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Growth Rate-Dependent Control of the *rrnB* P1 Core Promoter in *Escherichia coli*

MICHAEL S. BARTLETT AND RICHARD L. GOURSE*

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

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We have extended our previous studies of the DNA sequences required for growth rate-dependent control of rRNA transcription in *Escherichia coli*. Utilizing a reporter system suitable for evaluation of promoters with low activities, we have found that the core promoter region of *rrnB* P1 (−41 to +1 with respect to the transcription initiation site) is sufficient for growth rate-dependent control of transcription, both in the presence and in the absence of guanosine 3′-diphosphate 5′-diphosphate (ppGpp). The core promoter contains the −10 and −35 hexamers for recognition by the sigma 70 subunit of RNA polymerase but lacks the upstream (UP) element, which increases transcription by interacting with the alpha subunit of RNA polymerase. It also lacks the binding sites for the positive transcription factor FIS. Thus, the UP element, FIS, and ppGpp are not needed for growth rate-dependent regulation of rRNA transcription. In addition, we find that several core promoter mutations, including −10 and −35 hexamer substitutions, severely reduce *rrnB* P1 activity without affecting growth rate-dependent control. Thus, a high activity is not a determinant of growth rate regulation of rRNA transcription.

In steady-state cultures of *Escherichia coli*, the synthesis of rRNA per total cell protein increases with the square of the growth rate of the cell, a relationship called growth rate-dependent control (24). Growth rate control acts at the level of transcription initiation from the P1 promoters of the seven rRNA (*rrn*) operons, while the downstream P2 promoters of these operons appear to be transcribed constitutively (15, 32). A feedback system senses the level of translationally competent ribosomes and controls transcription initiation at *rrn* P1 promoters and some tRNA promoters in response to the need for protein synthesis (7, 17, 21, 27, 37). It has been suggested that this feedback system is the mechanism by which growth rate control of transcription is achieved (15, 27), although alternative hypotheses have been proposed (2, 3, 18–20), and the topic remains controversial.

Another system, stringent control, also acts at the level of transcription initiation from the *rrn* P1 promoters (16, 23, 33). In this system, rRNA synthesis is rapidly and severely inhibited by the production of guanosine 3′-diphosphate 5′-diphosphate (ppGpp) in response to aminoacyl-tRNA limitation (6, 34), although the mechanism by which ppGpp acts to reduce transcription is still unclear.

Less is understood about the *trans*-acting factors involved in growth rate regulation of *rrn* P1 transcription. Since ppGpp concentrations are inversely proportional to the growth rate of the cell under most conditions, ppGpp was proposed to be responsible for growth rate-dependent control (6, 31). However, it was found that rRNA transcription increases normally with growth rate even in strains lacking ppGpp (12) and that transcription from rRNA promoters can vary without corresponding changes in ppGpp levels (8). The FIS protein, another *trans*-acting factor that affects *rrn* P1 transcription initiation (30, 38), has also been considered as a growth rate regulator of rRNA transcription, since the levels of FIS vary under different growth conditions (1, 26). However, rRNA promoters lacking FIS binding sites and strains lacking FIS still exhibit growth rate-dependent control (15, 30).

We have investigated previously the *cis*-acting DNA sequences required for the high transcriptional activity and regulation of *rrnB* P1. *rrnB* P1 has an extended promoter structure (Fig. 1), consisting of a core region and an upstream (UP) element. The core region contains −10 and −35 consensus hexamers homologous to those shown to be recognized by the sigma 70 subunit of RNA polymerase (10). The UP element, located between −40 and −60 with respect to the transcription initiation site, interacts with the alpha subunit of RNA polymerase (29) and increases transcription approximately 30-fold in vitro and in vivo (28). The transcription factor FIS binds to three sites between −60 and −154 (30). FIS stimulates rRNA transcription about 10-fold, most likely by interacting directly with RNA polymerase (5, 14, 25).

