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Regulation of the *Escherichia coli rrnB* P2 Promoter

Heath D. Murray, J. Alex Appleman, and Richard L. Gourse*

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

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The seven rRNA operons in *Escherichia coli* each contain two promoters, *rrn* P1 and *rrn* P2. Most previous studies have focused on the *rrn* P1 promoters. Here we report a systematic analysis of the activity and regulation of the *rrnB* P2 promoter in order to define the intrinsic properties of *rrn* P2 promoters and to understand better their contributions to rRNA synthesis when they are in their natural setting downstream of *rrn* P1 promoters. In contrast to the conclusions reached in some previous studies, we find that *rrnB* P2 is regulated: it displays clear responses to amino acid availability (stringent control), rRNA gene dose (feedback control), and changes in growth rate (growth rate-dependent control). Stringent control of *rrnB* P2 requires the alarmone ppGpp, but growth rate-dependent control of *rrnB* P2 does not require ppGpp. The *rrnB* P2 core promoter sequence (−37 to +7) is sufficient to serve as the target for growth rate-dependent regulation.

To meet the requirement for protein synthesis as nutritional conditions change, bacteria modulate ribosome synthesis primarily by controlling transcription initiation from rRNA promoters. *Escherichia coli* contains seven rRNA operons (*rrnA*, *-B*, *-C*, *-D*, *-E*, *-G*, and *-H*), each of which has two promoters, *rrn* P1 and *rrn* P2, separated by ~120 bp (16). The *rrn* P1 and *rrn* P2 promoters have many sequence characteristics in common: near consensus −10 and −35 hexamers, separated by 16 bp, that bind the σ subunit of RNA polymerase (RNAP) (16); an A+T-rich region upstream of the −35 hexamer (UP element) that increases transcription by binding the C-terminal domains of the α subunits of RNAP (27, 28); and a G+C-rich region (the discriminator) (38) between the −10 hexamer and the transcription start site that is required for proper regulation (4, 8, 15, 25) (see Fig. 1 for the *rrnB* P2 sequence).

Regulation of rRNA transcription initiation is often analyzed in terms of the responses of rRNA promoters to three experimental situations. First, *rrn* P1 activity changes in proportion to the steady-state growth rate (growth rate-dependent control [12, 22; reviewed in reference 13]), coordinating the number of ribosomes with the need for protein synthesis. Second, *rrn* P1 activity changes in inverse proportion to changes in rRNA gene dose (feedback regulation [14; reviewed in reference 13]), maintaining ribosome synthesis homeostatically. Third, *relA*-dependent inhibition of *rrn* P1 activity is observed following amino acid starvation (stringent control [reviewed in reference 6]), preventing an overinvestment of energy in ribosome synthesis when the substrates for protein synthesis are unavailable.

The literature is less clear about the regulation of the *rrn* P2 promoters. Early studies indicated that *rrn* P2 promoters are growth rate dependent (but less so than *rrn* P1 promoters) and not stringently controlled (31–33), but subsequent studies suggested the opposite, that *rrn* P2 promoters are not growth rate dependent (12, 40) and are stringently controlled (11, 15, 19). Studies employing *rrnB* P2-*lacZ* or *rrnB* P2-*cat* fusions as reporters (12, 40) suggested that *rrn* P2 promoters are more active than *rrn* P1 promoters, in contrast to conclusions based on direct measurements of RNAs transcribed from *rrn* P1 and *rrn* P2 promoters (7, 20, 31, 40). Furthermore, inhibition of *rrn* P2 promoters by transcription originating upstream from the *rrn* P1 promoters (promoter occlusion [11]) qualifies conclusions about the intrinsic activity and regulation of *rrn* P2 derived from examination of constructs containing both promoters (7, 19, 20, 31–33).

Here we systematically reinvestigated the activity and regulation of *rrnB* P2 by using newly constructed reporters of *rrnB* P2 promoter activity, as well as direct measurement of transcripts by primer extension. We found that *rrnB* P2 is less active during steady-state growth than we concluded previously and that it is regulated in response to growth rate, amino acid starvation, and rRNA gene dose. In addition, we show that stringent control of *rrnB* P2 requires ppGpp but that growth rate-dependent regulation of *rrnB* P2 does not require ppGpp. The *rrnB* P2 core promoter sequence is sufficient to serve as the regulatory target for growth rate-dependent control.

MATERIALS AND METHODS

Media and growth conditions. Cells were grown at 30°C in Luria-Bertani (LB) medium, brain heart infusion medium (Difco), or either M9 (21) or morpholinepropanesulfonic acid (23) minimal medium containing 0.4% glucose or glycerol, with or without 0.8% Casamino Acids (Difco) plus tryptophan (40 μg/ml). When required, ampicillin (Sigma) was included at 100 μg/ml. To induce a stringent response, the serine analog serine hydroxamate (1 mg/ml; Sigma) was added to an exponentially growing culture supplemented with amino acids (37).

Strains. The strains and plasmids used in this study are listed in Table 1. Antibiotic resistance cassettes replacing the *relA* and *spoT* genes were transduced into λ lysogens by using phage P1*vir* (10). All promoter derivatives were generated by PCR from plasmids containing a wild-type promoter(s). Primers for the PCR were designed to include an *Eco*RI site at the upstream junction of the promoter sequence and a *Hin*dIII site at the downstream junction. The PCR products were cleaved with *Eco*RI and *Hin*dIII, ligated into plasmid pRLG770 (29), sequenced, and then cloned into bacteriophage λ derivatives containing *lacZ* to form promoter-*lacZ* fusions as previously described (26). In all *lacZ* fusion constructs (referred to as system 1 [26]), the DNA sequence downstream from the indicated promoter fragment is identical and derives from *trp-lac* DNA sequences. Promoter constructs are named by the endpoints of the DNA fragments used for their construction, where +1 is the transcription start site. *rrnB* P2 utilizes multiple transcription start sites; +1 refers to the most frequently utilized

* Corresponding author. Mailing address: Department of Bacteriology, University of Wisconsin, 1550 Linden Dr., Madison, WI 53706. Phone: (608) 262-9813. Fax: (608) 262-9865. E-mail: rgourse@bact.wisc.edu.

