Stringent control and growth-rate-dependent control have nonidentical promoter sequence requirements

(transcription initiation/guanosine 3'-diphosphate 5'-diphosphate/rRNA regulation)

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ABSTRACT *Escherichia coli* uses at least two regulatory systems, stringent control and growth-rate-dependent control, to adjust rRNA output to amino acid availability and the steady-state growth rate, respectively. We examined transcription from *rrnB* P1 promoters containing or lacking the cis-acting UP element and FIS protein binding sites after amino acid starvation. The "core promoter" responds to amino acid starvation like the full-length wild-type promoter; thus, neither the UP element nor FIS plays a role in stringent control. To clarify the relationship between growth-rate-dependent regulation and stringent control, we measured transcription from growth-rate-independent promoters during amino acid starvation. Four *rrnB* P1 mutants defective for growth-rate control and two other growth-rate-independent promoters (*rrnB* P2 and pS10) still displayed stringent regulation. Thus, the two systems have different promoter determinants, consistent with the idea that they function by different mechanisms. Two mutations disrupted stringent control of *rrnB* P1: (*i*) a multiple base change in the "discriminator" region between the −10 hexamer and the transcription start site and (*ii*) a double substitution making the promoter resemble the $E\sigma^{70}$ consensus promoter. These results have important implications for the mechanisms of both stringent control and growth-rate-dependent control of rRNA transcription.

Transcription of the seven rRNA operons in *Escherichia coli* is extraordinarily strong yet negatively regulated in response to nonoptimal nutritional conditions. The stringent control system inhibits rRNA transcription rapidly and specifically upon amino acid starvation (1). In contrast, the growth-rate-dependent control (GRDC) system ensures that rRNA synthesis relative to total cell protein is proportional to the square of the steady-state growth rate and is not strictly a function of amino acid availability (2).

Stringent control is mediated by the nucleotide guanosine 3'-diphosphate 5'-diphosphate (ppGpp) whose intracellular levels increase dramatically during amino acid starvation (3). Ribosome-associated RelA protein synthesizes ppGpp when uncharged tRNA occupies the ribosome's acceptor site (1). Neither the mode of action of ppGpp nor the promoter sequence determinants of stringent control are well understood, but a G+C-rich region between the −10 hexamer and the transcription start site of rRNA promoters (the "discriminator") is thought to be important (4–6).

The effector of GRDC remains controversial. *relA* mutants still display GRDC (1). However, basal ppGpp levels persist in *relA* strains because *spoT* encodes a second ppGpp synthetase (7). These basal levels correlate inversely with rRNA transcription (3)—hence, the suggestion that ppGpp regulates rRNA transcription in steady-state growth and during amino acid starvation (3, 8). Basal ppGpp levels clearly have func-

tional significance, since *relA spoT* strains lacking ppGpp are polyauxotrophic (7). However, rRNA transcription relative to the amount of cell protein increases normally with growth rate in these strains (9, 10), arguing that ppGpp is not essential for GRDC.

Each rRNA operon has two promoters: P1 is the major promoter at moderate-to-high growth rates, and P2 is thought to be constitutively expressed at low levels (2). *rrnB* P1 has three components: a "core" region containing the −10 and −35 hexamers, the UP element [the region from positions −40 to −60 contacting the RNA polymerase (RNAP) α subunit (11, 12)], and three sites in the region from positions −60 to −150 that bind FIS protein (13). FIS is not required for GRDC or stringent control (13).

Here, we use a primer-extension assay to explore the promoter sequence requirements for stringent control. We show that the FIS binding sites, the UP element, and specific transcribed sequences are dispensable for stringent control. We confirm the importance of the "discriminator" sequence and also identify a nondiscriminator mutation in *rrnB* P1 that disrupts stringent control. We find that several promoters display stringent control although they are not growth-rate-dependent. These results provide a starting point for investigating the molecular basis of the action of ppGpp during the stringent response and, in conjunction with our previous results, are most consistent with models for GRDC in which ppGpp does not play a major role.

