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# Carbon starvation and growth rate-dependent regulation of the *Escherichia coli* ribosomal RNA promoters: Differential control of dual promoters

(RNA control/transcription/guanosine 3'-diphosphate 5'-diphosphate)

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**ABSTRACT** **We studied the effects of carbon starvation and of varying the growth rate on the activity of each of the two tandem ribosomal RNA promoters from the *rrnA* operon of *Escherichia coli*. The cellular abundance of plasmid-encoded transcripts arising at promoters P1 and P2 and terminating at the ribosomal RNA terminator in promoter–terminator fusions, together with transcript turnover rates, was used to estimate promoter activities. The rate of synthesis of the P1-promoted transcript was found to increase exponentially with growth rate and predominate at fast growth rates. The activity of the downstream promoter (P2) changed only slightly at different growth rates. Upon carbon starvation, little or no activity of the upstream promoter was detectable, while P2 activity persisted. We interpret this to mean that the dual promoters are differentially regulated so as to have separate adaptive and maintenance functions. This model simplifies most features of rRNA regulation known in *E. coli*.**

The expression of the seven ribosomal RNA operons of *Escherichia coli* is regulated by two separate mechanisms: stringent RNA control and growth-rate control (for reviews see refs. 1 and 2). Stringent RNA control is provoked by limiting aminoacyl tRNA availability for protein synthesis. This leads to binding of codon-specified uncharged tRNA to ribosome acceptor sites, which activates the *relA*$^+$ gene product to catalyze the synthesis of guanosine 3'-diphosphate 5'-diphosphate (ppGpp). The accumulation of ppGpp itself or some related regulatory signal is thought to inhibit rRNA operon (*rrn*) transcription. The expression of *rrn* operons is also controlled by cellular growth rates. Except for slow growth rates (slower than a doubling every 2 hr), the rate of rRNA synthesis and accumulation increases faster with increasing growth rate than does mRNA synthesis. Under these conditions, basal levels of ppGpp can be correlated inversely with growth rate (3, 4). During slow growth and during carbon source starvation, rRNA is overproduced despite elevated levels of ppGpp, and substantial degradation of rRNA occurs (5–8). Thus, regulation of rRNA transcription is not invariably inhibited by ppGpp.

*In vitro* studies have resulted in proposals of a variety of mechanisms by which *rrn* transcription regulation could occur (1, 2). *In vivo* studies have localized regulatory determinants with increasing precision. Fusions of *rrn* promoter regions with portions of the galactose operon have shown that the target for both stringent and growth rate control is within the promoter region (9, 10). Probably all of the *E. coli rrn* operons have dual promoters, although one remains that has not had its sequence determined. We have been able to measure the activity of each of the two *rrnA* promoters *in vivo* by using plasmids containing *rrn* promoter–terminator fusions (11, 12). With rapidly growing cells, the upstream P1 promoter was found to be about three times more active than the downstream P2 promoter. During the stringent RNA control response, P1 was about 90% inhibited in *relA*$^+$ but not in *relA* strains, indicating stringent control of P1 activity. In contrast, P2 was judged to be only moderately (50%) inhibited in *both relA*$^+$ and *relA* hosts and, therefore, was not under stringent control. Furthermore, stringent regulation of P1 activity persisted even when the P2 promoter and the downstream regions extending to the mature 16S RNA gene were deleted (11, 13).

Here we describe measurements of the relative activities of the *rrnA* P1 and P2 promoters as a function of growth rate variation and during glucose starvation. We have found that the P1 promoter is strongly dependent on growth rate and can be progressively inactivated as growth slows to the point where, as during glucose starvation, it is only marginally detectable. The downstream P2 promoter behaves very differently; its activity is only weakly dependent on growth rate and remains quite active during glucose starvation. We suggest that the downstream promoter behaves as a constitutive maintenance promoter whose activity is relatively insensitive to regulation by either the stringent or growth-rate control mechanisms. This behavior can account for the excessive synthesis of rRNA in very slowly growing cells and in glucose starved cells. The adaptive nature of the upstream promoter can account for the response of *rrn* operon expression at moderate to fast growth rates.

## MATERIALS AND METHODS

**Cells, Growth, and RNA Extraction.** The pPS1-bearing CF 747 (*relA*) strain was grown at 32°C in 3-(*N*-morpholino)propanesulfonic acid minimal medium supplemented with minimal amino acid requirements present at 20 μg/ml and uridine present at 10 μg/ml as described (11). To achieve different growth rates, this minimal medium was either unsupplemented or supplemented with Casamino acids to 0.1% and 0.4%. The most rapid growth was achieved in Luria broth containing 0.2% glucose. When cell densities reached an absorbance at 600 nm of 0.25–0.35 in rapidly shaken cultures, samples were taken for RNA extraction or rifampicin was added prior to sampling as before (11). Carbon exhaustion was achieved by growing the cells in 0.02% glucose; after growth had stopped and samples had been taken (as in Fig. 5), glucose was resupplemented at 0.2%.

