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Changes in *Escherichia coli* rRNA Promoter Activity Correlate with Changes in Initiating Nucleoside Triphosphate and Guanosine 5′ Diphosphate 3′-Diphosphate Concentrations after Induction of Feedback Control of Ribosome Synthesis

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rRNA synthesis is the rate-limiting step in ribosome synthesis in *Escherichia coli*. Its regulation has been described in terms of a negative-feedback control loop in which rRNA promoter activity responds to the amount of translation. The feedback nature of this control system was demonstrated previously by artificially changing ribosome synthesis rates and observing responses of rRNA promoters. However, it has not been demonstrated previously that the initiating nucleoside triphosphate (iNTP) and guanosine 5′-diphosphate 3′-diphosphate (ppGpp), the molecular effectors responsible for controlling rRNA promoters in response to changes in the nutritional environment, are responsible for altering rRNA promoter activities under these feedback conditions. Here, we show that most feedback situations result in changes in the concentrations of both the iNTP and ppGpp and that the directions of these changes are consistent with a role for these two small-molecule regulators in feedback control of rRNA synthesis. In contrast, we observed no change in the level of DNA supercoiling under the feedback conditions examined.

In all cells examined, from prokaryotes to humans, expression of the products that make up the translation apparatus (rRNA, tRNA, ribosomal proteins, and associated factors) is tightly regulated. In *Escherichia coli*, several potentially overlapping regulatory systems have been identified as contributors to the control of rRNA and tRNA expression. Together, these regulatory systems match the protein synthetic potential to the demand for protein synthesis, no matter how the demand is altered (e.g., by nutritional shifts or starvations, by changes in growth phase, or by inhibitors of translation). Dissecting the roles of individual regulatory factors in this complex network has long posed a major experimental challenge (17, 25, 37).

E. coli has seven rRNA operons (*rrn*), each of which contains two promoters, *rrn* P1 and *rrn* P2. During moderate to rapid growth, the *rrn* P1 promoters provide the majority of rRNA transcription in the cell. Sequences upstream of the −35 hexamer of *rrn* P1 promoters account for much of the strength of these promoters.

Fis (factor for inversion stimulation) was originally identified for its role in site-specific inversion (23); however, it was shown subsequently to participate in other cellular processes as well, including activation of *rrn* P1 promoters (34). Each of the seven *rrn* P1 promoters has binding sites for Fis upstream of the core promoter element (three to five sites, depending on the operon), and activation by Fis increases promoter activity four- to eightfold (19). Between the Fis sites and the −35 hexamer of the core promoter is an A+T-rich sequence called the UP element. The C-terminal domain of the alpha subunit (αCTD) of RNA polymerase binds specifically to the UP element (33), increasing *rrn* P1 promoter activity 20- to 50-fold, depending on the operon (19). While these upstream promoter elements are essential for the strength of *rrn* P1 promoters, promoter constructs that lack the binding sites for Fis and αCTD (core promoters; −41 to + 1 with respect to the transcription start site) retain their characteristic regulatory properties (6, 24).

At least two small molecules regulate *rrn* P1 core promoter activity. Guanosine 5′-diphosphate 3′-diphosphate (ppGpp) was originally identified in cells that were starved for amino acids (7, 8), and subsequent experiments have shown that ppGpp also regulates rRNA transcription under other growth conditions (e.g., nutrient shifts, entry into stationary phase, response to translation inhibitors) (26, 30, 36). ppGpp is a direct negative regulator that decreases the half-life of the open complexes formed at all promoters (3). Since this kinetic step is rate limiting for transcription initiation at *rrn* P1 promoters, changes in ppGpp concentration affect rRNA expression.

In addition to being regulated by ppGpp, *rrn* P1 promoters are controlled by changes in the concentration of their initiating nucleoside triphosphate (iNTP). In vitro, *rrn* P1 promoters require unusually high concentrations of their respective iNTP for maximal transcription (4, 13, 36). In vivo, changes in iNTP concentrations directly control *rrn* P1 activity during progression through stationary phase, outgrowth from stationary phase, when translation is inhibited, and when the iNTP concentration is altered by mutations in NTP synthesis pathways (30, 36). As with ppGpp, direct effects of iNTP concentration on transcription initiation are limited to promoters that make short-lived open complexes. Thus, the unusual intrinsic kinetic characteristics of *rrn* P1 promoters result in their specific control by changes in the concentrations of small molecules (2, 3).

In bacteria, operons that are not being translated actively

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are normally subject to premature transcriptional termination. In addition to being subject to the mechanisms described above, the long, untranslated rRNA operons escape these polarity effects as a result of an antitermination system (10). This antitermination system uses host factors originally identified for their roles as N-utilization substances in λ phage antitermination (Nus factors) (9, 39, 42).