MATERIALS AND METHODS

Plasmids and Lysogens. The names of mutant promoters (Table 1) include the wild-type (WT) base, the position of the change, and the mutant base (e.g., G−34T). Most promoter fragments were taken from previously existing constructs (9, 14, 16, 17) and inserted into pKM2 (18). pRLG2915, pRLG2927, and pRLG2932 were constructed as described (14) by using synthetic oligonucleotides. Single-copy λ lysogens containing promoter–lacZ fusions were constructed as described (14). The promoters in all constructions were sequenced.

Cell Growth, Starvation, and RNA Extraction. Every promoter was assayed in parallel in both MG1655 [*relA*+ (10)] and CF1651 [MG1655 *relA251* (19)]. *relA*+ and *relA*− cultures grew at similar rates [μ (doublings per hour) ≈ 1.6]. Overnight cultures of "test" strains containing the plasmid of interest, plus a third culture (reference cells; see below), were grown at 37°C in Mops minimal medium (20) containing 0.4% glucose, uracil (50 μg/ml), thiamine (10 μg/ml), ampicillin (100 μg/ml), and all 20 amino acids except serine (each at 40 μg/ml). After dilution to an A_{600} of 0.015 to 0.040 and growth to an A_{600} of 0.4 to 0.5, each test culture was split. One-half received serine hydroxamate (Sigma) to 400 μg/ml to induce amino

Abbreviations: GRDC, growth-rate-dependent control; RNAP, RNA polymerase; WT, wild type; M.U., Miller unit(s).
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Table 1. Promoter fragments used in this study

Promoter name	Endpoint positions	Plasmids/ strains
rrnB P1 (WT)	−88, +1	pRLG1478; RLG1231*
rrnB P1 (WT)	−115, +50	pRLG1479†
rrnB P1 (WT)	−41, +1	pRLG1482
rrnB P1 (C−19T)	−48, +1	pRLG1488
rrnB P1 (G−6T)	−48, +1	pRLG1490
rrnB P1 (G−34T)	−48, +1	pRLG1494
rrnB P1 (T−33A)	−48, +1	pRLG2903; RLG2935
rrnB P1 (C−1T,C−15G)	−48, +1	pRLG2905; RLG1242*
rrnB P1 (CGC−5 → −7ATA)	−88, +1	pRLG2915; RLG2931
rrnB P1 (A ins−22)	−48, +1	pRLG2916
rrnB P2 (WT)	−70, +2	pRLG2925; RLG1263
rrnB P1 (T−33A,A ins−22)	−50, +2	pRLG2927; RLG2940
rrnB P1 (CCC−15 → −17TGA)	−154, +50	pRLG2932
rrnB P1 (T ins−23)	−48, +1	pRLG2934
rrnB P1 (WT)	−48, +1	pRLG2948
pS10 (WT)	−486, +14	pRLG2950; RLG2971
λpL (WT)	−1313, +2	pRLG2952
rrnB P1 (WT)	−88, +50	pRLG2967
rrnB P1 (WT)	−46, +1	pRLG2974; RLG2978‡
rrnB P1 (C−1T)	−46, +1	RLG2975; RLG2979‡

Endpoint positions are reported with respect to the transcription initiation site. Plasmid number of the pKM2 derivative is listed first, followed by lysogen strain number in those cases where the promoter fragment was also cloned into a λRS205/459 [system I] lysogen (14). *From ref. 10. †From ref. 15. ‡These lysogens are λRS468 derivatives [system II (12)].

acid starvation; water was added to the untreated half. Reference cells were untreated. After 20 min of continued incubation, samples were removed for immediate RNA isolation essentially as described (21). Test culture cells (3 ml), reference cells (3 ml), and lysis solution (1.5 ml) were boiled for 90 sec; purified RNA was dissolved in 100 μl of 10 mM Tris·HCl (pH 8.0).

Primer-Extension Assay for Stringent Control. The promoters under investigation transcribe short unstable RNAs that allow detection of decreases in RNA abundance during starvation. The primer (22) hybridizes to the transcript 50–70 bases downstream of the HindIII site forming the junction between the promoter fragment and plasmid vector DNA [within sequences originating from phage λ common to all plasmid constructs (Fig. 1)]. Total RNA (5 μg) was mixed with 0.25 pmol of γ-^{32}P-end-labeled primer, Sequenase reaction buffer (United States Biochemical) to 1× final concentration, and water to 10–15 μl. Hybridization and primer extension were performed as described (22), except that extension was for 30 min, and 200 units of Moloney murine leukemia virus reverse transcriptase was used. After electrophoresis (22), bands were visualized by autoradiography and quantified by radioanalytic imaging. No products were detected from cells lacking pKM2 plasmid derivatives (data not shown).

