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Control of rRNA Transcription in *Escherichia coli*

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INTRODUCTION

In nature, *Escherichia coli* cells experience constantly changing environments. In their host, they are exposed to a variety of nutritional conditions, while in their water environment, they encounter extreme starvation. It is in this context of continual environmental change that microbes have evolved rRNA control mechanisms that are highly integrated with the growth requirements of the cell. Most of this regulation occurs at the level of transcription initiation, and in general, synthesis of *E. coli* rRNA is maintained in proportion to the cell's requirement for protein synthesis. Should the organism find itself in a richer nutritional environment, it possesses mechanisms for rapidly increasing its rRNA synthesis to take advantage of this new opportunity to elevate its rates of protein synthesis and growth. Likewise, if the nutrient supply becomes limiting, elaborate strategies exist that quickly curtail wasteful rRNA synthesis. At least four different aspects of rRNA expression have been examined that bear on these changes in rRNA synthetic capacity. These are the well-known stringent response, a phenomenon known as growth rate-dependent regulation, and two other mechanisms, upstream activation and antitermination. While the first two topics clearly involve the modulation of expression, the second two, upstream activation and antitermination, are thought to function in a more passive fashion but might have the potential to regulate in specific circumstances. This review will examine the background and recent advances in the study of these four control mechanisms.

E. coli rRNA is synthesized from seven noncontiguous operons (*rrnA-E* and *rrnG-H*), which are asymmetrically distributed about the origin of replication (*oriC*) on one half of the circular chromosome (72) (Fig. 1). Three species of rRNA are made, and the order of their genes within *rrn* operons is promoter–16S RNA–23S RNA–5S RNA. Several different tRNA genes are found in the spacer region between the 16S and 23S RNAs and at the distal ends of some of the *rrn* operons (Fig. 2). Consequently, the gross anatomies of the seven operons are not strictly identical. From experiments with total cellular RNA extracts (i.e., the product of all seven operons), it was concluded that the rRNA genes are cotranscribed from large precursor molecules, which are cleaved into their component parts by specific RNases (5, 6, 68). The organization of the *16S*, *23S*, and *5S* genes into polygenic operons ensures the produc-

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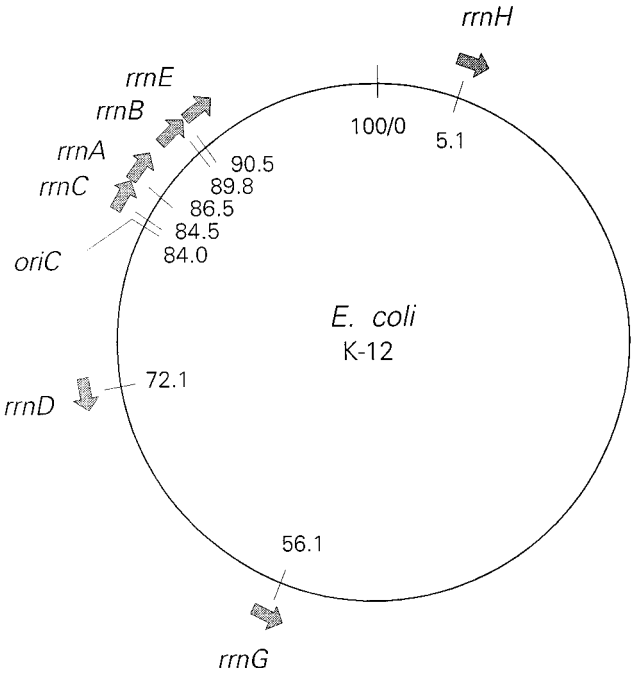

FIG. 1. Location in minutes of the seven *rrn* operons on the chromosome of *E. coli* K-12. Shaded arrows indicate the direction of transcription. The origin of replication, *oriC*, is shown at 84.0 min.

tion of equimolar amounts of the three rRNA species. While it is the general rule, this grouping of rRNA genes is not universal, and several examples in which not all rRNA genes are linked have been noted in the *Bacteria* (96, 119, 212, 291) and in the *Archaea* (265, 334).