**Plasmid Copy Number Determination.** Cells were grown in the various media in the presence of 20 μCi (1 Ci = 37 GBq) of $H_3{}^{32}PO_4$ acid and 3 mM potassium phosphate. They were

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Abbreviation: ppGpp, guanosine 3'-diphosphate 5'-diphosphate.

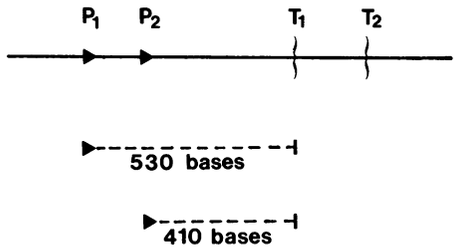


FIG. 1. Transcripts encoded by *rrn* promoter–terminator fusions. The construction of plasmid pPS1, including the sequence of the fusion region has been described (11). The P1- and P2-promoted transcripts arise from tandem promoters as shown and terminate *in vivo* at the first ribosomal terminator encountered, giving transcript lengths as shown.

harvested and lysed by the Clewell method (14). The sarkosyl lysates were electrophoresed for 15–20 hr in 0.7% agarose at 3 V/cm. After ethidium bromide staining, the bands corresponding to plasmid (pPS1) and chromosomal DNA were localized and excised, and radioactivity was measured.

## RESULTS

***In Vivo* Abundance of P1 and P2 Transcript at Different Growth Rates.** We have used previously the pPS1 plasmid that contains a fusion of the *rrnA* P1 and P2 promoter region to the *rrnB* terminator region (11, 12). *In vivo* this fusion gives rise to a 530-base-long P1–T1 transcript and a 410-base-long P2–T1 transcript as shown in Fig. 1. Terminations at the T2 site are observed *in vitro* in minor amounts (15) but are not seen *in vivo* (11). Fig. 2 shows an ethidium bromide-stained electropherogram of RNA extracted from cells growing at rates varying from 0.7 to 1.5 doublings per hr. We previously determined that RNA chains marked P1 and P2 on Fig. 2 do not comigrate with other RNA species and that their steady-state abundance can be measured by densitometry relative to the 5S RNA content of the sample (11). Even without quantitation, it is evident from Fig. 2 that the P1–T1/P2–T1 abundance ratio changed as a function of growth rate. As growth rates increased, the steady-state abundance of the P1–T1 transcript became an increasingly dominant portion of the total activity of the plasmid *rrnA* promoter region.

**Parameters Varying with Growth Rate That May Affect Measurement of P1 and P2 Activities.** We cannot relate steady-state transcript abundance to promoter activity unless the rates

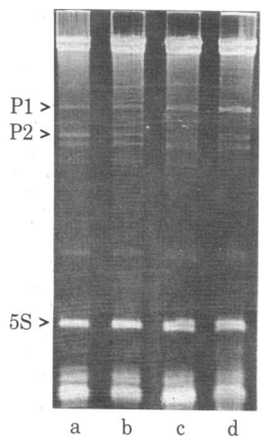


FIG. 2. P1–T1 and P2–T1 transcript abundance at different growth rates. RNA was extracted from cells growing exponentially and electrophoresed on 7 M urea/5% acrylamide gels as described (11). Lanes: a–d, ethidium bromide-stained samples from cells having growth rates of 0.7, 1.0, 1.2, and 1.5 doublings per hr, respectively. The positions of the P1- and P2-specified transcripts and cellular 5S RNA are indicated.

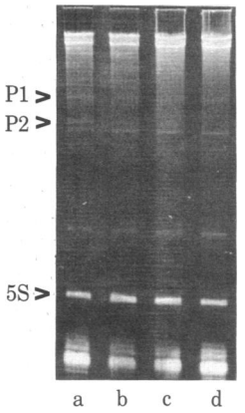


FIG. 3. P1–T1 and P2–T1 transcript stability after rifampicin addition. Plasmid-bearing cells were grown in minimal medium, and rifampicin was added to the exponential culture. Lanes: a–d, 0, 3, 6, and 9 min after rifampicin addition. The electrophoresis conditions are as for Fig. 2.

of decay are known. Addition of rifampicin, an inhibitor of RNA polymerase initiation, has been found with fast-growing cells to result in exponential decay of P1–T1 and P2–T1 transcripts with half-lives of about 2–3 min. The metabolic lability of both transcripts has been halved by either amino acid starvation or by chloramphenicol addition (11). An analogous rifampicin addition experiment (Fig. 3), but with cells growing at 0.7 doublings per hr, showed exponentially growing cells (lane a) and transcripts present 3, 6, and 9 min (lanes b–d, respectively) after rifampicin addition at 0.10 mg/ml. Both P1- and P2-specified transcripts had markedly reduced intensities after even 3 min of rifampicin exposure (lanes a and b). This suggests that this slow growth rate does not change transcript lability as compared to rapidly growing cells. This inference was borne out when transcript abundance was normalized to 5S RNA abundance in this experiment and with similar trials with cells grown at intermediate growth rates (data not shown).