DNA sequences between −48 and +1 are sufficient for growth rate-dependent control of *rrnB* P1 (9, 15). This region contains the core promoter and a portion of the UP element

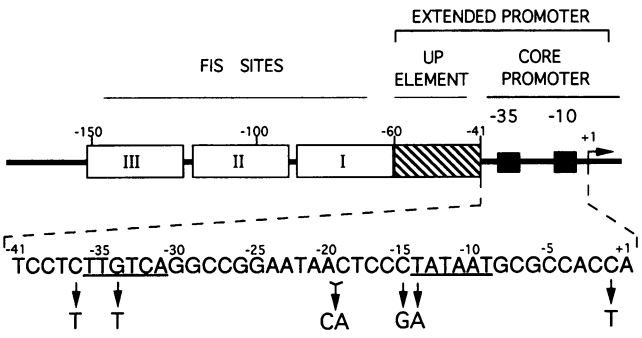

FIG. 1. Schematic representation of the *rrnB* P1 promoter. The core promoter, UP element, and binding sites for FIS are indicated. The DNA sequence from −41 to +1 is shown, with arrows indicating the mutations described in this paper.

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

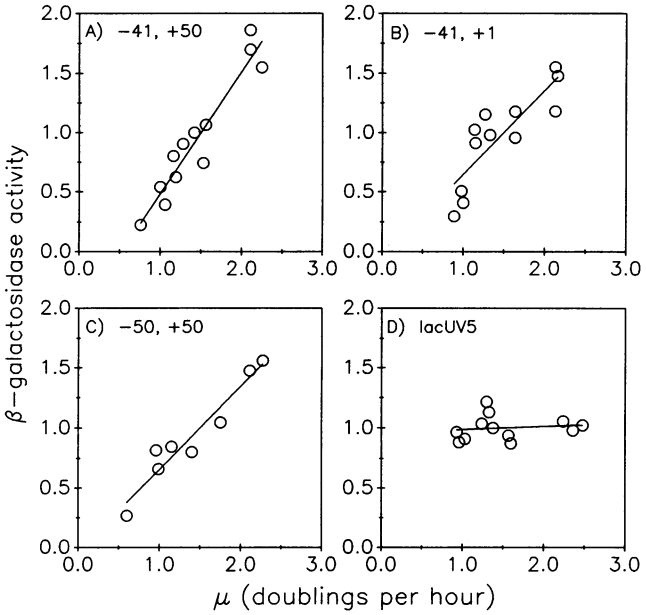

FIG. 2. Sequences upstream of the core promoter are not required for growth rate-dependent control of *rrnB* P1. Promoter-*lacZ* fusions (system II) were constructed essentially as described previously (35), with minor modifications described elsewhere (28). Promoter fragments were cloned into a plasmid intermediate (pMSB1; constructed by insertion of a *Hin*dIII linker, 5′-CCCAAGCTTGGG-3′ [New England Biolabs], into the *Sma*I site of pRS1553) and then introduced into a lambda phage (λRS468) by homologous recombination. Promoter sequences in *E. coli* LL309 monolysogenic for the recombinant phage were verified by PCR amplification and sequencing (25), using primers described previously (28). The lambda phage carrying the *lacUV5* promoter (λRS74) was described previously (35). Activities of *lacZ* fusions were measured at 37°C after growth in different media supporting a range of growth rates, as described previously (9). Slopes were normalized at a growth rate (μ) of 1.5 doublings per h so that the regulation of promoters with different absolute activities could be compared graphically, as described previously (9). (The growth rate at which activities are normalized is arbitrary and was chosen at a value in the range of growth rates obtained with a specific strain background. Plots of different promoters should be compared directly only with appropriate controls in the same strain background, with the same fusion system, and at the same growth temperature). Plots were drawn with SigmaPlot (Jandel Scientific, Sausalito, Calif.), and linear regressions and standard errors were calculated with Statistix II (NH Analytical Software, Roseville, Minn.). (A) Promoter activity (in MU) at a growth rate of 1.5 doublings per h, 318; slope of linear regression line (*m*), 1.03; standard error of the calculated slope (SE), 0.10. (B) MU = 323; *m* = 0.71; SE = 0.14. (C) MU = 2,054; *m* = 0.69; SE = 0.08. (D) MU = 1,924; *m* = 0.02; SE = 0.06. *rrnB* sequence endpoints are indicated for each panel (−41 to +50 and so on).