Sequence comparison suggests that some of the seven *E. coli rrn* operons could be functionally distinct. Information from extensive hybridization experiments also suggests both large and small differences. The *rrnB*, *-C*, *-E*, and *-G* operons all have a gene for $tRNA^{Glu-2}$ in their spacer region, while *rrnA*, *-D*, and *-H* have genes for $tRNA^{Ile-1}$ and $tRNA^{Ala-1B}$ (211) (Fig. 2). Also, the *rrnB* and *rrnG* operon spacers contain a 106-nucleotide sequence of potential secondary structure called the ribosomal spacer loop. The ribosomal spacer loop is absent from the spacer regions of the *rrnC*, *rrnE*, and *rrnB* operons of some descendants of the original K-12 strain (*rrnB1*), in which the ribosomal spacer loop is replaced by a shorter sequence (20 nucleotides) (120). Some *rrn* operons also differ in the genes at their distal ends. The *rrnC* operon has genes for $tRNA^{Asp}$ and $tRNA^{Trp}$ (the sole gene for this tRNA in *E. coli*). The *rrnD* operon has a distal $tRNA^{Thr-1}$ gene, and the *rrnH* operon has one for $tRNA^{Asp-1}$ (210). The *rrnD* operon is also unique in possessing two distal 5S RNA sequences (67). In addition to the above-mentioned heterogeneities, the individual *rrn* operons contain many small sequence differences, which occur in regions specifying the structural RNAs (e.g., reference 138), promoters, spacers, and terminators (120, 296). Whether any of these heterogeneities cause differences in the regulation or function of particular rRNAs has yet to be established.

Multiple *rrn* operons are found in the genomes of many organisms, and although the advantages conferred on *E. coli* by *rrn* operon redundancy are still only partially understood, the phenomenon is clearly important for the survival of *E. coli* and many other bacterial species. Experiments in which multiple *E. coli rrn* operons have been inactivated show that nearly optimal growth rates are obtained on complex medium with only five intact *rrn* operons (49). However, all seven *rrn* operons are necessary for rapid adaptation to certain nutrient and temperature changes (50). We have concluded from these experiments that a major function of multiple *rrn* operons is to allow *E. coli* to commence synthesis of ribosomes faster upon encountering more favorable growth conditions (50). Other possible roles of *rrn* operon multiplicity might be that ribosomes derived from specific *rrn* operons are required to translate certain mRNAs or that specific *rrn* operons are expressed under special physiological conditions. These speculations seem plausible considering the operon heterogeneities mentioned above. Although no evidence of such roles has yet been demonstrated in *E. coli* (51), growth stage-specific ribosomes have been demonstrated in the malaria-causing *Plasmodium berghei* parasite. *P. berghei* growing in its mosquito host makes *18S* rRNA predominantly from one gene (type C) but transcribes *18S* from a structurally distinct gene (type A) in the mammalian host (351, 366). The purpose of this interesting adaptation is unknown but could be necessitated by different temperatures encountered in the parasite's two hosts.

The *E. coli rrn* operons are transcribed to an exceptional degree, accounting for more than half of the cell's total RNA synthesis under rapid growth conditions. The promoter regions of all seven operons have been sequenced, and they all have the same general structure (Fig. 3). Each operon has tandem σ^{70} promoters, *P1* and *P2*, separated by about 100 bp. The *P2* promoter in turn is separated by a 200-bp leader region from the beginning of the mature 16S rRNA. None of the core promoter sequences (defined as -41 to $+1$ with respect to the transcription initiation site [14]) has a perfect consensus σ^{70} promoter sequence in terms of either its -35 and -10 sequences or its spacing between these two regions. In general, these deviations from the consensus tend to reduce the strength of the *rrn* core promoters, but some are responsible for increased control over *rrn* synthesis (14, 66). Ross and coworkers have subsequently shown that the exceptional