Plasmid copy number variation with growth rate is a possibility suggested by the observations of Steuber and Bujard (16). Using cells uniformly labeled with $^{32}P$, we found that the ratio of radioactivity recovered in pPS1 plasmid/chromosomal DNA indeed decreased with increasing growth rate (Table 1). However, the amount of chromosomal DNA per cell is known to increase with increasing growth rate (see ref. 17). When the DNA content per cell was calculated by extrapolation (17) to the growth rates used here and taken into account, the plasmid copy number per cell did not change appreciably over this range of growth rate variation (Table 1).

Table 1. pPS1 plasmid copy number at different growth rates

| Growth rate, doublings/hr | DNA ratio: cpm plasmid/ cpm chromosome | Genomes per cell | Plasmid copy no. per cell |
|---|---|---|---|
| 0.7 | 0.185 | 1.6 | 197 |
| 1.0 | 0.165 | 1.9 | 209 |
| 1.2 | 0.132 | 2.2 | 193 |
| 1.5 | 0.106 | 2.5 | 177 |

At growth rates shown, the ratio of plasmid DNA to chromosomal DNA was determined as described. The number of chromosomal genome equivalents of DNA per cell were calculated from the data of Kjeldgaard and Gausing (17) by extrapolation to the observed growth rates. The plasmid copy number was obtained as the product of column 2 and column 3 multiplied by the ratio of molecular weights of *E. coli* DNA to pPS1 DNA.

**The Differential Response of the Dual Promoters to Changing Growth Rate.** Densitometry of stained gels followed by normalization of the P1–T1 and P2–T1 transcript abundance to the 5S RNA content of each sample (Fig. 2) allowed quantitative estimates of the differential response of the two promoters. We thought this sort of quantitation was appropriate because host 5S RNA is an abundant species that is metabolically stable except under unusual conditions (18). The pPS1 fusion plasmid does not encode a complete 5S RNA gene and does not contribute to the cellular pool of 5S RNA (11). However, for measurements of growth rate control, we took into account that cellular 5S RNA itself is under growth rate control because 5S RNA genes are present on *rrn* operons and are cotranscribed with rRNA genes (19). Accordingly, we corrected P1–T1 and P2–T1 transcript abundance to the known growth-rate dependence of rRNA gene expression by using the data shown by Churchward *et al.* (20) in their figure 2 for a prototrophic strain. The activities of the dual promoters are shown together and individually in Fig. 4 as a function of growth rate. The behavior of the two promoters was contrasting. P1 activity changed in a manner characteristic of growth-rate control of total rRNA, whereas P2 activity was relatively unresponsive to growth-rate changes. The sum of P1 and P2 activities was also characteristic of classical growth-rate control at these moderate to fast growth rates because P1 predominated as growth rate increased.

**Glucose Starvation Effects on Dual Promoter Activity.** As already mentioned, at slow growth or with no growth at all, as during energy source exhaustion, overproduction of *rrn* transcripts occurs despite high levels of ppGpp (5–8). This feature of stable RNA regulation previously has eliminated simple models of ppGpp as a negative regulator. Glucose starvation was chosen as a simple means of provoking this response. Exhaustion of glucose resulted in the virtual disappearance of the P1–T1 transcript, whereas the P2–T1 transcript persisted, although at levels that were somewhat reduced compared to levels just prior to growth limitation (Fig. 5). It should be noted that ppGpp accumulated as expected under these conditions (data not shown). Resupplementation of depleted cells with glucose resulted in resumption of growth and the reappearance of the P1–T1 transcript activity (Fig. 5, lanes 4–6).