Normalization Procedure. For stringent control assays, a transcript from unstarved reference cells added at the time of cell lysis provides an internal control correcting for RNA recovery, primer hybridization, and reverse transcriptase efficiency. Reference and test promoter fragment endpoints
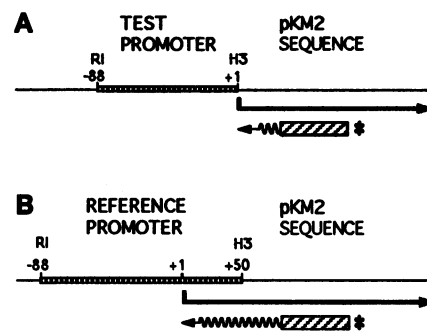

FIG. 1. Schematic of primer-extension assay for stringent control. Heavy arrows indicate mRNA. Asterisks mark primer-extension products. Test (A) and reference (B) promoter sequences are flanked by EcoRI (RI) and HindIII (H3) restriction sites. For test promoters with downstream endpoints other than +50, rrnB P1 WT (−115, +50) was the reference promoter. For test promoters with +50 endpoints, rrnB P1 WT (−88, +1) was the reference promoter.

yielded distinct primer-extension products (Fig. 1). Test band radioactivity was normalized to that of the reference band. In control experiments, normalized ratios were unaffected over a 20-fold range of primer concentration (data not shown).

β-Galactosidase Assays for GRDC of Transcription. Measurements were performed on promoter–lacZ fusions as described (23), except defined medium contained uracil (50 μg/ml), since MG1655 is partially defective for pyrimidine synthesis (24). Media used to vary μ were as follows: (i) AC minimal [7 g of K$_2$HPO$_4$/3 g of KH$_2$PO$_4$/0.05 g of MgSO$_4$/1 g of (NH$_4$)$_2$SO$_4$/H$_2$O to 1 liter] containing uracil (50 μg/ml), thiamine (10 μg/ml), 0.4% glucose (μ ≈ 0.5); (ii) medium i containing 0.5% Casamino acids and 0.4% glycerol instead of glucose (μ ≈ 0.9); (iii) medium i containing 0.5% Casamino acids (μ ≈ 1.1); (iv) LB (μ ≈ 1.3); (v) brain–heart infusion (Difco) (μ ≈ 1.5). Uracil was omitted in a few experiments; GRDC was unaffected.

RESULTS

Primer-Extension Assay for Stringent Control. Transcription from rrnB P1 containing sequences from positions −88 to +1 decreased ≈90% in a relA-dependent fashion after amino acid starvation induced by serine hydroxamate treatment, as expected for a promoter subject to stringent control (Fig. 2,
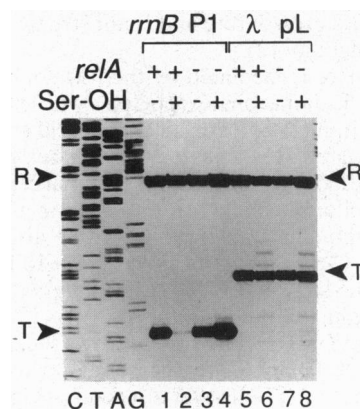

FIG. 2. Transcription from rrnB P1 and λpL in relA$^+$ and relA$^−$ strains after amino acid starvation. Ser-OH, serine hydroxamate treatment; T, test bands; R, reference bands. Lanes: 1–4, test promoter, rrnB P1 (−88, +1); 5–8, test promoter, λpL; 1, 2, 5, and 6, MG1655 (relA$^+$); 3, 4, 7, and 8, CF1651 (relA$^−$); C, T, A, and G, DNA sequence markers. The longer length of the λpL product derives from Kpn I–HindIII polylinker sequences in the plasmid vector not present in the rrnB P1 promoter construct.