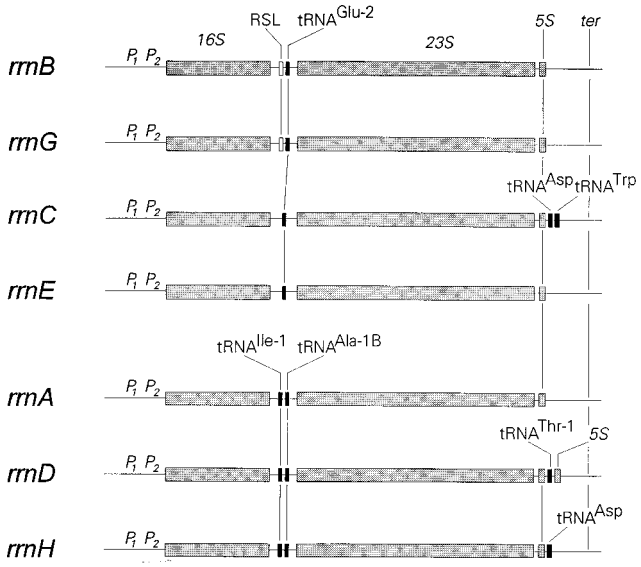

FIG. 2. Structure of the seven *E. coli rrn* operons. *P1* and *P2* are the tandem operon promoters. Shaded boxes represent the *16S*, *23S*, and *5S* genes. Spacer and distal tRNAs are indicated as solid boxes, and the small open boxes in the 16S-23S spacer regions of *rrnB* and *rrnG* indicate the ribosomal spacer loop (RSL) (120). The complex terminator region (*ter*) at the end of each operon consists of a rho-independent terminator(s) followed by a rho-dependent terminator (3, 304).

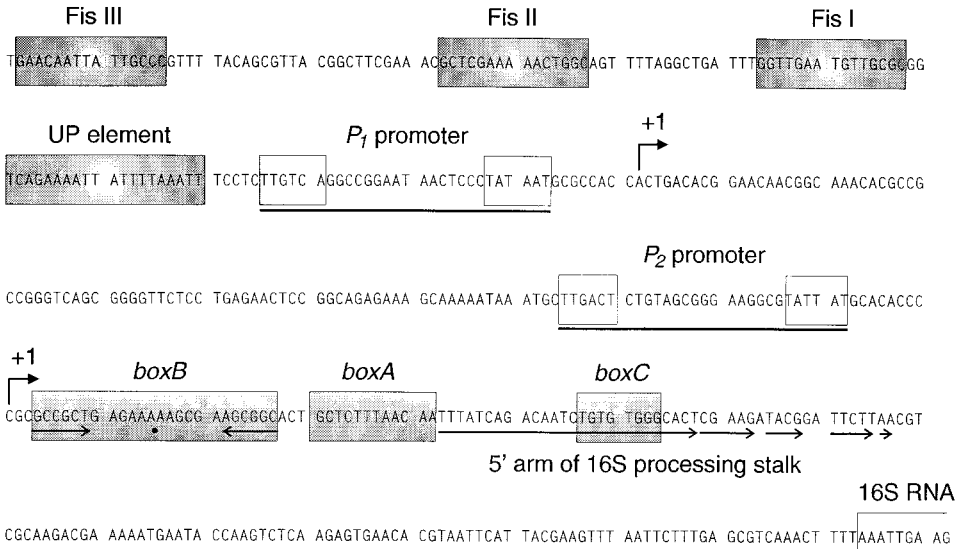

FIG. 3. Sequence and general features of the *rrnB* promoter region. Open boxes are used to represent the −10 and −35 regions of the *P1* and *P2* promoters and the beginning of the 16S RNA structural sequence. The three Fis binding sites and the UP element are indicated by radially shaded boxes. A Fis binding consensus sequence (G/T-YR-A/T-YR-C/A) has been defined (137) and modified [GNt(c/t)(A/g)a(a/t)(T/A)(g/a)t(T/c)(g/a)aNC] (81). The *boxB-boxA-boxC* elements of the *rrn* antitermination system are indicated by vertically shaded boxes. The arrows under *boxB* represent the hyphenated symmetry of the proposed stem-loop secondary structure, while the right-pointing arrows extending from *boxA* indicate the extent of the "left arm" of the 16S processing stalk.