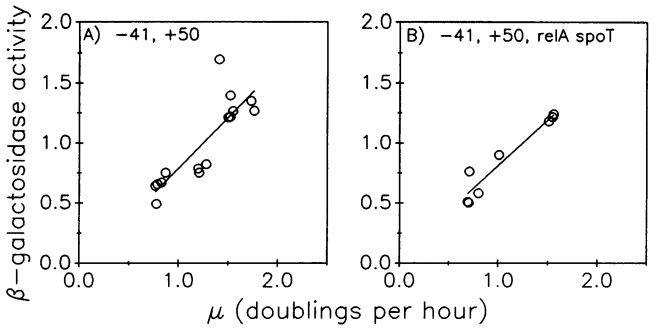

FIG. 3. ppGpp and activation by FIS are dispensable for growth rate-dependent control of *rrnB* P1. Lysogens containing system II fusions in MG1655 were constructed as described in the legend to Fig. 2, and *relA spoT* derivatives were made by P1 transduction as described previously (12). *relA spoT* strains require amino acid supplementation for growth (36), so they were always tested for amino acid auxotrophy before and after growth rate-dependent control experiments to verify the genotypes, as described previously (12). Growth rate control experiments were performed at 30°C. Slopes were normalized at a μ of 1.25 doublings per h and were processed as described for Fig. 2. (A) Wild-type strain. MU (at μ = 1.25) = 591; *m* = 0.85; SE = 0.15. (B) *relA spoT* strain. MU = 805; *m* = 0.75; SE = 0.08. For unknown reasons, the absolute activities of *rrnB* P1 core promoter-*lacZ* fusions are higher in MG1655 than in some other strains we have tested (see also Fig. 4 legend and reference 30). The fusions display normal growth rate dependence.

that increases transcription from the core promoter approximately eightfold (28). Several mutations within *rrnB* P1 that eliminate growth rate-dependent control were identified previously: a change at −33 from T to A, [T-33A], creating a consensus −35 hexamer; insertions increasing the spacer length between the −10 and −35 hexamers to the consensus 17 bp; and a double mutation, [C-1T, C-15G] (9). Each of these mutations increased promoter activity, even at the highest growth rates. Some mutations reducing promoter activity also appeared to abolish growth rate-dependent control, but their effects were difficult to interpret because of the high background activity of the promoter-*lacZ* fusion system used (9). In this study, we further investigated the promoter determinants of growth rate control of rRNA transcription.

Growth rate-dependent control of the *rrnB* P1 core promoter. The promoter-*lacZ* fusion system previously used for investigating growth rate-dependent control of *rrnB* P1 (which we refer to as system I [28]) yields about 40 Miller units (MU) of β-galactosidase activity when there is no promoter inserted upstream of the *lacZ* gene (11). While this background did not interfere with analysis of growth rate regulation of *rrnB* P1 promoters with high activity (i.e., promoters containing at least some upstream sequences) (9, 15), the low signal/noise ratio compromised analysis of regulation of *rrnB* P1 promoters completely lacking upstream sequences or containing mutations in the −10 and −35 consensus hexamers. Therefore, in order to analyze *rrnB* P1 derivatives with low activities, we used here the promoter-*lacZ* system developed by Simons and colleagues (35), in which terminators prevent readthrough transcription originating upstream of the promoter insertion site and in which the distance between the promoter insertion site and the *lacZ* gene is only about 100 bp. In this system, which we refer to as system II (28), promoterless *lacZ* constructs yielded less than 1 MU of β-galactosidase activity (data not shown), well below the activity of even our weakest promoter derivatives at all growth rates (see figure legends for activities of promoters). However, we found that strong promoters (e.g., *rrnB* P1 promoters containing the intact UP element) could not be cloned into the plasmid intermediate used to construct fusions in this system (4; for a discussion, see reference 28).

Growth rate-dependent control of several *rrnB* P1 derivatives and a *lacUV5* control promoter was determined by using system II *lacZ* fusions (Fig. 2). The activities of two P1 promoter derivatives lacking *rrnB* sequences upstream of −41 (−41 to +50 and −41 to +1) were growth rate dependent; the activities increased with increasing growth rate in a manner similar to that of a promoter containing a portion of the UP