Artificial manipulation of ribosome synthesis rates was used previously to demonstrate that a feedback mechanism(s) plays a role in the control of rRNA transcription. For example, when the rRNA gene dose was increased (21) or decreased (9, 43), when rRNA was overexpressed from inducible promoters (18), when the *fis* gene was deleted or the *rpoA* gene was mutated (4, 33, 34), or when the rRNA antitermination system was disrupted (39), corresponding changes in rRNA core promoter activities always restored overall rRNA synthesis rates—and therefore ribosome synthesis rates—to the level appropriate for the nutrient condition. Thus, feedback mechanisms balance rRNA promoter activities with the need for protein synthesis.

While it was demonstrated previously that changes in the concentrations of iNTPs and ppGpp control transcription from *rrn* P1 promoters in response to changes in nutritional conditions (30), it has not been demonstrated that the concentrations of these same molecules change under the conditions described above that were used previously to show that rRNA synthesis is feedback regulated. We show here that in all but one of these conditions, both the ppGpp and iNTP concentrations change, and in the remaining situation, the iNTP concentration alone changes. In all cases, changes in iNTP and ppGpp concentrations are in the direction consistent with a role for these small molecules in feedback regulation of rRNA expression. Thus, the results suggest that both ppGpp and iNTP concentrations serve as feedback regulators, linking protein synthesis and *rrn* P1 promoter activity.

MATERIALS AND METHODS

Strains. Promoter-*lacZ* fusions were constructed in strain VH1000 (MG1655 *lacZ lacI pyrE*$^+$ [13]). Promoter fragments were generated by PCR using oligonucleotides containing *Eco*RI sites upstream and *Hin*dIII sites downstream of the promoter sequence. Restriction fragments were then fused to *lacZ* on plasmids and recombined into bacteriophage λ, and the phage was lysogenized in single copy at the λ attachment site (λ system II) (32, 40). Strains and plasmids are listed in Table 1.

Mutations were introduced into lysogens by standard methods. The Δ*fis* insertion-deletion (*fis-767*) (22) was introduced by P1 transduction (29) with selection for kanamycin resistance. The *nusB5* allele was introduced by P1 transduction with selection for tetracycline resistance from a linked Tn*10* (12, 39). Tet^r colonies were screened for slow growth at 25°C (*nus* mutants are cold sensitive) (41). pHTf1α derivatives expressing either wild-type *rpoA* or *rpoA*-R265A were introduced by transformation (15). The *rrn* gene dose was increased by introduction of a multicopy plasmid containing a copy of the *rrnB* operon (plasmid pNO1301). Effects of increased gene dose on expression from promoter-*lacZ* fusions were quantified by comparison with expression from the same fusion in a strain containing a control plasmid, pNO1302, that codes for nonfunctional 16S and 23S rRNAs (pNO1302 carries rRNA genes containing large deletions) (21). Strains were grown at 37°C with aeration in the media as indicated in the figure legends. At least two fresh independent transductants or transformants were used for each experiment to reduce the chance of selecting for suppressor mutants.

NTP and ppGpp measurements. Cultures were grown in media described in the figure legends to mid-log phase (optical density at 600 nm [OD_{600}] of ~0.3). Promoter activities (see below) and nucleotide concentrations were determined from identical cultures, except that the cultures used for NTP measurements contained 20 μCi of $KH_2{}^{32}PO_4$/ml (Perkin Elmer Life Sciences). ATP and ppGpp were measured by thin-layer chromatography following formic acid extraction (20). Reported values represent the averages of extracts from at least three different cultures.

TABLE 1. Strains and plasmids

Name	Genotype and promoter endpoints in *lacZ* fusion	Source or reference
Strains		
RLG4755	VH1000/λ *rrn* P1 −41 to +50	This work
RLG4998	VH1000/λ *lacUV5* −59 to +36	3
RLG6228	VH1000/λ *rrn* P1(dis) −41 to +50	This work
RLG6241	VH1000 *nusB5*/λ *rrn* P1 −41 to +50	This work
RLG6243	VH1000 *nusB5*/λ *lacUV5* −59 to +36	This work
RLG6245	VH1000 *nusB5*/λ *rrn* P1(dis) −41 to +50	This work
RLG6247	VH1000 Δ*fis*/λ *rrn* P1 −41 to +50	This work
RLG6249	VH1000 Δ*fis*/λ *lacUV5* −59 to +36	This work
RLG6251	VH1000 Δ*fis*/λ *rrn* P1(dis) −41 to +50	This work
Plasmids		
pRLG770	pBR322 derivative	34
pHTf1-αwt	2- to 3-fold overexpression of wild-type *rpoA*	15
pHTf1-αR265A	2- to 3-fold overexpression of R265A *rpoA*	15
pNO1301	pBR322 containing intact *rrn* operon	21
pNO1302	pBR322 containing disrupted *rrn* operon	21