TABLE 1. Strains, plasmids, and promoter constructs used in this study

Strain or plasmid	Genotype or description	Source
Strains		
VH1000	MG1655 *pyrE lacI lacZ* (RLG3499)	9
CF1693	*relA251*::*kan spoT207*::*cam* (RLG857)	10, 39
RLG3848	VH1000 λ*rrnB* P1(−61 to +50)-*lacZ*	This work
RLG3851	VH1000 λ*rrnB* P2(−37 to +7)-*lacZ*	This work
RLG3863	VH1000 λ*rrnB* P2(−52 to +7)-*lacZ*	This work
RLG3866	VH1000 λ*rrnB* P2(−37 to +7)-*lacZ relA spoT*	This work
RLG3871	VH1000 λ*rrnB* P1P2(−152 of P1 to +7 of P2)-*lacZ*	This work
RLG3897	VH1000 λ*rrnB* P2(−112 to +7; C-5A, A-4T, C-3A)-*lacZ*	This work
RLG3898	VH1000 λ*rrnB* P2(−112 to +7; Cins−15)-*lacZ*	This work
RLG3914	VH1000 λ*rrnB* P2(−112 to +21)-*lacZ*	This work
RLG3915	VH1000 λ*rrnB* P2(−112 to +7; C+5G,C+7G)-*lacZ*	This work
RLG4296	VH1000 λ*rrnB* P2(−68 to +7; C+5G,C+7G)-*lacZ*	This work
RLG4757	VH1000 λ*rrnB* P1(−152 to +50)-*lacZ*	This work
RLG4993	VH1000 λ*lacUV5* (−59 to +36)-*lacZ*	This work
RLG5014	VH1000 λ*rrnB* P2(−112 to +7)-*lacZ*	This work
RLG6982	VH1000 λ*rrnB* P2(−112 to +7)-*lacZ relA*	This work
Plasmids		
pRLG770	General transcription vector	29
pRLG3858	pRLG770 *rrnB* P1 (−152 of P1 to +7 of P2)	This work
pRLG3859	pRLG770 *rrnB* P2 (−52 to +7)	This work
pRLG3865	pRLG770 *rrnB* P2 (−37 to +7)	This work
pRLG3890	pRLG770 *rrnB* P2 (−112 to +7; C-5A, A-4T,C-3A)	This work
pRLG3893	pRLG770 *rrnB* P2 (−112 to +7; Cins−15)	This work
pRLG3895	pRLG770 *rrnB* P2 (−112 to +21)	This work
pRLG3896	pRLG770 *rrnB* P2 (−112 to +7; C+5G,C+7G)	This work
pRLG4871	pRLG770 *rrnB* P2 (−112 to +7)	This work
pNO1301	pBR322 expressing an intact *rrnB* operon	14
pNO1302	pNO1301 with 2.4-kb deletion within 16S and 23S genes	14

site, a C residue 7 bp downstream from the −10 hexamer (Fig. 1; H.D.M. and R.L.G., data not shown).

The *rrnB* P2-*lacZ* fusion described previously (12) was constructed by cleavage of *rrnB* DNA with *Fnu*DII, ligation with a *Hin*dIII linker, and then fusion of this fragment to a λ phage arm containing *lacZ*. This resulted in an *rrnB* P2 promoter with the downstream DNA sequence 5′-CCCGGGGAAGCTT. . .-3′, beginning with position +1. This sequence differs from the wild-type *rrnB* P2 sequence by C-to-G transversions at +5 and +7 (Fig. 1).

In vivo β-galactosidase assays. Cultures of lysogens containing promoter-*lacZ* fusions were started (A_{600} of ~0.02) from fresh colonies and shaken at 30°C for about 4 generations to an A_{600} of ~0.35, and β-galactosidase activity was measured following sonication as previously described (12, 21).

In vivo primer extension. Cultures of lysogens containing promoter-*lacZ* fusions were started from fresh colonies (starting A_{600} of ~0.02) and shaken at 30°C for about 4 generations to an A_{600} of ~0.35. RNA was extracted by a boiling lysis procedure, and reverse transcription was performed as previously described (15, 35). Note that the absolute activities of promoters making different RNAs are not comparable, since the efficiency of extension by reverse transcriptase varies on different templates. To ensure that any differences in RNA levels observed at different growth rates did not result from changes in RNA half-life, half-lives were measured directly after addition of rifampin. No differences in RNA half-life were detected (data not shown). Furthermore, control promoters whose activities did not change with growth rate when examined by using promoter-*lacZ* fusions but that had the same mRNA sequences as the growth rate-dependent promoters examined here did not change with growth rate when examined by primer extension (data not shown).

Feedback assays. A multicopy plasmid containing an intact *rrnB* operon coding for functional 16S, 23S, and 5S rRNAs (pNO1301) or an *rrnB* operon coding for nonfunctional 16S and 23S rRNAs (pNO1302) was transformed into lysogens carrying promoter-*lacZ* fusions. Cultures were shaken at 30°C in M9 medium supplemented with glucose and ampicillin to an A_{600} of ~0.35, and β-galactosidase activity was measured as described above. As described in the figure legends, the activity of a promoter-*lacZ* fusion in a strain containing pNO1301 and that of a promoter-*lacZ* fusion in a strain containing pNO1302 were compared and the ratio was then normalized to that obtained from a control strain containing a *lacUV5* promoter-*lacZ* fusion transformed with the same plasmids (12).

RESULTS

Sequence determinants for *rrnB* P2 activity. In order to investigate the behavior of *rrnB* P2 systematically, we created a series of *rrnB* P2-*lacZ* fusions containing promoter fragments with different upstream endpoints and the same downstream endpoint: −112 to +7, −52 to +7, and −37 to +7 (Fig. 1). We measured the activities of these fusions at a relatively low growth rate (i.e., when most of the rRNA transcription in the cell originates from the *rrn* P2 promoters [see below and reference 31]). *rrnB* P2 promoters with upstream endpoints of −112 and −52 had similar activities and were fourfold more active than the −37 to +7 construct (Fig. 2). The stimulatory effect of the −37 to −52 region is consistent with the previous finding that *rrnB* P2 contains an UP element; i.e., that the *rrnB* P2 sequence upstream of the −35 hexamer stimulates transcription both in vivo and in vitro (27, 28). These results also suggest that, unlike the *rrn* P1 promoters, which are activated by the transcription factor Fis (29), *rrnB* P2 does not require

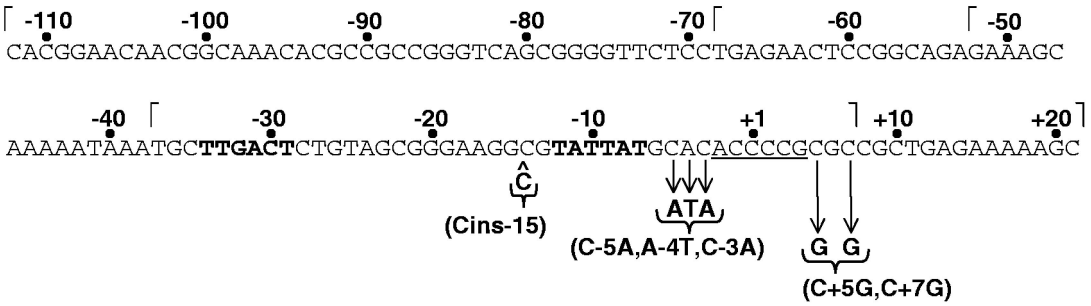

FIG. 1. Sequence of the *rrnB* P2 promoter region from −112 to +21. The most frequent *rrnB* P2 transcription start site is designated +1. The −10 and −35 hexamers are indicated in bold, start sites are underlined, and the upstream (−112, −68, −53, and −37) or downstream (+7 and +21) endpoints used for construction of *lacZ* fusions are indicated by ⌈ or ⌉, respectively. The −15C insertion is indicated by the symbol ^, and substitutions are identified below the arrows.

