedly oversimplified) emphasizing these differences is shown in Figure 8 and discussed further below. Characterization of the promoters from the other eight operons will be required to confirm that these characteristics are typical of *B. subtilis* rRNA promoters. In this context, we note that additional rRNA operons have been studied by R Rudner (City University of NY, personal communication).

Role of upstream sequences in B. subtilis rRNA promoters

Stimulation of *rrn* core promoter activity by upstream sequences in *B. subtilis* is moderate compared to that in *E. coli*. No *fis* gene was apparent from examination of the *B. subtilis* genome sequence, consistent with the lack of evidence for an activator (Figure 2). Most or all of the modest effect of the *rrn* upstream sequences could derive from αCTD–UP element interactions. UP elements are common in *B. subtilis* promoters (Fredrick *et al*, 1995; Helmann, 1995; Meijer and Salas, 2004).

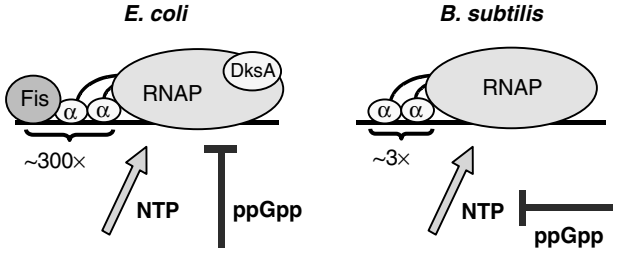


Figure 8 Schematic diagram illustrating mechanisms contributing to *rrn* P1 promoter activity in *E. coli* versus *B. subtilis*. The transcription factor Fis and RNAP αCTD binding to UP element DNA account for the unusually high activity of *rrn* P1 promoters from *E. coli*, but not *B. subtilis*. Changing NTP and ppGpp concentrations regulate rRNA promoter activities in both bacteria, but in *B. subtilis* ppGpp may inhibit rRNA transcription indirectly by reducing GTP levels. For the sake of simplicity (and since its effect on *B. subtilis* rRNA promoters was not examined), H-NS is not pictured.

Binding of *B. subtilis* RNAP to *rrn* core promoters apparently is efficient enough that αCTD binding has relatively small additional impact on recruitment of RNAP to the promoter. However, *rrn* UP elements might stimulate weaker core promoters if fused as chimeric constructs. *B. subtilis* rRNA core promoters contain −10 and −35 hexamers with excellent matches to the proposed consensus sequences for recognition by regions 4.2 and 2.3–2.4 of the σ subunit of RNAP, and in contrast to *E. coli* rRNA core promoters, they also contain sequences characteristic of extended −10 elements, have A+T-rich sequences between the −10 hexamer and the transcription start site, and some have consensus −10/−35 spacer lengths (17 bp). These properties likely account for the intrinsic strength of *B. subtilis* rRNA promoters.

GTP sensing by rRNA promoters

Our data suggest that *B. subtilis* rRNA promoters are regulated directly by the concentration of the iNTP, namely GTP (Figure 8). Changes in GTP concentrations have also been reported to regulate gene expression in several other systems in *B. subtilis*, but by completely different mechanisms than that described here (Ochi *et al*, 1982; Ratnayake-Lecamwasam *et al*, 2001; Inaoka and Ochi, 2002; Inaoka *et al*, 2003).

We found that GTP concentrations changed with growth phase and nutritional conditions in *B. subtilis*. rRNA promoter activities correlated with these changes *in vivo* and were dependent on the concentration of GTP *in vitro*. rRNA promoter activity also correlated with a decrease in GTP concentration during entry into stationary phase (data not shown). Furthermore, a *B. subtilis rrnB* P1 promoter mutant requiring lower GTP levels for maximal transcription *in vitro* than wild-type *rrnB* P1 displayed much less pronounced changes in activity with growth rate and phase than the wild-type promoter (data not shown).

The detailed mechanism underlying regulation of *B. subtilis* rRNA promoters by GTP concentration remains to be determined. Future studies will define the *B. subtilis* rRNA promoter sequences required for regulation and whether open complex half-life is a crucial determinant of sensitivity to the iNTP concentration. The G+C-rich sequence element between the −10 element and the transcription start site, referred to as the discriminator region (Travers, 1984), is essential for regulation of *E. coli* rRNA promoters by the iNTP and by ppGpp (Barker and Gourse, 2001; Murray *et al*, 2003b; Murray and Gourse, 2004). However, this element is A+T rich in *B. subtilis* rRNA promoters. Future studies will address how the promoter remains sensitive to the iNTP concentration despite the A+T-rich character of its discriminator sequence. Interestingly, the *B. subtilis* rRNA promoters formed longer-lived open complexes with *E. coli* RNAP than with *B. subtilis* RNAP when challenged with heparin (Figure 5D and data not shown). This may simply reflect a greater sensitivity of *B. subtilis* RNAP to heparin (Whipple and Sonenshein, 1992).