Promoter activity in vivo. λ monolysogens containing promoter-*lacZ* fusions were grown in the media described in the figure legends for three to four generations to an OD_{600} of ~0.3. Cultures were placed on ice for >30 min and lysed by sonication, and β-galactosidase activity was measured (5). In order to focus on the transcription initiation-specific effects of the following feedback situations, we express the *rrnB* P1-specific effect as the ratio of the observed change in *rrnB* P1 activity divided by the observed change in the activity of the unregulated control promoter, *rrnB* P1(dis) (see Fig. 1 and Results). The *rrnB* P1 and *rrnB* P1(dis) promoter constructs lacked binding sites for Fis in order to eliminate confounding effects from potential changes in Fis concentration. Reported values are the averages of two separate assays from each of at least two independent cultures.

Measurement of plasmid supercoiling. λ monolysogens were transformed with a pBR322 derivative, pRLG770 (34). Wild-type, *nusB5*, and Δ*fis* cultures were grown as described in the figure legends for Fig. 2 and 4A, with the addition of ampicillin (100 μg/ml). At an OD_{600} of ~0.5, plasmids were extracted using a Qiaprep spin miniprep kit (Qiagen, Valencia, Calif.). Approximately 400 ng of plasmid DNA was loaded onto a 1% agarose 1× Tris-boric acid-EDTA-buffered gel containing 25 μg of chloroquine/ml (Sigma Chemical Co., St. Louis, Mo.), and electrophoresis was at 30 V for 20 h in 1× Tris-boric acid-EDTA containing 25 μg of chloroquine/ml. Under these conditions, supercoiled plasmids migrate slower than relaxed plasmids (11). The locations of supercoiled and relaxed plasmids were confirmed by comparison with plasmids extracted from cells treated with (10 μg/ml for 30 min) and without the gyrase inhibitor norfloxacin (Sigma Chemical Co.) (27). Gels were washed three times for 10 min each time in distilled water to remove chloroquine, stained in 5 μg of ethidium bromide/ml, and visualized using UV light. Fresh transformants of independent transductants were used in each experiment.

RESULTS

Rationale. Homeostatic control systems function by continually monitoring specific signals and making corresponding small regulatory adjustments. Since these small fluctuations are too subtle to detect under normal steady-state growth conditions, larger perturbations to the system must be introduced in order to identify the regulatory mechanisms that participate in the control circuit. Previous studies demonstrated that disturbances in rRNA expression induce feedback control mechanisms that restore normal ribosome synthesis by altering *rrn* P1 core promoter activities (16, 18, 21, 33, 34, 39). However, the molecular signals responsible for this feedback have remained unclear.

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                -30        -20        -10        +1
rrnB P1         cttgtcaggccggaataactcccTATAATgcgccaccA
rrnB P1(dis)    cttgtcaggccggaataactcccTATAATgatacaccA
lacUV5          tttacactttatgcttccggctcTATAATgtgtggA
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FIG. 1. Sequences of core promoters used in this study. −10 and −35 hexamer sequences are in bold. The +1 NTP is in bold capital letters. Sequence endpoints of DNA fragments used to construct promoter-*lacZ* fusions are indicated in Table 1. The *rrnB* P1(dis) promoter (24) is identical to *rrnB* P1 except for a 3-bp substitution (underlined) that causes the promoter to form a longer-lived open complex, resulting in a loss of regulation (3). This promoter makes the same transcript as *rrnB* P1.

We examined four situations that were shown previously to alter *rrn* P1 core promoter activity (feedback conditions), presumably because they generated an imbalance between ribosome synthesis and the demand for protein synthesis. Three of these feedback conditions were caused by mutations in genes coding for factors involved in rRNA synthesis, *nusB* (39), *rpoA*, (33), or *fis* (3, 34). The fourth condition was caused by an increase in the *rrn* gene dose (21). We measured changes in the concentrations of ppGpp and iNTPs, the small molecules known to regulate *rrn* P1 core promoter activities in response to changes in the nutritional environment, during each of the situations that induced feedback.

The effects of each of these situations on promoter activity were detected using promoter-*lacZ* fusions as reporters (see Materials and Methods). We used three promoter constructs: *rrnB* P1, a well-characterized representative of the *rrn* P1 promoters, and two control promoters, *rrnB* P1(dis) and *lacUV5* (Fig. 1). The control promoters are relatively insensitive to changes in ppGpp and iNTP concentrations in vivo and in vitro, because they make long-lived open complexes (3).