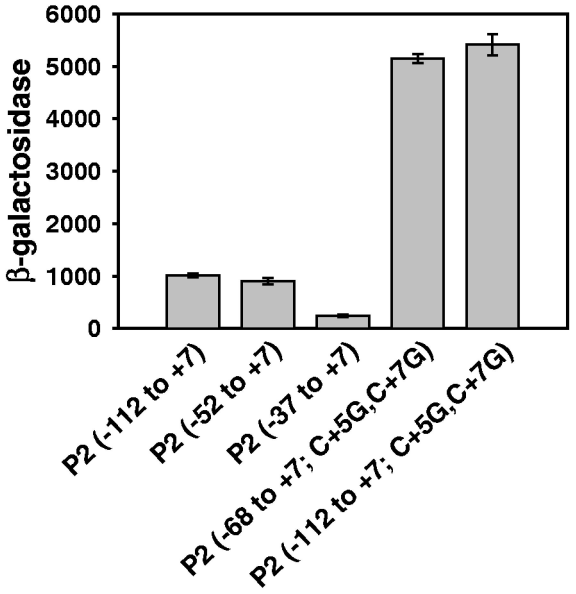

FIG. 2. Relative activities of *rrnB* P2 promoter variants. β-Galactosidase activities from single-copy *rrnB* P2 promoter-*lacZ* fusions were measured in M9 minimal medium containing 0.4% glycerol as a carbon source. The *rrnB* P2(−68 to +7; C+5G,C+7G) fusion is the construct reported in reference 12. The average and standard deviation of at least three independent experiments are shown for each promoter.

activation by a transcription factor that binds upstream of the UP element.

The *rrnB* P2-*lacZ* fusion used in our previous studies (12, 15) was five- to sixfold more active than the P2(−112 to +7) or P2(−52 to +7) fusion described above (Fig. 2). This construct [now referred to as P2(−68 to +7; C+5G,C+7G)] contains a different upstream endpoint and C-to-G transversions at positions +5 and +7 (i.e., downstream of the transcription start site [Fig. 1 and Materials and Methods]). Since sequences upstream of −52 did not appear to affect transcription, we tested whether the difference in activity between the original and new constructs resulted from the substitutions at +5 and +7 by introducing these transversions into the P2(−112 to +7)-*lacZ* fusion. The P2(−112 to +7; C+5G,C+7G) construct was five- to sixfold more active than the wild-type P2(−112 to +7) promoter (Fig. 2).

In theory, the increase in β-galactosidase activity could have arisen from either an increase in mRNA half-life or a more favorable RNAP-promoter interaction. We found that the C+5G,C+7G mutations did not alter mRNA half-life in vivo, but the mutations did alter characteristics of the open complex formed with RNAP in vitro (data not shown; see Discussion). Therefore, we now consider our previously constructed *rrnB* P2 promoter-*lacZ* fusion, P2(−68 to +7; C+5G,C+7G), to be a mutant that does not accurately reflect wild-type promoter interactions with RNAP. As a result, we reevaluated our previous conclusions concerning the regulation of the *rrnB* P2 promoter (12, 15).

The *rrnB* P2 promoter is subject to growth rate-dependent control. To evaluate growth rate dependence of transcription from the *rrnB* P2 promoter, cultures were grown in various media supporting different steady-state growth rates and promoter activities were determined. The activities of promoter-*lacZ* fusions containing the *rrnB* P2 −37 to +7, −52 to +7, or −112 to +7 sequence increased at least threefold over a fourfold range of growth rates (Fig. 3A to C). Promoter-*lacZ* fusions with an *rrnB* P2 downstream endpoint of +21 (with an upstream endpoint of either −112 [Fig. 3D] or −37 [data not shown]) exhibited regulation similar to that of *rrnB* P2 promoters with a downstream endpoint of +7, suggesting that these junctions represent wild-type promoter behavior. These regulation patterns are quite different from those exhibited by the control *lacUV5* promoter (Fig. 3E) or by the mutant promoter construct P2(−112 to +7; C+5G,C+7G) (Fig. 3F and reference 12; see also reference 40). These results suggest that sequences upstream from −37 or downstream from +7 are not required for growth rate-dependent regulation of *rrnB* P2 and that the substitutions at +5 and/or +7 affect properties of the *rrnB* P2 promoter that are important for its regulation (see Discussion).

Previous studies of *rrnB* P1 revealed a number of features in the core promoter important for regulation, including the length of the spacer (16 bp) between the −10 and −35 hexamers and the identity of the sequence in the discriminator region between the −10 hexamer and the transcription start site (4, 8). We constructed mutations in the *rrnB* P2 spacer or discriminator region to determine whether changing these features would alter regulation of this promoter. Either a 1-bp insertion in the *rrnB* P2 spacer, P2(−112 to +7; Cins−15), or a 3-bp substitution in the discriminator region, P2(−112 to +7; C−5A,A−4T,C−3A), significantly reduced growth rate regulation of the promoter (Fig. 3G and H), suggesting that similar sequence features in *rrn* P1 and *rrn* P2 might contribute to their regulation.

Growth rate-dependent regulation of the *rrnB* P2 promoter in tandem with *rrnB* P1. In their natural setting in rRNA operons, the *rrn* P2 promoters are located about 120 bp downstream from the *rrn* P1 promoters. To measure the activities of *rrnB* P2 when it is alone or in tandem with *rrnB* P1, we directly measured RNA synthesis by using quantitative primer extension. Transcription from the isolated *rrnB* P2 promoter increased as a function of growth rate (Fig. 4A), in agreement with the results obtained with *lacZ* fusions. However, in the presence of transcription from *rrnB* P1 (promoter activity illustrated in Fig. 4B), *rrnB* P2 promoter activity did not increase with growth rate and actually decreased slightly (Fig. 4A). These results are consistent with previous indications (11) that when the *rrn* P2 promoter is in its natural setting, its activity is occluded by transcription originating from *rrn* P1. Occlusion is most apparent when *rrnB* P1 activity is greatest, i.e., at high growth rates, and is sufficient to mask the intrinsic growth rate dependence of *rrnB* P2.