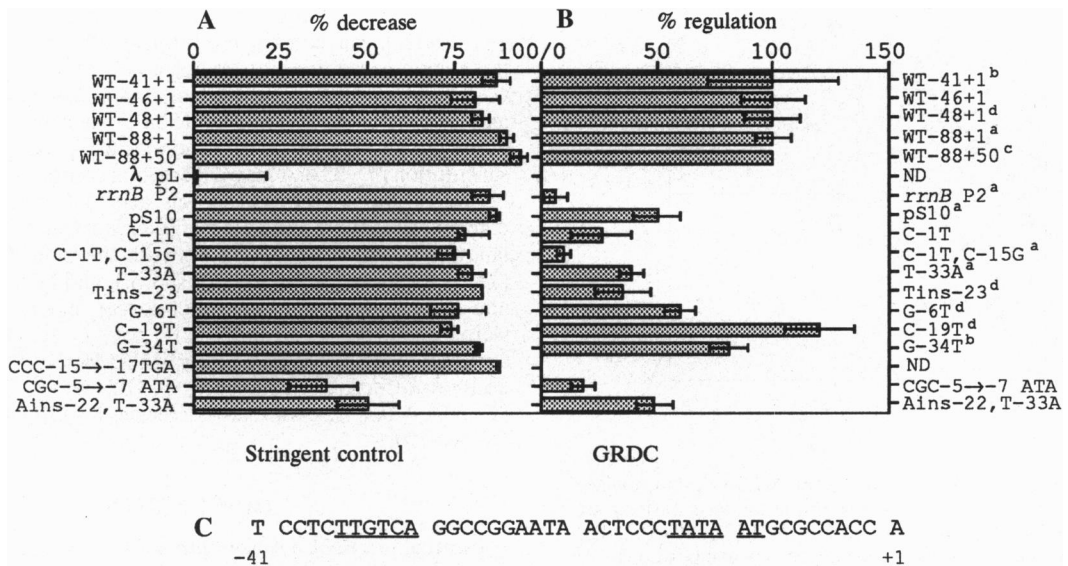FIG. 3. Quantitation of stringent control and GRDC. (*A*) Percent decrease after amino acid starvation: [1 − (transcription with serine hydroxamate/transcription without serine hydroxamate)] × 100% in a *relA*+ strain. Error bars are the range (at least two assays). 100%, No remaining transcription after starvation; 0%, transcription unchanged after starvation. (*B*) Percent GRDC: (slope mutant/slope WT) × 100% (average of at least three assays). Mutant and WT slopes were always determined in the same strain background. The standard error was calculated as described (23). Some data were taken from the following previously published work: a, slopes expressed relative to the slope of *rrnB* P1 (−88, +1); b, data from ref. 9; c, data from ref. 16; d, data from ref. 23. Statistical information for β-galactosidase activities in MG1655. M.U. (Miller units), activity at μ = 1.0; S, slope; SE, standard error of the slope. WT (−88, +1): M.U., 4886; S, 1.372; SE, 0.075. WT (−46, +1): M.U., 2067; S, 0.998; SE, 0.097. *rrnB* P2: M.U., 6568; S, 0.088; SE, 0.073. pS10: M.U., 3111; S, 0.686; SE, 0.131. [C−1T]: M.U., 2232; S, 0.256; SE, 0.086. [C−1T,C−15G]: M.U., 2984; S, 0.139; SE, 0.042. [T−33A]: M.U., 5783; S, 0.531; SE, 0.062. [CGC−5 → −7ATA]: M.U., 7096; S, 0.242; SE, 0.071. [A ins−22,T−33A]: M.U., 5963; S, 0.678; SE, 0.097. ND, not determined. Absolute activities from system I and II fusions should not be compared directly, since the same promoter in system II is 13-fold more active than in system I (12). (*C*) WT *rrnB* P1 core promoter sequence. The −10 and −35 hexamers are underlined.

lanes 1–4). The nonstringent λpL promoter showed little or no decrease in transcription (Fig. 2, lanes 5–8). Quantitation of these results and of the stringent and GRDC experiments described below is displayed in Fig. 3.