strength of the *rrn P1* promoters is caused by sequences outside the core promoter region, primarily in their upstream flanking sequences (274, 275). This activation of the *rrn* promoter regions by their flanking sequences will be discussed under "Upstream Activation." In vitro, *P1* and *P2* promoters from *rrnA* and *rrnE* (100), *rrnB* (102), and *rrnD* (359) show roughly equal activity. However, in vivo, expression from *P1* is generally greater because of its ability to be transactivated, and *P2* in its native context is thought to be a relatively low-level, unregulated promoter (281, 282). Interestingly, when detached from *P1*, *P2* shows many of the regulatory characteristics of the *P1* promoter, including an increased promoter strength (to 70% of that of *P1*) and sensitivity to the stringent response (89, 150).

In addition to the well-studied *P1* and *P2* promoters, other promoters have been identified upstream of some of the operons which may also have an influence on *rrn* expression. Two extra promoters, *P3* and *P4*, were noted by Boros et al. (27) about 1 kb upstream of the *rrnB* operon, and RNA polymerase (RNAP) molecules originating from these promoters are thought to be capable of transcribing into *rrnB*. mRNAs from *P3* and *P4* encode a 289-amino-acid protein involved in vitamin B_{12} metabolism (9), but the significance of transcription into *rrnB* is unknown. Upstream of the *rrnG* operon is the gene for the heat shock protein ClpB (164, 306), and a significant amount of transcription from its promoter is thought to continue through a rather weak terminator into *rrnG* (304). However, the in vivo significance of this readthrough is also not known. Finally, sequences highly homologous to consensus binding sites for the heat shock sigma factor (σ^{32}) are interdigitated with the sigma-70 binding sites of each of the *rrn P1* promoters. Gourse's group has shown that the heat shock RNAP Eσ^{32} can bind to these sequences in *rrnB* and initiate transcription in vitro (222). The role of these promoters, presumably, is to maintain *rrn* expression during the stress response, although this has not yet been directly demonstrated.

Most of the regulation of *E. coli* rRNA expression occurs at the level of transcription initiation, although recent evidence suggests that the maintenance of a high elongation rate is also important for the completion of full-length transcripts (see "Growth Rate-Dependent Regulation" and "Antitermination" below). Of the two major promoters, *P1* is the more highly regulated in its native context. This promoter is controlled in relation to amino acid availability (the stringent response) and in relation to growth rate. Also, upstream of *P1* are several binding sites for the transcription activator Fis and an AT-rich sequence, the UP element, which constitutes an extension of the promoter and has intrinsic activation capability through contacts with the α-subunit of RNAP (263, 274). Finally, transcription from both *P1* and *P2* passes through an antitermination sequence similar to the N-mediated antitermination sites in bacteriophage lambda (183). In this review, we will describe what is known about each of these regulatory mechanisms as they pertain to *E. coli* rRNA transcription and, where possible, draw parallels to what is known about other prokaryotic and eukaryotic systems.

STRINGENT CONTROL

The stringent response is the term used to describe the elaborate set of adjustments that the cell makes in response to starvation for amino acids (for a comprehensive review, see reference 40). Historically, two hallmarks of the response have been noted: a rapid shutdown of stable RNA (rRNA and tRNA) synthesis (26, 280, 309) and a correspondent accumulation of two unusual nucleotides, originally termed magic spots I and II and subsequently identified as guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (37, 38). [Together, pppGpp and ppGpp are hereafter designated (p)ppGpp.] Recently another important characteristic of the stringent response has been observed: the elongation rate of RNAP molecules transcribing mRNA decreases (340, 343). When cells are suddenly deprived of an essential amino acid, the normally low levels of (p)ppGpp present in rapidly growing cells begin to increase a few seconds after starvation commences, reach a maximum between 5 and 15 min later, and then decrease to a new steady-state level 10- to 20-fold higher than the original basal value (37, 78, 190). The synthesis of

(p)ppGpp during amino acid starvation is catalyzed by the RelA protein (also known as the stringent factor) in response to a decrease in the ratio of aminoacylated (charged) to nonaminoacylated (uncharged) tRNA in the cell (273). The binding of an uncharged tRNA to the acceptor site on the ribosome triggers the synthesis of (p)ppGpp, which in turn inhibits transcription of stable RNA operons in an as yet poorly understood fashion.