We next measured whether a *lacZ* fusion to a DNA fragment containing the intact *rrnB* P1P2 region would report the sum of the two isolated promoter activities (Fig. 4C). The isolated *rrnB* P1 and *rrn*B P2 promoters were each growth rate dependent (although to different extents [31]). As expected from the results shown in Fig. 4A, the activity of the tandem *rrnB* P1P2-*lacZ* fusion at low growth rates reflected the sum of the activities from the isolated promoters (Fig. 4C, dashed line), since occlusion of *rrnB* P2 is negligible when *rrnB* P1 is least active. In contrast, at high growth rates, the activity of the

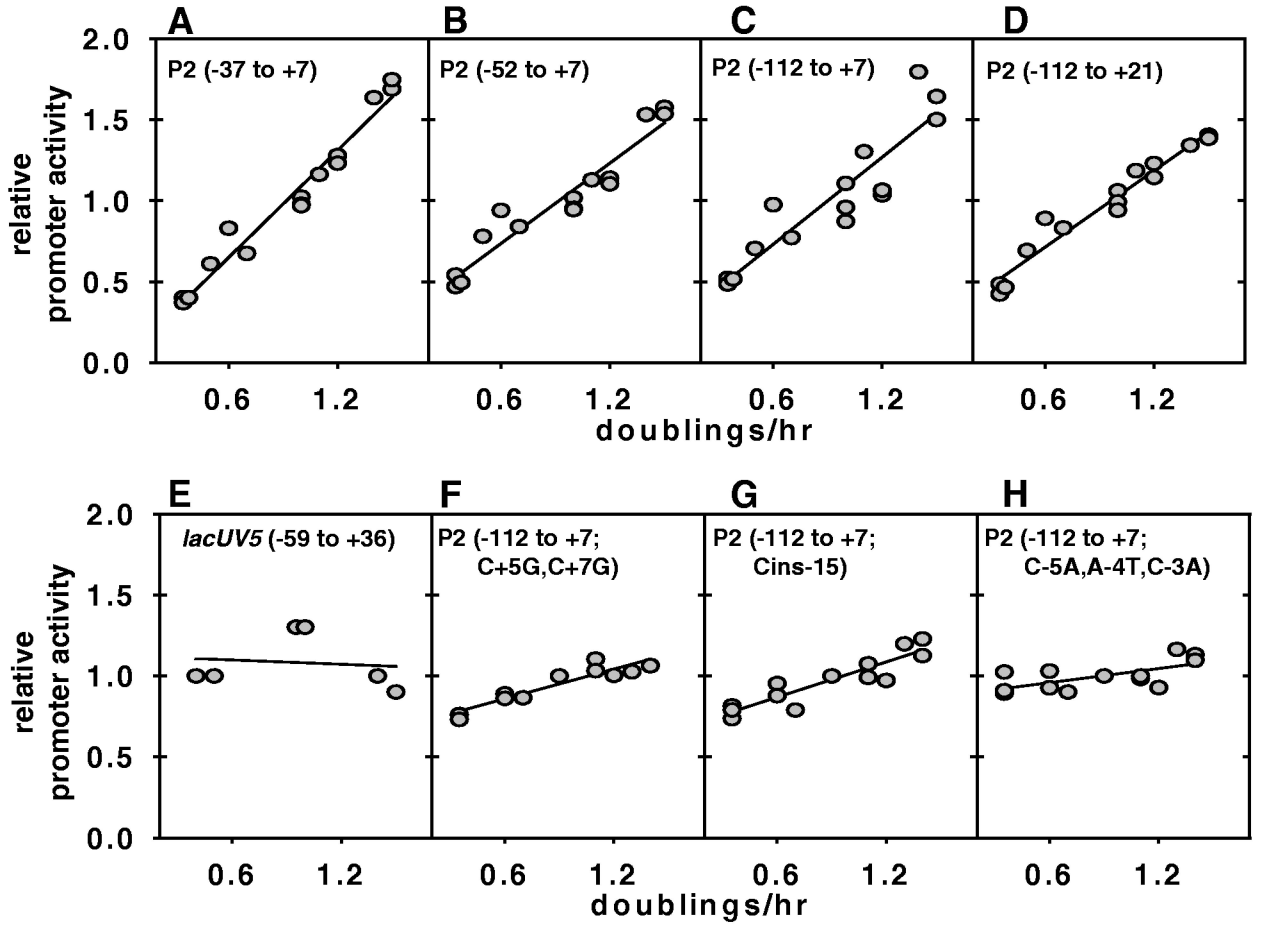

FIG. 3. Promoter activities as a function of growth rate. β-Galactosidase activities were measured at different growth rates, obtained by growing cells in different media as described in Materials and Methods: M9 medium with 0.4% glycerol, M9 medium with 0.4% glucose, M9 medium with 0.4% glycerol plus 0.8% Casamino Acids plus tryptophan, M9 medium with 0.4% glucose plus 0.8% Casamino Acids plus tryptophan, and LB medium. Linear regressions were drawn by using SigmaPlot 5.0 (Jandel Scientific). The endpoints of the wild-type and mutant *rrnB* P2 promoter fragments used to construct the fusions are indicated in the panels. The *lacUV5* promoter-*lacZ* fusion shown in panel E has been described previously (12). To enable visual comparison of the slopes, the activity of each promoter was normalized to a value of 1.0 at a growth rate of 0.9 doubling per hour (8). Strain designations and observed promoter activities (in Miller units) at a growth rate of 0.9 doubling per hour are as follows: A, RLG3851, 784 ± 34 U; B, RLG3863, 1,749 ± 72 U; C, RLG5014, 1,958 ± 236 U; D, RLG3914, 4,189 ± 252 U; E, RLG4993, 408 ± 66 U; F, RLG3915, 7,211 ± 300 U; G, RLG3898, 3,167 ± 281 U; H, RLG3897, 2,240 ± 104 U. Data from at least two independent experiments are shown for each construct.

rrnB P1P2 fusion was substantially lower than that of the sum of the two isolated promoters (Fig. 4C, dashed line), consistent with the observed occlusion of *rrnB* P2 by *rrnB* P1 under these growth conditions (Fig. 4A). We conclude that the *lacZ* fusion to the tandem rRNA promoters accurately reports expression from the intact *rrnB* P1P2 promoter region, describing the sum of the activities from the *rrnB* P1 promoter and the occluded *rrnB* P2 promoter.