The *rrnB* P1 Core Promoter Is Sufficient for Stringent Control. Transcription from the *rrnB* P1 core promoter (Fig. 3C), which is 300-fold less active than the WT promoter with the UP element and FIS sites (12), was inhibited by amino acid starvation to the same extent as the full-length promoter (Figs. 3 and 4, lanes 1–4). Thus, stringent control requires neither FIS nor the UP element. An *rrnB* P1 mutant with even lower activity than the WT core promoter [G−34T (9)] retained
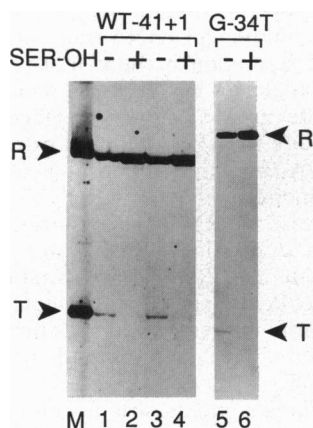
stringent control (Figs. 3 and 4, lanes 5 and 6), also suggesting that high promoter strength is not a requirement for stringent control.

Several Promoters Display Stringent Control but Not GRDC. Several previously identified *rrnB* P1 mutations abolish GRDC (9, 16, 23). To clarify the relationship between GRDC and stringent control, we measured the response of four such promoters to amino acid starvation: a double substitution [C−1T,C−15G], a single base-pair substitution [C−1T], an insertion creating a consensus 17-bp spacing
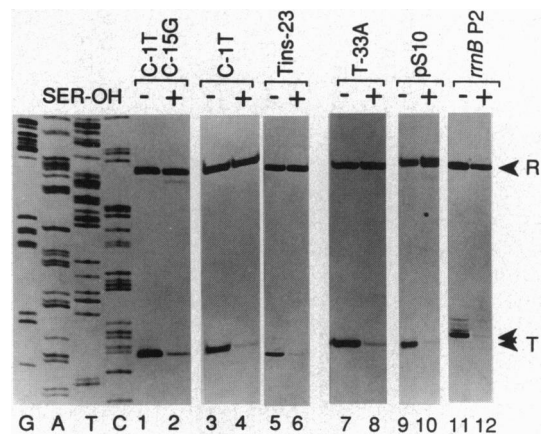

FIG. 4. Response of weak *rrnB* P1 promoters to amino acid starvation in MG1655. Lanes: 1–4, WT (−41, +1), duplicate experiments; 5 and 6, [G−34T]; M, size marker consisting of primer extensions from WT (−88, +1) and WT (−115, +50). Ser-OH, serine hydroxamate. R, reference product; T, test product.

FIG. 5. Stringent response of six growth-rate-independent promoters in strain MG1655. Lanes: 1–8, *rrnB* P1 mutants; 1 and 2, [C−1T,C−15G]; 3 and 4, [C−1T]; 5 and 6, [T ins−23]; 7 and 8, [T−33A]; 9 and 10, pS10; 11 and 12, *rrnB* P2. Ser-OH, serine hydroxamate. Results are compiled from several different gels. Markers on each gel confirmed that all promoters started at the appropriate site. Promoters with spacer insertions ([T ins−23], lanes 5 and 6) and [A ins−22] (data not shown) had additional minor starts. *rrnB* P2 (lanes 11 and 12) also had several initiation sites as reported (25).
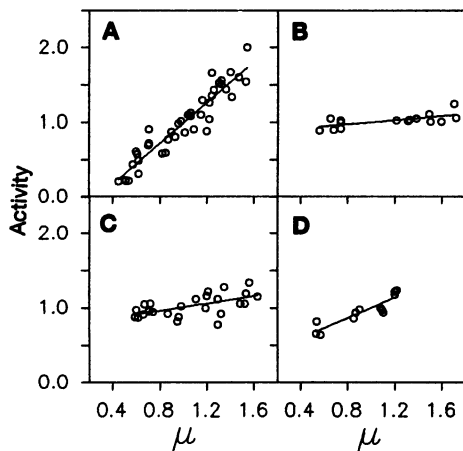
FIG. 6. GRDC of *rrnB* P1 mutants in MG1655. Normalized β-galactosidase activities of single-copy promoter–*lacZ* fusions are plotted against μ (doubling time in hours). (*A*) WT (−88, +1). The slope of WT *rrnB* P1 depends only on sequence between positions −41 and +1 (9, 16, 23). (*B*) [C−1T,C−15G]. (*C*) [CGC−5 → −7ATA]. (*D*) [T−33A,A ins−22].

between the −10 and −35 hexamers [T ins−23], and a single base-pair substitution creating an Eσ⁷⁰ −35 consensus hexamer [T−33A]. All were stringent (Fig. 5). Two other growth-rate-independent promoters, the S10 ribosomal protein operon promoter pS10 and *rrnB* P2, also displayed stringent control (Fig. 5). Thus, the promoter determinants for response to the two regulation systems are not the same.