Role of ppGpp in control of rRNA transcription

The ubiquity of *relA* in bacteria and the recent discovery of ppGpp in chloroplasts (Givens *et al*, 2004; Takahashi *et al*, 2004) attest to ppGpp's importance as a regulator of gene expression. It is possible that ppGpp affects *B. subtilis* RNAP

directly under conditions not examined here, but it is also conceivable that effects of ppGpp on *B. subtilis* rRNA promoters are always mediated by changes in GTP concentration. Further studies will be required to address this issue. The reduction in GTP concentration from increases in ppGpp might be ascribable both to consumption of GTP during ppGpp biosynthesis and to direct inhibition of IMP dehydrogenase, the first enzyme in GTP biosynthesis (Lopez *et al*, 1981). Considering the evolutionary conservation of at least some of the amino-acid residues in RNAP that contact ppGpp (Artsimovitch *et al*, 2004), it is surprising that *B. subtilis* RNAP appears insensitive to ppGpp. However, we note that *B. subtilis* and *E. coli* RNAP differ in other important aspects of gene regulation as well (Mencia *et al*, 1998; Artsimovitch *et al*, 2000).

Selection for G at the +1 position in B. subtilis rRNA promoters

The mechanism of regulation of rRNA transcription may have driven the evolution of *B. subtilis* rRNA promoter sequence. Comparisons of the sequences downstream from the likely −10 elements from both promoters in all 10 *B. subtilis* rRNA operons (data not shown), and extrapolation from the primer extension results with *rrnB* and *rrnO* (Figure 1), suggest that all *B. subtilis* rRNA promoters initiate with GTP. Since ATP concentration increases slightly in at least some conditions where an increase in rRNA transcription would be disadvantageous (e.g. amino-acid starvation), and ATP concentration decreases slightly in at least some conditions where a decrease in rRNA transcription would be disadvantageous (e.g. upshift), the apparent choice of G residues at the +1 position in rRNA promoters is likely not a chance event. The fact that the activity of the *rrnB* P1+1A variant increased slightly under conditions where ATP concentration increased slightly (Figure 6A and C) suggests that there might be promoters with kinetic characteristics in common with rRNA promoters that have evolved with A residues at the +1 position in order to respond positively to changes in ATP concentration in these conditions.

DksA homologs in B. subtilis

DksA was recently identified as a transcription factor crucial for regulation of rRNA promoters in *E. coli* (Figure 8; Paul *et al*, 2004a). DksA binds in the secondary channel of *E. coli* RNAP (Perederina *et al*, 2004), stabilizes interactions of RNAP with ppGpp, and decreases open complex lifetime, putting rRNA promoters into a kinetic range *in vivo* where they are susceptible to changes in ppGpp and NTP concentrations (Paul *et al*, 2004a). Although there are no *B. subtilis* ORFs with strong similarity to *dksA*, we singly deleted the three most homologous ORFs, *yteA, yocK,* and *ylyA*. We detected no differences in *rrnB* P1 promoter activity under a variety of growth conditions in these mutant strains (data not shown). Either there is no *B. subtilis* DksA homolog, or these gene products have redundant functions (masking effects of single disruptions), or a factor analogous to DksA is not recognizable from sequence analysis. We cannot exclude the possibility that a factor is needed to mediate the interaction of RNAP with ppGpp, but the failure of ppGpp to exert an effect on transcription *in vivo* from *rrnB* P1 promoters lacking a G at the +1 position, even under conditions where the *relA* gene is required for regulation, suggests that effects of ppGpp may be indirect.

Concluding remarks

In both *E. coli* and *B. subtilis*, the sequences of rRNA promoters, the properties of RNAP, the responses of NTPs and ppGpp concentrations to changes in growth conditions, and the responses of rRNA promoters to changes in these small molecules have all coevolved to achieve similar responses in rRNA expression. However, each organism has solved the requirements for proper rRNA promoter strength and regulation in its own unique manner. As has become apparent from studies on the mechanisms of regulation of other genes in *B. subtilis* and *E. coli*, there is more than one strategy for solving a similar regulatory problem (e.g. Henkin and Yanofsky, 2002; Yanofsky, 2003).