***rrnB* P2 is stringently controlled but does not require ppGpp for growth rate-dependent control.** To determine whether the *rrnB* P2 promoter is stringently controlled, we used primer extension to measure its expression following amino acid starvation in a wild-type strain. Figure 5A shows that transcription from wild-type *rrnB* P2 was strongly inhibited, as reported previously for *rrnB* P1 (11, 15, 19). The *rrnB* P2 promoter with an altered discriminator sequence, P2(−112 to +7; C−5A, A−4T,C−3A), was inhibited only slightly (15, 38), and inhibition of wild-type *rrnB* P2 did not occur in a *relA* mutant (Fig. 5A). Thus, the results confirm previous conclusions (11, 15, 19, 35) that *rrnB* P2 is stringently controlled by the *relA* product, ppGpp.

ppGpp concentrations change inversely with growth rate (6, 30). To determine whether ppGpp is required for growth rate-dependent control of *rrnB* P2, we measured the activity of an *rrnB* P2-*lacZ* fusion at different growth rates in a wild-type strain and in a strain lacking the two genes capable of ppGpp synthesis, *relA* and *spoT*. Growth rate regulation of *rrnB* P2 was very similar in the mutant and wild-type strains (Fig. 5B), indicating that a regulator(s) other than ppGpp is sufficient to maintain normal levels of transcription from *rrnB* P2 in steady-state growth. However, we have not ruled out the possibility that ppGpp, when present, contributes to this regulation.

Increased rRNA gene dose reduces transcription from *rrnB* P2. Total rRNA synthesis is gene dose independent, because transcription from individual rRNA operons is reduced in inverse proportion to the increase in rRNA gene number (14). It

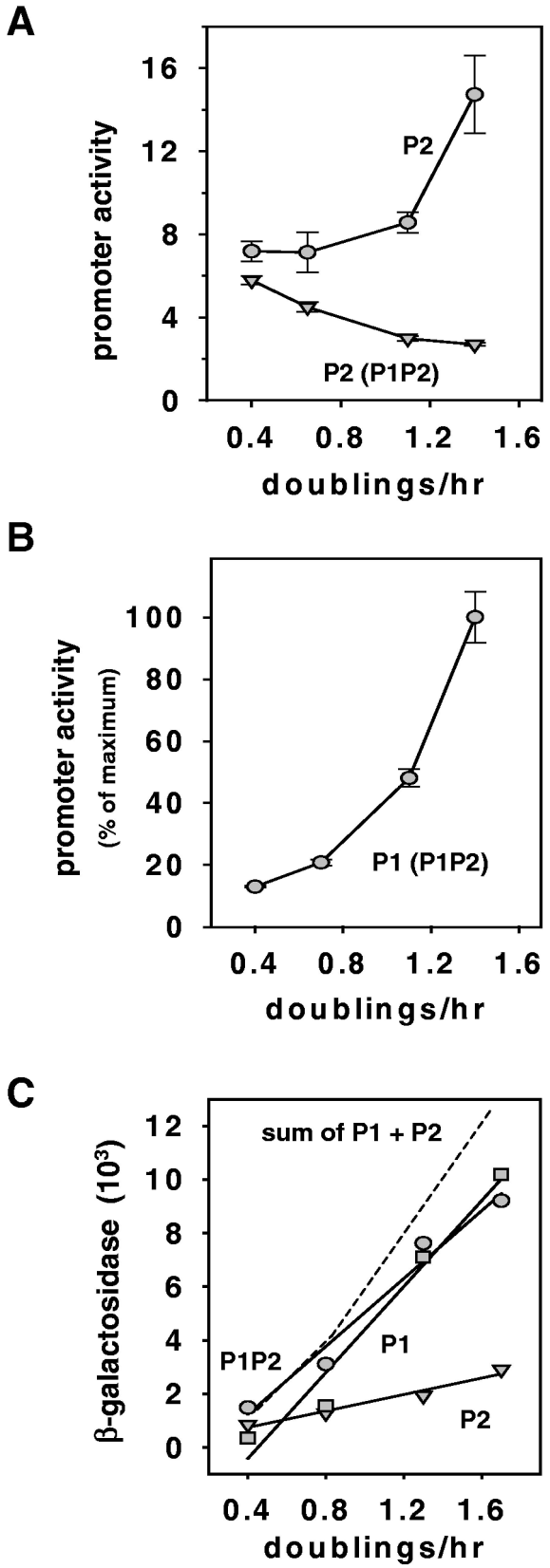

FIG. 4. Transcription from *rrnB* P2 in the presence and absence of *rrnB* P1. (A) RNA transcribed from *rrnB* P2 promoter-*lacZ* fusions was measured directly by primer extension from lysogens grown in morpholinepropanesulfonic acid medium supplemented with 0.4% glycerol, 0.4% glucose, or 0.4% glucose plus 0.8% Casamino Acids plus tryptophan or in LB medium. Symbols: ●, RLG5014, *rrnB* P2(−112 to +7); ▼, RLG3871, *rrnB* P1P2(−152 of P1 to +7 of P2). The average and standard deviation of at least three independent experiments are shown. Promoter activities are expressed in arbitrary units. (B) RNA transcribed from *rrnB* P1, in the context of the *rrnB* P1P2(−152 of P1 to +7 of P2) promoter-*lacZ* fusion, was measured directly by primer extension from lysogens grown in the same media used for the experiment whose results are shown in panel A. The average and standard deviation of at least three independent experiments are shown. Promoter activity is expressed as a percentage of maximum promoter activity. Absolute promoter activity should not be compared to that of *rrnB* P2 (see Materials and Methods). (C) β-Galactosidase activities from the same promoter-*lacZ* fusions described in panels A and B and from an *rrnB* P1-*lacZ* fusion. Symbols: ●, RLG3871, *rrnB* P1P2(−152 of P1 to +7 of P2); ■, RLG4757, *rrnB* P1(−152 to +50); ▼, RLG5014, *rrnB* P2(−112 to +7). The average of two independent experiments is shown for each promoter. The dashed line is a plot of the sum of the activities from the isolated *rrnB* P1-*lacZ* and *rrnB* P2-*lacZ* fusion constructs.

was shown previously that *rrnB* P1 promoter-*lacZ* fusions were inhibited by the presence of extra rRNA operons on multicopy plasmids, while the mutant *rrnB* P2 promoter-*lacZ* fusion P2(−68 to +7; C+5G,C+7G) was unaffected (12). To determine whether the wild-type *rrnB* P2 promoter is feedback regulated, we compared the activities of wild-type P2(−112 to +7) and two mutant promoter-*lacZ* fusions, P2(−112 to +7; C+5G, C+7G) and P2(−112 to +7; C−5A,A−4T,C−3A), in the presence of a multicopy plasmid harboring an intact *rrnB* operon or control plasmids (see the legend to Fig. 6 and reference 12). The wild-type *rrnB* P2 and *rrnB* P1 promoters were inhibited about 30 and 50%, respectively, while the mutant *rrnB* P2 promoters were inhibited less than 10% by the presence of extra rRNA operons (Fig. 6). Thus, *rrnB* P2 promoter activity is feedback regulated, although to a lesser extent than *rrnB* P1.