RelA—(p)ppGpp Synthetase I

The *relA* gene is located at 60 min on the *E. coli* chromosome and encodes the 77-kDa (p)ppGpp synthetase I enzyme (44, 123, 252, 253). *relA* mutants have the so-called relaxed response and continue to synthesize rRNA and tRNA during amino acid starvation. The RelA protein is found associated with ribosomes in cell extracts, although the fraction of ribosomes that bind RelA is unclear; estimates of 1 molecule of RelA per 200 ribosomes (253) and 1 molecule of RelA for every ribosome (285) have been reported. However, more recent experiments suggest that normally only a few percent of the ribosomes have a bound RelA (153). The purified RelA enzyme cannot synthesize (p)ppGpp in the absence of ribosomes but can be activated by methanol, detergents, or specific proteins, suggesting that the activation signal in vivo is probably a ribosome-induced conformational change (43, 253, 318). *relC* mutations, which alter the gene for the large ribosomal subunit protein L11 (*rplK*), cause a relaxed phenotype both in vivo and in vitro, suggesting that wild-type L11 may relay the signal to RelA that uncharged tRNAs are present (84, 246, 262). This is not, however, the only way that *relC* might act, and several other possible modes of action can be considered: *relC* mutations might alter binding of uncharged tRNA or recognition of the 3′ end of the uncharged tRNA. Interestingly, the *relC* mutation does not alter binding of RelA to ribosomes (84).

When bound to wild-type ribosomes, RelA synthesizes (p)p pGpp in vitro upon addition of mRNA, a codon-specific uncharged tRNA, ATP, and GTP (122, 254). The synthesis reaction is the transfer of pyrophosphate from ATP to GTP or GDP to yield pppGpp or ppGpp, respectively (44, 185, 314, 317). When there are insufficient amounts of a particular charged tRNA in the cell to satisfy the requirement for protein synthesis, transient stalling of the ribosome at the "hungry" codons occurs. The uncharged tRNA binds directly to the A site without elongation factor EF-Tu, and (p)ppGpp synthesis occurs without any movement of the ribosome at the elongation step (122, 123). This process was first noted more than 25 years ago by Cashel and Gallant, who referred to it as ribosome idling (38). It is thought that the idling ribosome undergoes cycles of tRNA binding, release, and (p)ppGpp synthesis until such time as the aminoacylated tRNA becomes available again (269, 270). On the basis of the kinetics of the reaction and the relative sizes of the intracellular GTP and GDP pools, pppGpp is thought to be the favored product in vivo (44, 253). Guanosine pentaphosphate can then be converted to ppGpp by the product of the *gpp* gene, pppGpp 5′-phosphohydrolase, an enzyme which is involved primarily in polyphosphate metabolism (301).

Interestingly, overexpression of the *relA* gene, even in cells that are not starved for amino acids, causes very slow growth rates, presumably due to increased synthesis of (p)ppGpp and inhibition of stable RNA expression (46, 252, 289, 322). This is consistent with the idling ribosome model, in which the more severe is the amino acid starvation, the more ppGpp is produced, and the greater is the observed inhibition of rRNA and tRNA synthesis. The overproduction of RelA achieved is about 100-fold, enough to increase the number of ribosomes bound to RelA from a few percent to 100% (289). Therefore, the increased (p)ppGpp production is likely the result of an increased fraction of RelA-bound ribosomes encountering random hungry codons. The inhibition of growth by RelA overproduction does not occur in *relC* mutants, presumably because signals cannot be conveyed from the ribosome to stimulate ppGpp production (289). Interestingly, the amino-terminal half of RelA appears to be a constitutively active protein, and its overexpression allows ppGpp production and growth inhibition independently of RelC.