Stringent control assays were performed in MG1655, a different strain than was used in some of our earlier studies on GRDC. Therefore, we repeated certain GRDC assays in MG1655. Fig. 6 shows representative results. In agreement with published studies (9, 16, 23), WT *rrnB* P1 is growth-rate-dependent (Fig. 6*A*), while the double substitution [C−1T,C−15G] is growth-rate-independent (Fig. 6*B*). Mutant promoters [C−1T] and [T−33A] and pS10 and *rrnB* P2 were also growth-rate-independent (Fig. 3).

Mutations Altering both Stringent Control and GRDC. We measured stringent control of several additional mutant *rrnB* P1 promoters that had not been assayed previously for GRDC. One
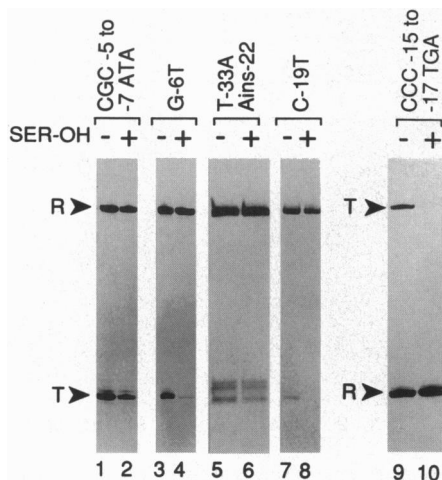

FIG. 7. Two *rrnB* P1 mutant promoters defective in stringent control. Ser-OH, serine hydroxamate; T, test product; R, reference product. The test product at right derives from a promoter fragment with a +50 endpoint; the reference product derives from a promoter with a +1 endpoint. Lanes: 1 and 2, [CGC−5 → −7ATA]; 3 and 4, [G−6T]; 5 and 6, [T−33A,A ins−22]; 7 and 8, [C−19T]; 9 and 10, [CCC−15 → 17TGA].

of these, a 3-bp substitution in the discriminator region [CGC−5 → −7ATA], was constructed because of previous reports that this region is required for stringent control (4–6). This mutant was impaired for stringent control, retaining ≈60% activity after amino acid starvation, but a single base-pair change in this region [G−6T] had little or no effect on stringent control. A double substitution [T−33A,A ins−22] creating consensus −10 and −35 hexamers and 17-bp spacing was also defective for stringent control, remaining ≈50% active after starvation (Figs. 3 and 7, lanes 1–6). However, promoters with the individual mutations [T−33A], [A ins−22] (data not shown), and [T ins−23] (Figs. 3 and 5) were stringent. Two other spacer-region mutations that were tested, [C−19T] and [CCC−15 → −17TGA], retained stringent control (Fig. 7, lanes 7–10). Both of the stringent-control-defective promoters, [CGC−5 → −7ATA] and [T−33A,A ins−22], were also defective for GRDC (Figs. 3 and 6 *C* and *D*).

DISCUSSION

Control of rRNA Transcription. We have shown that stringent control of *rrnB* P1 requires only DNA sequences within the core promoter and that the promoter sequence determinants of stringent control and GRDC are not identical. Whereas some sequences are required for both control systems, others are important for GRDC but not stringent control (Fig. 3). Interpreting these results and previous observations that strains lacking ppGpp still display GRDC (9, 10) and that basal ppGpp levels do not always correlate inversely with transcription (26–28), we conclude that differences in promoter sequence requirements are most consistent with models where GRDC and stringent control work by different mechanisms.