Feedback control of *rrn* P1 promoters in a *nusB5* strain. Since rRNA transcripts are not translated, they would be subject to premature termination of transcription (polarity) if it were not for an antitermination system (see above). We observed fourfold more *rrnB* P1 promoter activity in a strain where rRNA antitermination was partially disrupted (*nusB5*) than in a wild-type strain (Fig. 2), whereas the activities of the two control promoters increased only ~twofold. Correlating with the twofold specific effect on *rrnB* P1 transcription initiation, there was a 45% increase in ATP concentration and a 40% decrease in ppGpp concentration (Fig. 2) (see also reference 39). These data are consistent with the model that increases in the iNTP concentration and decreases in the ppGpp concentration contribute to feedback derepression of *rrn* P1 promoters when rRNA transcription elongation is disrupted.

Feedback control of *rrn* P1 promoters in strains expressing variants of αCTD. Previous work showed that when UP element function was disrupted by overexpression of α variants defective in DNA binding, core *rrnB* P1 promoter activity increased (33). Overexpression of a DNA binding-defective α subunit (R265A) resulted in a 3.8-fold increase in *rrnB* P1 activity (Fig. 3), while the activities of the *lacUV5* and *rrnB* P1(dis) promoters increased only 2 and 2.5-fold, respectively, yielding an *rrnB* P1-specific increase of ~1.5-fold. A 20% increase in ATP concentration and a 25% decrease in ppGpp concentration accompanied this increase in *rrnB* P1 transcription initiation. These data are consistent with the model that changes in both iNTPs and ppGpp concentrations contribute to feedback control of rRNA expression induced by defects in UP element function.

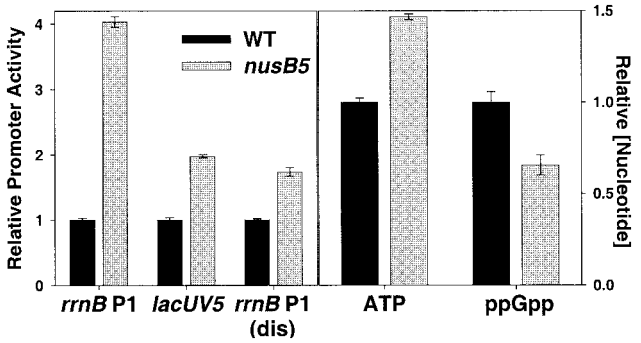

FIG. 2. Feedback derepression in a *nusB5* strain. Promoter activities in wild-type and *nusB5* strains were measured from single-copy promoter-*lacZ* fusions using β-galactosidase assays. Promoter activities are expressed relative to the average activity measured in a wild-type strain. Absolute promoter activities in the wild-type strain were as follows: *rrnB* P1, 96 Miller units (MU); *lacUV5*, 1,519 MU; *rrnB* P1(dis), 1,295 MU. Strains were grown at 37°C in morpholinepropanesulfonic acid (MOPS) minimal medium supplemented with 0.4% glucose, 10 μg of thiamine/ml, and 1 mM KH_2PO_4. ATP and ppGpp were extracted from identical cultures grown in the presence of 20 μCi of $KH_2{}^{32}PO_4$/ml. Nucleotide concentrations are expressed relative to the concentration in the wild-type strain. Error between measurements from independent cultures is indicated. The growth rates of the wild-type and mutant strains were 1.20 and 0.73 doublings/hour, respectively.

Feedback control of *rrn* P1 promoters in a Δ*fis* strain. The Fis protein binds to sites upstream of *rrn* P1 promoters and