SpoT—(p)ppGpp Synthetase II and (p)ppGpp 3′-Pyrophosphohydrolase

relA null mutants still accumulate (p)ppGpp, for example, during glucose starvation, indicating the presence of at least one other pathway for the synthesis of (p)ppGpp (118, 178, 354). [The (p)ppGpp accumulation and inhibition of rRNA synthesis during carbon source starvation are, however, not as rapid as during the stringent response (205).] This second activity, called (p)ppGpp synthetase II (PSII) (77, 270), is abolished in *spoT* null mutants (131, 355). The *spoT* locus (82 min) encodes an 80-kDa protein first identified as the major enzyme for (p)ppGpp degradation (4, 124–126, 316). Thus, the SpoT protein is thought to act as a bifunctional enzyme catalyzing either (p)ppGpp synthesis or hydrolysis. A finite possibility remains, however, that SpoT and PSII are distinct proteins and that PSII requires SpoT for either its activity or its expression. Although SpoT was originally thought to be associated with the ribosome (126), it can hydrolyze (p)ppGpp when purified from ribosomes (126, 316), and it has recently been shown that SpoT is found free in the cytoplasm (77, 98). The hydrolase reaction is dependent on Mn^{2+} ions and catalyzes the removal of pyrophosphate from ppGpp or pppGpp (124–126). A preferential inhibition of the degradation but not the synthesis reaction by metal chelators indicates that the metal cofactor requirements for the two reactions are probably different. It also appears that each reaction is inhibitory of the other. Thus, the degradation of (p)ppGpp by the *spoT* gene product is not thought to be the simple reversal of an equilibrium synthesis reaction but rather to occur at an alternative site on the enzyme, although this has yet to be proven (131, 355). It has been shown that the RelA and the SpoT proteins have significant homology throughout their lengths (199), which may reflect their common (p)ppGpp synthetase capacity (355). However, the signal that regulates this activity in the two proteins is thought to be different: RelA-mediated (p)ppGpp synthesis is largely in response to amino acid starvation, while SpoT-mediated accumulation of (p)ppGpp is actually inhibited by amino acid starvation (172, 173) and instead appears to respond to fluctuations in the intracellular energy source pools (85, 92). In *relA spoT* double mutants ($ppGpp^0$ strains), no (p)ppGpp is made, proving that only these two proteins are involved in (p)ppGpp synthesis (131, 355). Steady-state levels of (p)ppGpp are achieved by an equilibrium of synthesis and degradation rates, and although both RelA and PSII/SpoT proteins probably contribute to basal levels of (p)ppGpp synthesis during balanced growth (40, 244), the contribution by PSII/SpoT is thought to be much more significant (77, 130). Since the half-life of ppGpp is approximately 20 s either during the stringent response or during steady-state growth, most of the control is thought to be at the level of ppGpp synthesis (190). The effects of ppGpp under balanced growth conditions

will be discussed under "Growth Rate-Dependent Regulation."

Mechanism of ppGpp Action

The first observed effect of the stringent response was the inhibition of stable RNA synthesis, which was subsequently attributed to the accumulation of ppGpp. However, attempts to measure the effect of ppGpp on *rrn* transcription in vitro have not always yielded consistent results. Some reports have suggested that ppGpp causes inhibition of RNAP binding or initiation at the *rrn P1* and *P2* promoters (100, 116, 162, 239, 312, 332, 335, 336), whereas others have concluded that ppGpp has no significant effect on either promoter (102, 121, 327). When inhibitory effects on transcription initiation have been observed, they have been interpreted as ppGpp-induced conformational changes in RNAP (60, 333), and an RNAP partitioning model has been proposed on this basis (278, 333). This model suggests that two forms of RNAP exist: a ppGpp-bound form and a free (unbound) form, where the ppGpp-bound form cannot initiate transcription at stringently regulated promoters. Intermediate factors had been proposed to help the ppGpp-RNAP interaction, since ppGpp appeared to cause inhibition of rRNA synthesis more consistently in crude rather than purified systems (266, 358). However, more recent data have suggested that ppGpp can bind directly to RNAP (264, 322). Genetic evidence has suggested that the RNAP β- (39, 186, 187, 322), β′- (39), and σ-subunits (29, 39, 133) are important for this interaction, with the β-subunit turning up consistently in most screens. Mutations in the other RNAP subunits may reflect their roles as transducers of the signal. Although ppGpp can apparently bind free RNAP in the absence of template DNA (264), there have also been reports suggesting that RNAP undergoes a conformational change upon binding stringent promoters which makes it susceptible to ppGpp interaction (157, 202).