In this interpretation, stringent control is mediated by ppGpp at the high concentrations found during amino acid starvation (3). However, we propose that the 10- to 100-fold lower basal ppGpp concentrations observed in steady-state growth (3) are either too low to affect RNAP during rRNA transcription or do not vary enough to account for differences in rRNA transcription over this range of growth rates. GRDC may be mediated by a feedback signal determined in some way by excess translational capacity (2, 17, 29), but the regulator's identity remains to be determined.

Other models making no mechanistic distinction between GRDC and stringent control perceive them as the same regulatory response displayed to different degrees. For example, ppGpp has been proposed to modify RNAP, partitioning it into two forms, an unmodified form transcribing rRNA promoters and a ppGpp-modified form preferring mRNA promoters (30). In this model, rRNA transcription shuts down after amino acid starvation because *relA*-produced ppGpp converts most of the cell's RNAP to the modified form, but during steady-state growth, *relA*-independent variations in ppGpp levels modify RNAP to different extents, causing the variations in rRNA transcription known as GRDC.

In passive or indirect models for GRDC (31), ppGpp is proposed to decrease the RNA chain elongation rate, sequestering RNAP in elongating transcription complexes and thereby reducing the free RNAP concentration. As with the partitioning model described above, promoters regulated indirectly by ppGpp should respond to both regulation systems, since no distinction in mechanism is made between stringent control and GRDC.

Both the partitioning and passive models could accommodate a class of stringently controlled but growth-rate-independent promoters if these promoters were insensitive to small changes in the concentration of unmodified RNAP induced by basal levels of ppGpp, yet could still respond to the high levels present during the stringent response. This theoretically might be achieved by a mutant promoter with in-

creased affinity for RNAP; such a mutation should increase promoter strength. However, some stringent but growth-rate-independent promoters are considerably weaker than WT *rrnB* P1 (Fig. 3). For example, the growth-rate-independent [C−1T] (−46, +1) mutant promoter is ≈30-fold weaker than the growth-rate-dependent WT *rrnB* P1 (−88, +1) promoter. Thus, the behavior of some of the stringent but growth-rate-independent promoters does not fit the pattern expected from such a class.

Promoter Sequences Required for Stringent Control. Although genetic evidence for ppGpp as the effector of the stringent response is compelling (1, 32), the mechanism by which it inhibits transcription is less clear. Several reports (refs. 4–6; M. Holmes, personal communication) suggest that the G+C-richness of the "discriminator" is important for stringent control. Our results agree with those observations: the 3-bp discriminator substitution [CGC−5 → −7ATA] dramatically reduced promoter response to amino acid starvation. The stringent promoters *rrnB* P2 and pS10 also have a discriminator-like sequence between the −10 region and the transcription start site (6, 33).

Mutations in different parts of the *rrnB* P1 promoter can disrupt stringent regulation (i.e., [T−33A,A ins−22] and the 3-bp discriminator substitution). Either or both of these mutations could represent "kinetic bypasses" that make the step affected (directly or indirectly) by ppGpp no longer rate limiting for transcription. Alternatively, one or both regions altered by these mutations could be part of a promoter motif that, when complexed with ppGpp-modified RNAP, is inactive for transcription. It will be interesting in this regard to determine the kinetic properties of stringent and nonstringent promoters.

It is unlikely that we have identified all promoter positions important for response to amino acid starvation, since we did not select for promoters defective in stringent control; rather, we chose most of the promoters in this study because of their known GRDC defects. This may be why we did not identify a promoter class competent for growth-rate regulation yet defective for stringent control.

Promoter Occlusion and *rrn* Regulation. Our results that *rrnB* P1 and pS10, but not a discriminator mutant, are stringent confirm previous reports (5, 6, 34). However, the finding that *rrnB* P2 is subject to stringent regulation conflicts with past observations (6, 21). Previous investigators measured P2 in its natural context downstream from P1 (21), whereas we worked with the P2 promoter alone. P2 may appear unregulated in the tandem context because of occlusion from the upstream P1 promoter (even though the isolated P2 promoter can respond to ppGpp), as proposed by Glaser and colleagues (35). In support of this hypothesis, P2 is a strong promoter when separated from P1 (Figs. 3 and 5, and unpublished data) but is weak in the presence of P1 (2).

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