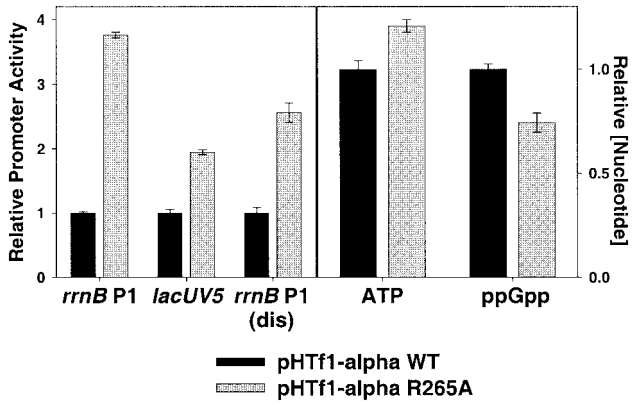

FIG. 3. Feedback derepression in a strain overexpressing *rpoA*-R265A. Promoter activities in strains overexpressing *rpoA*-R265A (from pHTf1-αR265A) were compared to activities in strains overexpressing wild-type α (pHTf1-αWT) using promoter-*lacZ* fusions described in Fig. 1 and 2. Promoter activity is expressed relative to the average activity measured in strains overexpressing wild-type α. Promoter activities in strains overexpressing wild-type α were as follows: *rrnB* P1, 60 MU; *lacUV5*, 808 MU; and *rrnB* P1(dis), 762 MU. Strains were grown as described in the legend to Fig. 2, with the addition of 100 μg of ampicillin/ml. ATP and ppGpp concentrations were measured as described in the legend for Fig. 2. Nucleotide concentrations are expressed relative to the concentration in the strain overexpressing wild-type α. The growth rates of the strains expressing the wild-type and mutant α were 0.84 and 0.65 doublings/hour, respectively.

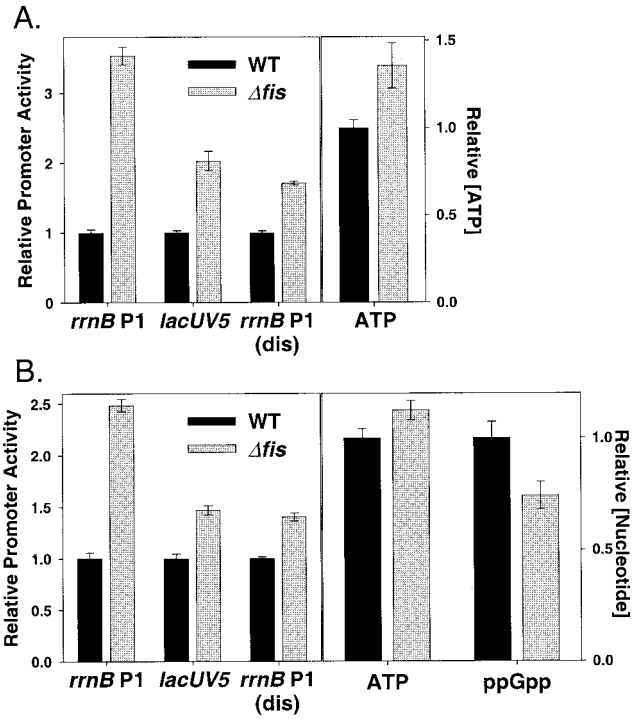

FIG. 4. Feedback derepression in a Δ*fis* strain. (A) Promoter activities and ATP and ppGpp concentrations in wild-type and Δ*fis* strains grown in MOPS medium supplemented with 0.4% glucose, 20 amino acids (80 μg/ml), 10 μg of thiamine/ml, and 1 mM KH_2PO_4 were measured as described in the legends for Fig. 2 and 3. (B) Cultures were grown as described in the legend for panel A but in the absence of amino acids. The growth rates of the wild-type and mutant strains were 1.71 and 1.58 doublings/hour, respectively, in panel A and 1.15 and 0.98 doublings/hour, respectively, in panel B.

activates transcription four- to eightfold when cells are growing logarithmically at moderate to high growth rates (19, 34). When the *fis* gene is deleted, full-length *rrn* P1 promoters lose this activation, and feedback derepression of core *rrn* P1 promoters restores rRNA synthesis to normal levels (3, 34).

At a high growth rate (~1.7 doublings per hour), when Fis makes a relatively large contribution to *rrn* P1 promoter activity, we observed 3.5-fold more core *rrnB* P1 activity in the Δ*fis* strain than in the wild-type strain (Fig. 4A), whereas the control promoters, *lacUV5* and *rrnB* P1(dis), increased only 2 and 1.7-fold, respectively. This approximately twofold *rrnB* P1-specific feedback derepression in the Δ*fis* strain was accompanied by a 35% increase in the cellular ATP concentration (Fig. 4A).

Since the ppGpp concentration was not measurable with confidence under these high growth rates using our detection methods, we also measured the effects of the Δ*fis* mutation on *rrnB* P1 activity in cells grown at a lower growth rate (in minimal medium supplemented with glucose but not amino acids), a situation where the ppGpp concentration was higher. Fis concentrations are lower at this more modest growth rate (~1 generation per hour), reducing the occupancy of Fis sites and thus the effect of Fis on *rrn* P1 promoter activity (1). Deletion of the *fis* gene increased *rrnB* P1 activity 2.5-fold under these conditions, whereas the control promoters increased only 50% (Fig. 4B). This ~1.6-fold *rrnB* P1-specific derepression was accompanied by a small but reproducible increase in ATP concentration (~12%) and a small but reproducible decrease in the ppGpp concentration (~25%) (Fig. 4B). These data are consistent with the conclusion that changes in the concentrations of both iNTPs and ppGpp contribute to the restoration of *rrn* P1 promoter activity in a Δ*fis* strain.