It had also been suggested, on the basis of in vitro data, that the inhibition of rRNA transcription occurs at the level of transcription elongation; ppGpp was proposed to induce RNAP pausing in the region of the *rrn P2* promoter and at a site in the *rrn* leader called t_L, also known as the turnstile attenuator (161, 163). However, recent studies have suggested that while ppGpp does inhibit the transcription elongation rate of mRNAs (343), the rRNA elongation rate, by virtue of the presence of the antitermination system (see below), is ppGpp independent in vivo (339, 340).

Using *rrn* promoter fusions in vivo, several groups have demonstrated differential stringent control of the *rrn P1* and *P2* promoters (107, 282, 361). It was found that *P1* activity was strongly inhibited during the stringent response but that *P2*, the weaker, more constitutive promoter in rapidly growing cells, was relatively insensitive to amino acid starvation in its native context. When uncoupled from *P1*, the *P2* promoter becomes stronger and is subject to stringent control, suggesting that it may be partially occluded in the tandem context (89, 150). When the *P2* promoter and t_L regions are deleted, stringent control of *P1* is unaffected (107). This cast the initial doubt on the significance of the in vitro data which had suggested a regulatory role for the t_L structure. The absence of the antitermination factors from the t_L in vitro system probably explains the conflicting results (107, 161, 163), although the use of linear as opposed to supercoiled templates and the absence of competing promoter templates have also been suggested as possible explanations (40).

In an effort to mimic the in vivo situation and to test the plausibility of the RNAP partitioning model mentioned above, the question of competing templates was addressed in vitro by Kajitani and Ishihama (155–157). In a mixed-template assay, they showed differential response to ppGpp of the *rrnE P1* and *P2* promoters. In this system, ppGpp was proposed to inhibit transcription initiation by increasing the rate of decay of open promoter complexes. Glass et al. (104) also studied the properties of two β-subunit mutants of RNAP described by Nene and Glass (217–219) which exhibited relaxed control in vivo (217–219). Although these enzymes were thought to be ppGpp resistant in vitro and the magnitude of the resistance to ppGpp appeared to reflect the degree of the stringent response deficiency shown by these mutants in vivo (104), it was subsequently shown that these strains do not make as much ppGpp as the wild type. An increased ppGpp-dependent pausing of the mutant RNAPs during mRNA transcription has been proposed to explain these results (13).