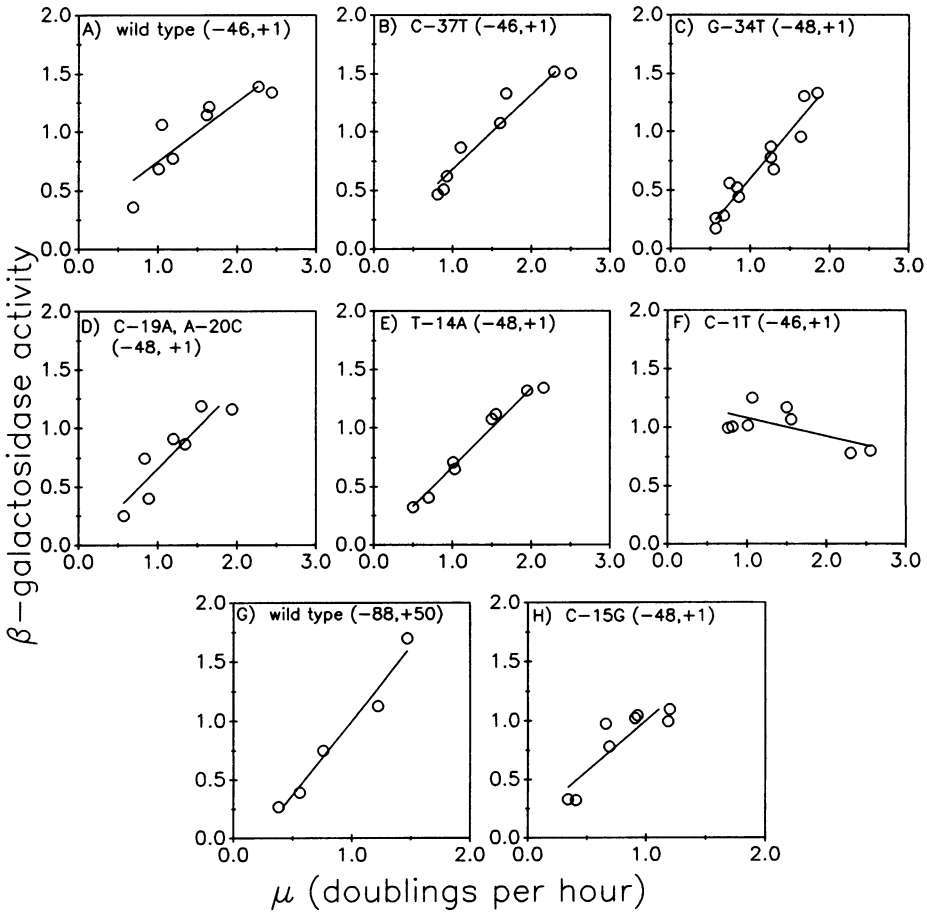

FIG. 4. Effects of mutations in the core promoter on growth rate-dependent control. Lysogens were constructed in LL309 as described in the legend to Fig. 2. The *rrnB* sequence endpoints for each promoter are indicated in parentheses. (A to F) System II fusions assayed at 37°C; activities were normalized at 1.5 doublings per h. (G to H) System I fusions assayed at 30°C; activities were normalized at 1.0 doublings per h. The lower temperature for growth and the reduced growth rate at which the normalization was done result from the fact that system I λ lysogens contain a temperature-sensitive repressor. (A) MU = 1,309; m = 0.51; SE = 0.11. (B) MU = 148; m = 0.64; SE = 0.07. (C) MU = 211; m = 0.81; SE = 0.08. (D) MU = 972; m = 0.68; SE = 0.15. (E) MU = 4.9; m = 0.67; SE = 0.05. (F) MU = 2,409; m = −0.15; SE = 0.07. (G) MU = 2,400; m = 1.26; SE = 0.13. (H) MU = 2,392; m = 0.89; SE = 0.20. The activities of system I and II fusions should not be compared directly, since the same promoter yields different absolute activities in the two systems, as discussed previously (28). The *rrnB* P1 promoter containing the [C-15G] mutation was constructed by subcloning a fragment from *Eco*RI (−48) to *Hha*I (−6) containing the mutation together with a fragment containing wild-type *rrnB* P1 sequences from −5 to +1. The promoter containing the −1 mutation was constructed by using synthetic oligonucleotides.

element (−50 to +50), characterized previously in system I (15) (Fig. 2A through C). The activity of a *lacUV5* promoter fused to *lacZ* by system II did not change appreciably at different growth rates (Fig. 2D), in agreement with previous results obtained by system I (15). Thus, the *rrnB* P1 core promoter (−41 to +1) is sufficient for growth rate control of rRNA transcription.

Growth rate control of the *rrnB* core promoter in strains lacking ppGpp. It has been shown previously that neither ppGpp nor FIS is essential for growth rate-dependent control (9, 12, 30). However, we could not rule out the possibility that these two systems are redundant; i.e., either system has the ability to confer growth rate control on the promoter, and the presence of one might compensate for the absence of the other. To address this, we measured the regulation of an *rrnB* P1 promoter (−41 to +50) that lacks the FIS binding sites responsible for activation in a strain that contains the *relA251* and *spoT207* deletions and therefore cannot synthesize ppGpp (12, 36). This promoter displayed regulation both in the presence (Fig. 2A and 3A) and in the absence (Fig. 3B) of ppGpp, indicating that some other regulatory factor besides ppGpp or FIS can mediate growth rate-dependent control. Furthermore, since the promoter also lacked the UP element, the unknown regulator does not function by affecting UP element-alpha subunit interactions.