Feedback control of *rrn* P1 promoters in strains with altered *rrn* gene dose. Previous studies have shown that feedback mechanisms decrease *rrn* P1 promoter activity when cells are transformed with multicopy plasmids encoding rRNA operons, keeping overall rRNA synthesis rates constant independent of the rRNA gene dose. We observed a 3.3-fold decrease in *rrnB* P1 core promoter activity in the presence of extra intact *rrn* operons (Fig. 5), consistent with results from previous studies (21). In contrast, *lacUV5* and *rrnB* P1(dis) activity decreased by only 1.8- and 1.6-fold, respectively. Thus, there was an approximately twofold *rrnB* P1-specific feedback response to the increase in rRNA gene dose. We observed a small but reproducible decrease in ATP concentration (20%) in response to the increased *rrn* gene dose, but we did not observe an increase in the ppGpp concentration (Fig. 5). Although it is likely that the changes in the iNTP concentration contribute to the decrease in *rrnB* P1 promoter activity, this is probably insufficient to account for the entire effect on transcription initiation. Thus, it is possible that all means of inducing feedback control do not utilize the identical set of regulatory mechanisms (see also references 9 and 43 and Discussion, below).

Changes in DNA supercoiling are not responsible for feedback control of *rrn* P1 promoters in Δ*fis* or *nusB5* strains. In the figures above, we correlated changes in the concentrations of two regulators of *rrn* P1 core promoters, iNTPs and ppGpp,

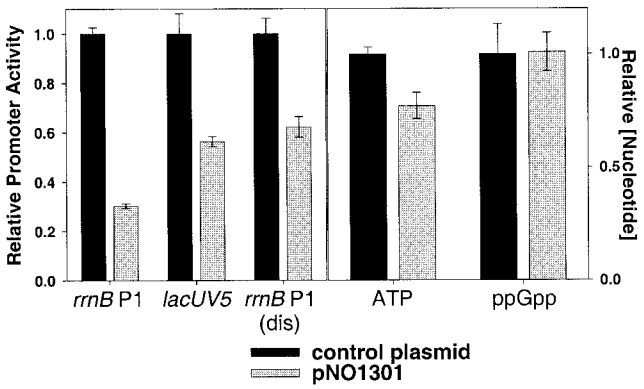

FIG. 5. Feedback derepression in a strain with increased *rrn* gene dose. By using β-galactosidase assays, promoter activities (from single-copy promoter-*lacZ* fusions) in strains carrying extra rRNA operons (plasmid pNO1301) (21) were compared to activities in strains containing a control plasmid (pNO1302) that does not make functional 16S or 23S rRNAs (21). Promoter activity is expressed relative to the average activity measured in strains containing the control plasmid. Growth and ATP and ppGpp extraction were performed as described in the legend for Fig. 3. Nucleotide concentrations are expressed relative to the concentration in the strain containing the control plasmid. Promoter activities in strains containing the control plasmid were as follows: *rrnB* P1, 137 MU; *lacUV5*, 1,789 MU; and *rrnB* P1(dis), 1,469 MU. The growth rate of the strain containing pNO1301 was 0.86 doublings/hour, and that of the strain containing pNO1302 was 1.11 doublings/hour.