DISCUSSION

***rrn* P2 promoters are regulated.** We have shown that the *rrnB* P2 promoter (in the absence of *rrnB* P1) has intrinsic regulatory characteristics similar to those of the *rrn* P1 promoters. *rrnB* P2 activity increases with growth rate, core promoter sequences are sufficient for this growth rate-dependent regulation, and ppGpp is not required for this control. Furthermore, *rrnB* P2 is feedback inhibited by the presence of extra rRNA operons and is stringently controlled (11, 15). Thus, our studies suggest that both the *rrn* P1 and *rrn* P2 promoters are coordinately regulated (although not to the same extent). As a consequence of being regulated to different degrees, the *rrn* P1 and *rrn* P2 promoters have different functions: the *rrn* P1 promoters are most responsible for the large amounts of rRNA required during rapid growth, while the *rrn* P2 promoters are responsible for most of the rRNA expression that occurs at low growth rates (Fig. 4B and reference 31).

In some previous studies (18, 41), changes in *rrnB* P2 promoter activity with growth rate and amino acid limitation were attributed to changes in the concentration of free RNAP. We suggest that this conclusion is not likely to be correct, since (i) UP elements reduce the concentration of RNAP required for transcription (26), yet growth rate-dependent regulation of *rrnB* P2 was not affected by deletion of its UP element (Fig. 3);

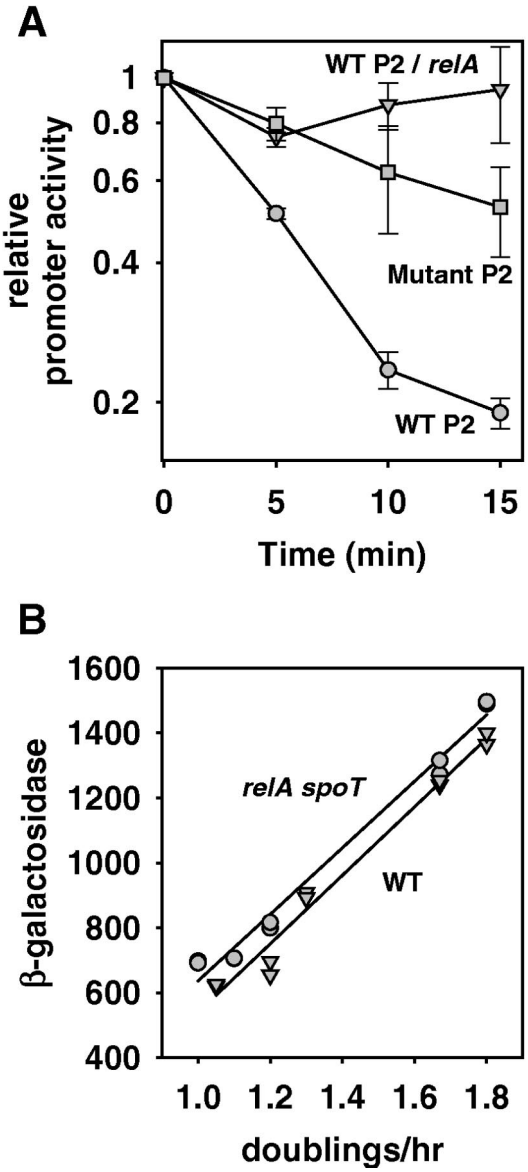

FIG. 5. *rrnB* P2 is stringently controlled but does not require ppGpp for growth rate-dependent regulation. (A) RNA transcribed from *rrnB* P2 promoter-*lacZ* fusions was measured directly by primer extension following amino acid starvation induced by serine hydroxamate addition to a culture growing exponentially in LB medium (A_{600} of ~0.3). The identity of the *rrnB* P2 promoter and the strain background are indicated for each sample. Symbols: ●, RLG5014, wild-type (WT) *rrnB* P2(−112 to +7); ■, RLG3987, mutant *rrnB* P2(−112 to +7; C−5A,A−4T,C−3A); ▼, RLG6982, wild-type *rrnB* P2(−112 to +7) in a *relA* strain. The average and standard deviation of three independent experiments are shown. (B) β-Galactosidase activity from an *rrnB* P2 core promoter-*lacZ* fusion, P2(−37 to +7), as a function of growth rate in wild-type and *relA spoT* mutant strains. Symbols: ▼, RLG3851, wild-type strain; ●, RLG3866, *relA spoT* mutant strain. Data from two independent experiments are shown for each strain.

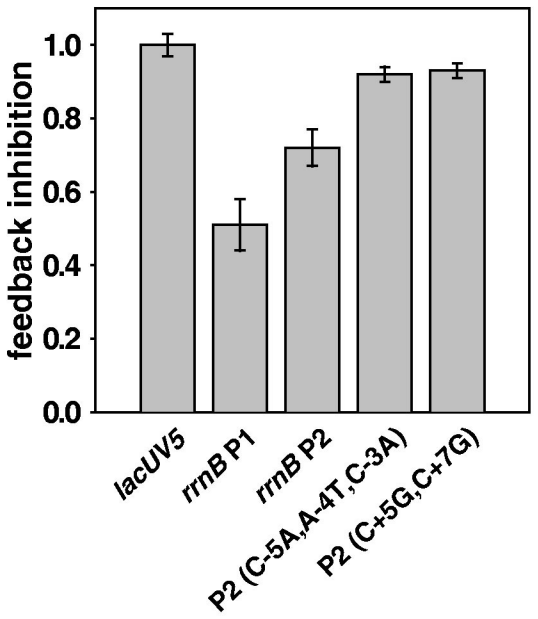

FIG. 6. *rrnB* P2 promoter activity is inhibited by the presence of extra rRNA operons. Lysogens containing promoter-*lacZ* fusions were transformed with a multicopy plasmid containing either an intact *rrnB* operon (pNO1301) or an *rrnB* operon containing a large deletion in the 16S and 23S rRNA genes (pNO1302). Promoter activity is expressed as the ratio of the β-galactosidase activity from a promoter-*lacZ* fusion in a strain containing pNO1301 to that in a strain containing pNO1302 and then normalized to the value obtained from a control *lacUV5* promoter as previously described (12). RLG4993, *lacUV5* (−59 to +36); RLG3848, *rrnB* P1 (−61 to +50); RLG5014, *rrnB* P2(−112 to +7); RLG3987, *rrnB* P2(−112 to +7; C−5A,A−4T,C −3A); RLG3915, *rrnB* P2(−112 to +7, C+5G,C +7G). The average results and standard deviations of experiments with three independent transformants are shown.