Materials and methods

Strains, strain construction, media and growth conditions, primer extension, protein purification, and methods for determination of mRNA half-life, NTP concentration, and ppGpp concentration are provided in Supplementary material.

Reporters of promoter activity

Promoter constructs were fused to *lacZ*, but activities were assayed by primer extension, rather than by β-galactosidase activity. When measured by primer extension, *rrn* P1 promoter activities increased proportionally to the growth rate, as expected (Figure 3), correlating with the increase in RNA:protein ratios observed with increasing growth rate (data not shown). At the highest growth rates (i.e. in cells grown in rich medium), β-galactosidase activities consistently underestimated rRNA promoter activities. Apparently, transcription initiation is not reported accurately by β-galactosidase activity in *B. subtilis* for promoters that initiate transcription at such high frequency. Previous conclusions about the relative activities of different promoters using *lacZ* fusions with different RNA leaders (e.g. Okamoto and Vold, 1992) might be compromised further by differences in mRNA half-life or translation efficiency.

RNA extraction

Preparation of RNA did not include centrifugation steps prior to cell lysis and extraction with phenol, limiting the potential for decay of the short-lived reporter mRNA. A recovery marker RNA (RM) was added at the time of extraction, controlling for differences in degradation during extraction and for variation between samples at later steps. The RM RNA was made from *B. subtilis* strain RLG6943 or *E. coli* strain RLG1100 from a template containing a binding site for the same primer used for extension of the test promoter transcripts, but differed from the test transcript in length at the 5′ end, allowing extension products to be distinguishable on gels (Josaitis *et al*, 1995). Typically, 1 ml of cells was pipetted directly into 2 ml phenol/chloroform (1:1) and 0.25 ml lysis buffer (50 mM Tris–HCl pH 8.0, 500 mM LiCl, 50 mM EDTA pH 8.0, 5% SDS). After brief vortexing, the RM (~20 μl) was added, followed by immediate sonication. Water was added to increase the aqueous volume to 6 ml to prevent precipitation of salts, followed by two extractions with phenol/chloroform, two precipitations with ethanol, and suspension of the pellet in 20–50 μl 10 mM Tris–HCl, pH 8.0.

In vitro transcription

Plasmid templates (*Eco*RI–*Hin*dIII promoter fragments in pRLG770) are listed in Supplementary Table S1. For Figure 4, multiple round transcription was performed (10 μl reactions, 15 min, 30°C, 10 nM RNAP, 1 nM supercoiled plasmid template, 40 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.1 μg/ml BSA, and 150 mM KCl). CTP, GTP, and ATP were 100 μM (except when ATP or GTP was varied from 10 to 2000 μM), and UTP was 10 μM plus 2 μM [α-^{32}P]UTP. For Figure 2, ATP, CTP, and GTP were 200 μM, and a range of RNAP concentrations was tested to determine a subsaturating concentration at which potential effects of the UP element would not be obscured. Reactions were initiated with RNAP, allowed to proceed

for 15 min at 30°C, terminated by addition of an equal volume of formamide loading buffer (95% formamide, 20 mM EDTA pH 8.0), electrophoresed on 7 M urea–5.5% polyacrylamide gels, and quantified by phosphorimaging.

For the single round assays testing effects of ppGpp (0.5 mM; TriLink Inc.) in Figure 5, RNAP was incubated with DNA for 10 min at 30°C at the indicated KCl concentrations. In Figure 5A, transcription was initiated with 200 μM each ATP, CTP, GTP, 10 μM UTP, 2 μM [α-^{32}P]UTP, and 600 nM dsDNA competitor containing a consensus promoter, as described (Paul *et al*, 2004a). In Figure 5B–D, the fraction of RNAP–promoter complexes remaining at various times after addition of 3 μg/ml heparin was determined by *in vitro* transcription as described (Barker *et al*, 2001), except that the KCl concentration was 30 mM and the NTP concentrations were 400 μM ATP and GTP, 200 μM CTP, 10 μM UTP, and 2 μM [γ-^{32}P]UTP. No transcript was detected when plasmid and heparin were preincubated before RNAP addition.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank T Gaal, W Ross, M Barker, H Murray, T Henkin, G Spiegelman, and R Rudner for helpful discussions and comments on the manuscript. This work was supported by RO1 GM37048 from the National Institutes of Health and by a Hatch grant from the United States Department of Agriculture.

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