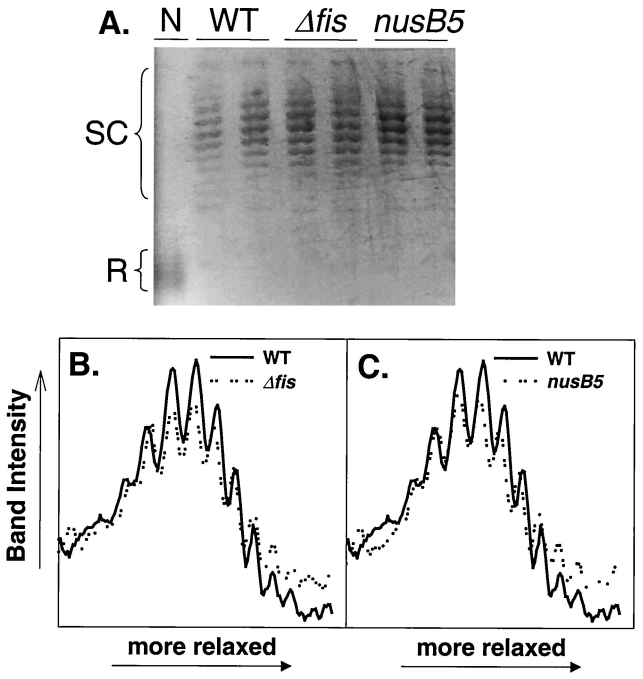

FIG. 6. Plasmid topology is not affected by the Δ*fis* or *nusB5* mutations. (A) pBR322-derivative pRLG770 (34) was extracted from wild-type (WT), Δ*fis*, and *nusB5* strains grown as described in the legend for Fig. 3. The samples in the two lanes shown for each strain derived from duplicate cultures. The DNA in lane N was isolated from a wild-type strain treated with the gyrase inhibitor norfloxacin (10 μg/ml for 30 min) prior to plasmid extraction and electrophoresis on chloroquine gels (see Materials and Methods). In these gels, more relaxed plasmids (R) migrate faster than supercoiled plasmids (SC), as reported previously (11). Topoisomers were quantified using ImageQuant 5.1 (Molecular Dynamics). Representative traces comparing plasmid DNA from Δ*fis* versus wild-type strains (B) and *nusB5* versus wild-type strains (C) are shown.

with changes in the activity of *rrnB* P1 in a variety of experimental situations, suggesting that these regulatory factors contribute to feedback control of rRNA expression. However, we cannot rule out that other mechanisms also make contributions to either the *rrn* P1-specific or the general effects on transcription observed in these situations. For example, changes in DNA supercoiling could theoretically contribute to the observed changes in promoter activity in the feedback situations discussed above, since the activities of many promoters (including *rrn* P1 promoters) increase with increases in negative supercoiling (44). To determine if the level of DNA supercoiling is different in Δ*fis* and *nusB5* strains compared to wild-type strains, we extracted plasmids from these strains during log phase and examined their mobilities on chloroquine gels. Cells were grown in minimal glucose medium (Fig. 6A) and in minimal glucose medium supplemented with amino acids (data not shown). We observed no significant difference in the degree of plasmid supercoiling between the Δ*fis* mutant and the wild-type strain or between the *nusB5* and the wild-type strain (Fig. 6B and C). We conclude that changes in supercoiling do not contribute significantly to the observed increases in *rrn* P1 promoter activities observed in these feedback situations (assuming that plasmid topology is reflective of the chromosomal topology near the rRNA operons). Our result with the Δ*fis* strain conflicts with that reported by another group who concluded that supercoiling levels increased in a Δ*fis* strain relative to a wild-type strain (38) (see Discussion).

DISCUSSION

Changes in iNTP and ppGpp concentrations contribute to feedback control of rRNA synthesis. We propose that changes in the concentrations of two small-molecule effectors, iNTPs and ppGpp, contribute to compensatory increases in the activities of *rrn* P1 promoters following treatments that would be expected to decrease rRNA output. In *nusB5*, *rpoA-R265A*, and Δ*fis* mutant strains, ATP concentrations increased and ppGpp concentrations decreased relative to the wild-type strain, apparently compensating (at least in part) for the decrease that would have been expected otherwise in the rRNA synthesis rate. We note that the degree of compensation in the mutant strains was not always sufficient to restore the level of promoter activity that would have been expected at the growth rate of the wild-type strain. In some cases, growth was significantly slower than in the wild-type strain (see figure legends). It is well established that rRNA core promoter activity correlates with cell growth rate (growth rate-dependent regulation) (6, 17), and in no case did a mutant strain grow faster than the wild-type strain (see figure legends). Therefore, the observed increases in *rrnB* P1 core promoter activity in the mutant strains cannot be attributed to an increase in growth rate.

A role for iNTPs and ppGpp under feedback conditions is consistent with the previous conclusion that the increase in *rrn* P1 promoter activity following inhibition of translation (from spectinomycin or chloramphenicol treatment) results from an increase in ATP concentration and a decrease in ppGpp concentration (36). Furthermore, the level of feedback derepression by variant *rrnB* P1 promoters in Δ*fis* strains correlated with the promoters' iNTP requirements in vitro (4). Taken together, these data suggest that both ppGpp and iNTP concentrations play roles in feedback control of rRNA transcription.

We have always observed that the activities of control promoters (as measured by promoter-*lacZ* fusions) change in parallel with *rrnB* P1, but to a lesser extent, under feedback conditions. Thus, the specific effect on rRNA transcription is superimposed on a more general effect on gene expression in these situations. Either the changes in iNTP and ppGpp concentrations have a smaller effect on the activities of these promoters that is superimposed on the specific effects on rRNA and rRNA-like promoters or feedback directly or indirectly introduces a general effect on some other step in reporter gene expression (e.g., transcription elongation, mRNA decay, or translation). We emphasize that *rrnB* P1 and *rrnB* P1(dis) make exactly the same transcript (Fig. 1), indicating that the specific effect of feedback conditions on reporter gene expression is on transcription initiation, not some later step in gene expression. These results are consistent with the model that the kinetic step in reporter gene expression responsible for the specific effect of feedback conditions on these promoters is open complex lifetime, the step affected by the concentration of the iNTP and ppGpp.