Recently, it was reported that although the rate of stable RNA synthesis (r_s) per cell protein increased normally with increasing growth rate in a *relA spoT* strain, expression from a different *rrnB* P1 promoter-*lacZ* fusion failed to display growth rate regulation (19). Although we cannot be certain about the cause of the difference in the regulatory capabilities of our fusions and that of Hernandez and Bremer (19), we speculate that the apparent loss of growth rate regulation in the *relA spoT* strain observed by those authors resulted from translational effects on the reporter system. Their *lacZ* fusions were constructed to contain an extremely poor ribosome binding site (18), resulting in β-galactosidase activities more than 100-fold lower than those obtained with a comparable promoter in our

fusions. We suggest that the weak ribosome binding site may not compete well for ribosomes in a *relA spoT* strain, in which there are increased mRNA levels at high growth rates (19). Thus, the β-galactosidase activity from this fusion might not correctly reflect regulation at the promoter level. We also note that the persistence of growth rate-dependent control in *relA spoT* strains in our experiments is unlikely to result from peculiarities of the fusion system used or from the chromosomal location of the fusions, since negative controls (non-growth rate-regulated promoters in the same fusion system at the same chromosomal location) did not display growth rate regulation in *relA spoT* strains (4, 12).

Effects of point mutations in the core promoter on growth rate-dependent control. We also tested growth rate regulation of a class of *rrnB* P1 mutants with reduced promoter activities, using system II promoter-*lacZ* fusions. A substitution just upstream of the −35 hexamer [C-37T] severely reduced promoter activity without affecting upstream activation (22), possibly by creating a T tract that alters the DNA structure of this region (13). The [T-14A] and [G-34T] mutations also severely reduced promoter activity (11), most likely by interfering with interactions with the sigma subunit of RNA polymerase, since the mutations are at highly conserved positions in the −10 and −35 hexamers, respectively. In fact, the activity of the [T-14A] construct was less than 2% of that of the wild-type core promoter. The double mutation [C-19A, A-20C] had a less dramatic effect on promoter activity but was also retested because previous results indicated that it might be defective in growth rate-dependent control (9). As system II fusions, each of these mutant promoters had activities well above background at all growth rates. Each promoter responded to growth rate-dependent control (Fig. 4A to E). Thus, promoters need not be strong to be growth rate regulated.

The double mutation [C-1T, C-15G] increased promoter strength and interfered with growth rate control of *rrnB* P1 (9). We therefore created *lacZ* fusions with promoters containing each of the mutations alone and tested their regulation (Fig. 4F and H). The [C-15G] promoter had approximately twofold-higher activity than the wild type, but growth rate control of the promoter was left intact. The [C-1T] mutation abolished growth rate control of the promoter.

The C-to-T change at position −1 is the only *cis*-acting mutation we have isolated thus far in which growth rate control is defective but the strength of the promoter at high growth rates is unchanged. Primer extension analysis of in vivo RNA from the [C-1T] promoter indicated that the transcriptional start site is the same as in the wild-type promoter (23). Formally, the mutation could interfere with repression or cause constitutive activation at low growth rates or simply alter the kinetic properties of the promoter such that it is no longer rate limited at the regulated step in transcription initiation. Experiments are in progress to determine whether the interaction of the [C-1T] promoter with RNA polymerase is altered in vitro in the absence of additional effectors.

Summary. We have shown that UP element interactions with the alpha subunit of RNA polymerase are not required for growth rate-dependent control of *rrnB* P1 transcription. The required sequences (−41 to +1) encompass the −10 and −35 consensus hexamers, which are recognized by the sigma subunit of RNA polymerase, but mutations that affect this interaction do not necessarily interfere with regulation. Furthermore, growth rate-dependent control of *rrnB* P1 persists in the simultaneous absence of ppGpp and FIS binding to the upstream activation sites, the two previously identified *trans*-acting mechanisms influencing rRNA transcription initiation. Thus, our data suggest that there is yet another regulator of rRNA transcription whose target is the core region of the promoter or RNA polymerase.

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