(ii) *rrnB* P2 activity was unaffected by conditions that should reduce the effective RNAP concentration within the cell (i.e., by the presence of multicopy plasmid pNO1302, whose transcription titrates out RNAP [reference 2 and data not shown]); and (iii) *rrnB* P2 activity was affected by conditions that do not alter the cell's RNAP concentration (i.e., by the presence of multicopy plasmid pNO1301 [Fig. 6 and references 2, 11, 26, and 30]). Thus, the activity of *rrnB* P2 is unlikely to be a valid indicator of the free cellular RNAP concentration.

Mechanism of regulation of *rrn* P2 promoters. The *rrnB* P2 promoter forms a relatively short-lived open complex with RNAP (H.D.M. and R.L.G., unpublished data), accounting for its sensitivity to inhibition by ppGpp (3). However, since *rrnB* P2 is growth rate dependent even in *relA spoT* mutant strains, mechanisms in addition to those involving ppGpp must contribute to control of the *rrn* P2 promoters. One mechanism contributing to regulation of the *rrn* P1 promoters involves sensing of changing initiating nucleoside triphosphate concentrations (4, 5, 9, 35). *rrnB* P2 responds to changing concentrations of its initiating nucleoside triphosphate, primarily CTP, in vitro and in vivo (H.D.M and R.L.G., unpublished data). We suggest, therefore, that when the *rrn* P2 promoters are responsible for most rRNA synthesis, ribosome production is determined, at least in part, by CTP levels.

The small DNA-binding proteins Fis and H-NS affect the activity of the *rrn* P1 promoters in vitro and in vivo (1, 29, 36). We tested *rrnB* P2 promoter activity in strains with *fis* or *hns* deleted in vivo and observed that *rrnB* P2 activity was increased in both mutants (data not shown). However, neither protein has been implicated in direct negative control of *rrn* P2 pro-

moters (e.g., see reference 36). Since *rrnB* P2 promoter activity is regulated homeostatically (Fig. 6), it is likely that the increase in *rrnB* P2 promoter activity in the strain lacking *fis* results indirectly from the loss of activation of *rrn* P1 (feedback derepression [13]). In contrast, the activities of numerous promoters have been shown to increase in *hns* strains (34). Further work is required to determine whether the effect of *hns* on *rrnB* P2 promoter activity is specific and/or direct.

The loss of regulation of the *rrnB* P2 promoter that resulted from the transversions at +5 and +7 was somewhat unexpected, since sequences downstream of +1 that affect regulation of *rrn* P1 promoters have not been identified (8). The C+5G and C+7G mutations increase the half-life of the *rrnB* P2 open complex in vitro (H.D.M. and R.L.G. unpublished), consistent with the model in which regulation of *rrnB* P2 results from its intrinsic kinetic characteristics. Since RNAP clamps down on the template in the downstream region of the transcription elongation complex (17) and this region is protected by RNAP in footprints of transcription initiation complexes (24), it is possible that direct contacts of RNAP with DNA are affected by the mutations at +5 and/or +7. We will address the molecular mechanism of regulation of *rrnB* P2 in a subsequent report.