Additional factors might contribute to feedback control of rRNA synthesis. When feedback inhibition of *rrn* P1 core promoter activity was induced by increasing the *rrn* gene dose, ATP concentration decreased, but ppGpp concentration stayed the same. It was also reported previously that depletion of rRNA operons resulted in an increase in *rrn* expression (at the level of both transcription initiation and elongation) without a corresponding decrease in ppGpp levels (9). There are at least two possible explanations for the lack of a change in ppGpp concentration under one or both of these conditions. First, it is possible that other regulatory factors play a role in the feedback response induced by a change in *rrn* gene dose, working in conjunction with the small change in iNTP concentration. Alternatively, it is possible that the change in iNTP concentration is the only regulatory signal responsible for the specific change in *rrn* P1 activity under these conditions but that the small change in iNTP concentration is sufficient to cause a greater relative change in *rrnB* P1 promoter activity. For example, if *rrnB* P1 were almost completely unoccupied by RNA polymerase, one might expect that a relatively small increase in ATP concentration could cause a relatively large fold increase in promoter activity, a situation that occurs during outgrowth of cells from stationary phase (30). However, this is probably not the case in exponential phase (36). Therefore, we favor the explanation that there may be additional mediators of feedback control of rRNA synthesis in *E. coli*. This conclusion is in agreement with that reached by Condon et al. and Voulgaris et al., who reported that the ppGpp concentration did not decrease (9) and the ATP concentration did not increase (43) in strains with a reduced number of rRNA operons. Furthermore, these investigators noted that the activities of certain *rrn* P1 promoter variants that were relatively insensitive to the iNTP concentration were still subject to feedback control in strains with a reduced *rrn* gene dose. Thus, there might be additional regulatory signals that are induced by certain feedback conditions.

Δ*fis* and *nusB* mutants do not display altered DNA supercoiling. We did not observe a difference in the degree of negative supercoiling displayed by the same plasmid in the Δ*fis* mutant, the *nusB5* mutant, and the wild-type strain. Johnson and colleagues also did not observe altered levels of supercoiling in strains lacking *fis* (28; R. Johnson, personal communication). These results conflict with results in a previous report in which it was concluded that plasmids are hypersupercoiled in Δ*fis* strains (38). We do not know the basis for this discrepancy. Since the altered concentrations of ppGpp and ATP induced in *fis* and *nusB* mutants are likely to be deleterious to cell growth in the long term, it is possible that mutations might ultimately arise in these strains that compensate for defects in rRNA synthesis by other means. One such class of mutations might increase the overall level of supercoiling in the cell. To decrease the likelihood of obtaining such second-site suppressors, we used several fresh independent transductants of the *fis*::*kan* and *nusB5* alleles in each experiment.

ppGpp and iNTPs are regulatory signals linking rRNA transcription to the overall amount of translation. Perturbation of one part of the machinery that controls rRNA transcription results in responses by other parts of the machinery to compensate for the original perturbation. Thus, the control of rRNA synthesis by small molecules demonstrates how the cell has evolved robust complex regulatory networks for control of essential biosynthetic processes.

We suggest that both ppGpp and iNTP concentrations continually fluctuate in response to slight variations in the availability of nutrients or in the activity of translating ribosomes and that *rrn* P1 promoters are poised to respond to these small changes, fine-tuning rRNA expression to the demand for protein synthesis in order to maintain homeostasis. However, these transient small changes in both the concentrations of small molecules and rRNA promoter activity are too small to detect with available methods. These homeostatic responses are detectable experimentally only when larger disruptions of ribosome synthesis are induced (see also references 30 and 36).

The synthesis of rRNA varies proportionally to the steady-state growth rate (growth rate-dependent control) (35). However, growth rate-dependent control of rRNA transcription is not lost in cells that cannot make ppGpp (14), and iNTP concentrations remain relatively constant at all growth rates (31; D. A. Schneider and R. L. Gourse, submitted for publication). Thus, although changing concentrations of ppGpp and iNTPs control the rapid responses of *rrn* P1 promoters to changes in growth phase, upshifts, and downshifts (30), they are not essential for growth rate-dependent control. Therefore, in steady-state situations, other regulators exist that are capable of conferring growth rate-dependent control of rRNA expression on bacterial cells.

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