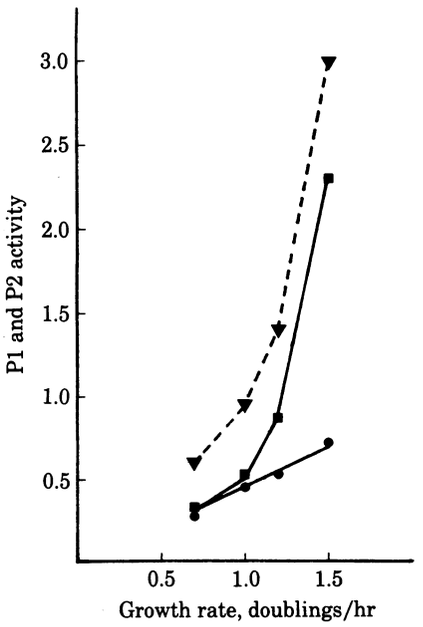


FIG. 4. P1 and P2 promoter activities as a function of growth rate. The abundance of the P1- and P2-promoted transcripts is normalized to the 5S RNA, plasmid copy number, and the growth-rate dependence of 5S RNA. Squares correspond to P1, circles to P2, and triangles to the sum of the P1 and P2 activities.

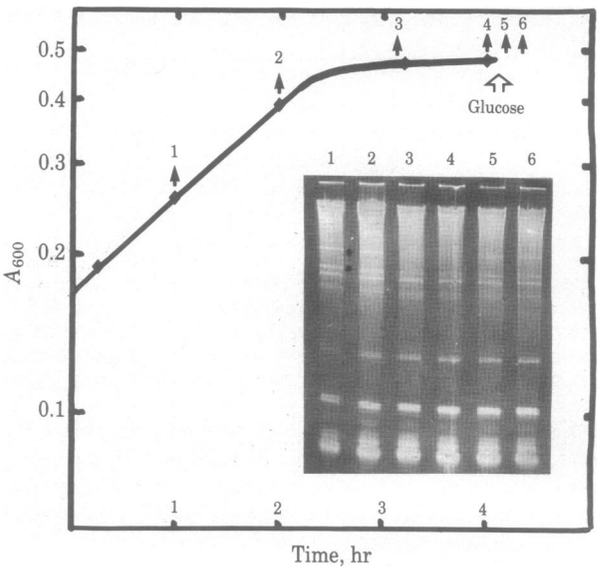


FIG. 5. Glucose exhaustion alters the abundance of P1 and P2 transcripts. Plasmid-bearing cells were inoculated in minimal medium containing 0.02% glucose, and they grew for four generations before growth limitation occurred. As indicated, glucose was added back to the culture at 0.2%. RNA samples were prepared at the times indicated by arrows and electrophoresed as in Fig. 2. The positions of the P1- and P2-specified transcripts are indicated with dots between gel lanes 1 and 2.

## DISCUSSION

Although the presence of two tandem promoters on *rrn* operons has been known for some time (21–25), until recently little experimental attention has been given to the possibility that their differential regulation might account for growth rate control and stringent RNA control. The experiments described here, as well as our previous work with *rrnA* promoter fusions *in vivo*, suggest that the dual promoters are differentially regulated over a wide range of environmental conditions (11, 12). The upstream promoter seems largely responsible for high levels of rRNA expression in rapidly growing cells. However, it also can be inhibited severely in amino-acid-starved, carbon-source-starved, or slowly growing cells as well as in cells recovering from stationary phase. The downstream promoter behaves as a relatively less-active, constitutive promoter that might provide maintenance rRNA synthesis, which we presume might be necessary for survival under adverse conditions (15). So far, we have found the P2 promoter inactive under only one physiological condition, stationary phase; in this instance, however, we cannot rule out the possibility of high rates of transcript decay (12). Maintenance and adaptive functions ascribed to separate tandem promoters have been found for the galactose operon (26).

The precise identification of the regulators involved in stable RNA control is uncertain (see refs. 1 and 2). Yet, we are tempted to speculate that it is the ppGpp independence of the P2 promoter, noted during the stringent response, that leaves P2 active even in slowly growing cells, where the ppGpp concentration is high (3). Although all exceptional RNA control conditions have yet to be examined with respect to differential promoter regulation, such as heat shock (27, 28), it is possible that ppGpp could be both a necessary and sufficient, negative

regulator of P1 activity (4). That would be consistent with our findings in a highly purified *in vitro* transcription system with supercoiled templates (15). Furthermore, studies of cellular stable RNA gene activity as a function of ppGpp concentration over a variety of conditions and in both relaxed and stringent strains show a sizeable (30%) fraction that is ppGpp resistant (4). This is intriguing because this residual fraction corresponds in rapidly growing cells to the contribution of P2 activity to the total activity, although other explanations are possible (4).

The *rrnA* promoter region used in these experiments is not identical to all *rrn* promoter regions and, therefore, might not be representative. However, the *rrnA* promoter region shows extensive homology with both *rrnB* and *rrnG* downstream of the −70 and −40 region of P1, respectively (22–25). Although *rrnA* behavior is likely to be similar for at least three of the seven operons, the possibility of operon-specific modulations remains to be rigorously explored. The differential regulation of the two *rrnA* promoters observed so far is sufficient to account for most known regulatory features of rRNA expression in a simplified manner.

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