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REFERENCES

1. **Afflerbach, H., O. Schroder, and R. Wagner.** 1998. Effects of the *Escherichia coli* DNA-binding protein H-NS on rRNA synthesis in vivo. Mol. Microbiol. **28:**641–653.
2. **Barker, M. M., T. Gaal, and R. L. Gourse.** 2001. Mechanism of regulation of transcription initiation by ppGpp. II. Models for positive control based on properties of RNAP mutants and competition for RNAP. J. Mol. Biol. **305:**689–702.
3. **Barker, M. M., T. Gaal, C. A. Josaitis, and R. L. Gourse.** 2001. Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation in vivo and in vitro. J. Mol. Biol. **305:**673–688.
4. **Barker, M. M., and R. L. Gourse.** 2001. Regulation of rRNA transcription correlates with nucleoside triphosphate sensing. J. Bacteriol. **183:**6315–6323.
5. **Bartlett, M. S., T. Gaal, W. Ross, and R. L. Gourse.** 1998. RNA polymerase mutants that destabilize RNA polymerase-promoter complexes alter NTP-sensing by *rrn* P1 promoters. J. Mol. Biol. **279:**331–345.
6. **Cashel, M., D. R. Gentry, V. H. Hernandez, and D. Vinella.** 1996. The stringent response, p. 1458–1496. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and J. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, vol. 1. ASM Press, Washington, D.C.
7. **de Boer, H., and M. Nomura.** 1979. In vivo transcription of rRNA operons in *Escherichia coli* initiates with purine nucleoside triphosphates at the first promoter and with CTP at the second promoter. J. Biol. Chem. **254:**5609–5612.
8. **Dickson, R. R., T. Gaal, H. A. deBoer, P. L. deHaseth, and R. L. Gourse.** 1989. Identification of promoter mutants defective in growth-rate-dependent regulation of rRNA transcription in *Escherichia coli*. J. Bacteriol. **171:**4862–4870.
9. **Gaal, T., M. S. Bartlett, W. Ross, C. L. Turnbough, Jr., and R. L. Gourse.** 1997. Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. Science **278:**2092–2097.
10. **Gaal, T., and R. L. Gourse.** 1990. Guanosine 3′-diphosphate 5′-diphosphate is not required for growth rate-dependent control of rRNA synthesis in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:**5533–5537.
11. **Gafny, R. S., S. Cohen, N. Nachaliel, and G. Glaser.** 1994. Isolated P2 rRNA promoters of *E. coli* are strong promoters that are subject to stringent control. J. Mol. Biol. **243:**152–156.
12. **Gourse, R. L., H. A. de Boer, and M. Nomura.** 1986. DNA determinants of rRNA synthesis in *E. coli*: growth rate dependent regulation, feedback inhibition, upstream activation, antitermination. Cell **44:**197–205.
13. **Gourse, R. L., T. Gaal, M. S. Bartlett, J. A. Appleman, and W. Ross.** 1996. rRNA transcription and growth rate-dependent regulation of ribosome synthesis in *Escherichia coli*. Annu. Rev. Microbiol. **50:**645–677.
14. **Jinks-Robertson, S., R. L. Gourse, and M. Nomura.** 1983. Expression of rRNA and tRNA genes in *Escherichia coli*: evidence for feedback regulation by products of rRNA operons. Cell **33:**865–876.
15. **Josaitis, C. A., T. Gaal, and R. L. Gourse.** 1995. Stringent control and growth-rate-dependent control have nonidentical promoter sequence requirements. Proc. Natl. Acad. Sci. USA **92:**1117–1121.
16. **Keener, J., and M. Nomura.** 1996. Regulation of ribosome synthesis, p. 1417–1428. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and J. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, vol. 1. ASM Press, Washington, D.C.
17. **Korzheva, N., A. Mustaev, M. Kozlov, A. Malhotra, V. Nikiforov, A. Goldfarb, and S. A. Darst.** 2000. A structural model of transcription elongation. Science **289:**619–625.
18. **Liang, S., M. Bipatnath, Y. Xu, S. Chen, P. Dennis, M. Ehrenberg, and H. Bremer.** 1999. Activities of constitutive promoters in *Escherichia coli*. J. Mol. Biol. **292:**19–37.
19. **Liebig, B., and R. Wagner.** 1995. Effects of different growth conditions on the in vivo activity of the tandem *Escherichia coli* ribosomal RNA promoters P1 and P2. Mol. Gen. Genet. **249:**328–335.
20. **Lund, E., and J. E. Dahlberg.** 1979. Initiation of *Escherichia coli* ribosomal RNA synthesis in vivo. Proc. Natl. Acad. Sci. USA **76:**5480–5484.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Miura, A., J. H. Krueger, S. Itoh, H. A. deBoer, and M. Nomura.** 1981. Growth rate-dependent regulation of ribosome synthesis in *E. coli*: expression of the *lacZ* and *galK* genes fused to ribosomal promoters. Cell **25:**773–782.
23. **Neidhardt, F. C., C. A. Bloch, and D. F. Smith.** 1974. Culture media for enterobacteria. J. Bacteriol. **119:**736–747.
24. **Ozoline, O. N., and M. A. Tsyganov.** 1995. Structure of open promoter complexes with *Escherichia coli* RNA polymerase as revealed by the DNase I footprinting technique: compilation analysis. Nucleic Acids Res. **23:**4533–4541.
25. **Pokholok, D. K., M. Redlak, C. L. Turnbough, Jr., S. Dylla, and W. M. Holmes.** 1999. Multiple mechanisms are used for growth rate and stringent control of *leuV* transcriptional initiation in *Escherichia coli*. J. Bacteriol. **181:**5771–5782.
26. **Rao, L., W. Ross, J. A. Appleman, T. Gaal, S. Leirmo, P. J. Schlax, M. T. Record, Jr., and R. L. Gourse.** 1994. Factor independent activation of *rrnB* P1. An "extended" promoter with an upstream element that dramatically increases promoter strength. J. Mol. Biol. **235:**1421–1435.
27. **Ross, W., S. E. Aiyar, J. Salomon, and R. L. Gourse.** 1998. *Escherichia coli* promoters with UP elements of different strengths: modular structure of bacterial promoters. J. Bacteriol. **180:**5375–5383.
28. **Ross, W., K. K. Gosink, J. Salomon, K. Igarashi, C. Zou, A. Ishihama, K. Severinov, and R. L. Gourse.** 1993. A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. Science **262:**1407–1413.
29. **Ross, W., J. F. Thompson, J. T. Newlands, and R. L. Gourse.** 1990. *E. coli* Fis protein activates ribosomal RNA transcription in vitro and in vivo. EMBO J. **9:**3733–3742.
30. **Ryals, J., R. Little, and H. Bremer.** 1982. Control of rRNA and tRNA syntheses in *Escherichia coli* by guanosine tetraphosphate. J. Bacteriol. **151:**1261–1268.
31. **Sarmientos, P., and M. Cashel.** 1983. Carbon starvation and growth rate-dependent regulation of the *Escherichia coli* ribosomal RNA promoters: differential control of dual promoters. Proc. Natl. Acad. Sci. USA **80:**7010–7013.
32. **Sarmientos, P., S. Contente, G. Chinali, and M. Cashel.** 1983. Ribosomal RNA operon promoters P1 and P2 show differential regulatory responses, p. 65–74. *In* D. H. Hamer and M. Rosenburg (ed.), Gene expression. Alan R. Liss, Inc., New York, N.Y.
33. **Sarmientos, P., J. E. Sylvester, S. Contente, and M. Cashel.** 1983. Differential stringent control of the tandem *E. coli* ribosomal RNA promoters from the *rrnA* operon expressed in vivo in multicopy plasmids. Cell **32:**1337–1346.
34. **Schmid, M. B.** 1990. More than just "histone-like" proteins. Cell **63:**451–453.
35. **Schneider, D. A., T. Gaal, and R. L. Gourse.** 2002. NTP-sensing by rRNA promoters in *Escherichia coli* is direct. Proc. Natl. Acad. Sci. USA **99:**8602–8607.
36. **Tippner, D., H. Afflerbach, C. Bradaczek, and R. Wagner.** 1994. Evidence for a regulatory function of the histone-like *Escherichia coli* protein H-NS in ribosomal RNA synthesis. Mol. Microbiol. **11:**589–604.
37. **Tosa, T., and L. I. Pizer.** 1971. Biochemical bases for the antimetabolite action of L-serine hydroxamate. J. Bacteriol. **106:**972–982.
38. **Travers, A. A.** 1984. Conserved features of coordinately regulated *E. coli* promoters. Nucleic Acids Res. **12:**2605–2618.
39. **Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser, and M. Cashel.** 1991. Residual guanosine 3′,5′-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J. Biol. Chem. **266:**5980–5990.
40. **Zacharias, M., H. U. Goringer, and R. Wagner.** 1989. Influence of the G\CGC discriminator motif introduced into the ribosomal RNA P2- and *tac* promoter on growth-rate control and stringent sensitivity. EMBO J. **8:**3357–3363.
41. **Zhang, X., and H. Bremer.** 1995. Control of the *Escherichia coli rrnB* P1 promoter strength by ppGpp. J. Biol. Chem. **270:**11181–11189.