DNA Targets for Stringent Control

The DNA targets for stringent control have been studied extensively with both rRNA and tRNA genes. A GC-rich region known as the discriminator, between the promoter −10 sequence and the +1 RNA transcription start site, has been noted as a common feature of promoters regulated by the stringent response (329, 331). When the GC-rich discriminator of the *tyrT* tRNA gene is replaced with an AT-rich sequence, the promoter becomes resistant to stringent control (174, 330). Interestingly, the promoter strength also increases; thus, suboptimal strength of the core promoter has also been proposed as a common feature of stringently regulated promoters (174). The conclusions of this study have been confirmed for the *P1* promoter of the *rrnB* operon (150). Although the discriminator sequence may be necessary for stringent control, it is probably not sufficient. The *rrnA* and *rrnE P2* promoters possess GC-rich discriminator sequences but are insensitive to stringent control (155–157, 281, 282). Furthermore, Zacharias et al. have shown that while a single base change in the discriminator sequence (GCAC to GCGC) immediately downstream of the −10 region was sufficient to put the *rrnB P2* promoter under stringent control, the same sequence failed to confer stringent control when downstream of the *tac* promoter (361). While this observation supports the idea mentioned above, that a nonconsensus promoter sequence may also be required for stringency, other researchers have found the detached *rrnB P2* promoter, with its native discriminator sequence, to be stringently regulated (89, 150). The GCGC discriminator sequence from −7 to −4 has also been implicated in the stringent control of the *thrT-tufB* operon, which contains four tRNA genes in addition to one of the genes for elongation factor EF-Tu. Changing each of the GC pairs to AT by oligonucleotide mutagenesis lessened the extent of ppGpp inhibition in vitro (204). Ohlsen and Gralla (234) have hypothesized that ppGpp may impair the ability of RNAP to melt DNA in vivo, consistent with in vitro observations by Kajitani and Ishihama (157). Thus, promoters with a consensus discriminator sequence may be more likely to be affected by stringent control because of the stronger base pairing of the GC pairs. In this light, it is interesting that an AT-rich sequence between the −10 and +1 nucleotides has been implicated in the positive control shown by some promoters, e.g., that of the *his* operon, during the stringent response (329, 331). However, the importance of the GCGC discriminator sequence was put into question by experiments in which the lambda P_L and T7 promoters were fused to the *rrnB* operon. The lambda promoter-*rrnB* construct, despite having a relatively poor GAGC discriminator sequence, was stringently regulated in vivo (356). Likewise, transcription by

T7 RNAP of the T7 gene 10 promoter fused to the *rrnB* operon was also regulated in response to amino acid starvation in this study, leading the authors to suggest that stringent control is not limited to the *E. coli* RNAP (356). However, in more recent experiments, the lambda P_L promoter did not appear to be stringently regulated in vivo (150).

While the role of ppGpp in the control of stable RNA synthesis during the stringent response is undisputed, the exact mechanism by which it exerts its effect is still unclear, largely because of the difficulty in identifying exactly where in the transcription cycle ppGpp acts and precisely what constitutes a stringent promoter. The observation by Vogel et al. (343) that the transcription elongation rate of mRNA slows from 42 nucleotides per second to only 20 nucleotides per second during the stringent response is consistent with an RNAP sequestering model (144), which was proposed to explain the role of ppGpp in growth rate-dependent regulation of RNA synthesis and will be considered in more detail in the next section. In this model, the slowing of RNAP during mRNA transcription makes RNAP selectively limiting for initiation of rRNA transcription, since it has been proposed that *rrn* promoters are more difficult to saturate with RNAP than are mRNA promoters (365). The sudden lack of available RNAP might explain the abrupt but transient arrest in rRNA synthesis at the onset of the stringent response. However, it remains to be seen whether the "stringent characteristics" of the *rrn* promoters can be linked to their saturability.

Stringent Control in Other Organisms

Although the stringent response has been studied for nearly 40 years, not much is known about the effects of amino acid starvation in organisms other than *E. coli. relA* mutants of *Salmonella typhimurium* (193), *Bacillus subtilis* (313), *Serratia marcescens* (24), and *Klebsiella pneumoniae* (267) have been isolated. The *spoT* gene has been identified in *S. typhimurium* (276), and SpoT hydrolase activity in *Bacillus subtilis* (18) and *Bacillus stearothermophilus* (77) has been reported. In organisms in which the stringent response has been examined, such as *S. typhimurium* (58, 59), *B. subtilis* (91, 229, 298, 313), and *Streptomyces coelicolor* (310), the anatomy of the response has been shown to be very similar to that of *E. coli*. Interestingly, the original *B. subtilis relA* mutant isolated (313) has lost its ability to respond to both amino acid and carbon source starvation (229), and an intragenic suppressor mutation which selectively restores the response to carbon limitation has been isolated (113). This suggests that the RelA and SpoT functions of the *E. coli* enzymes may be combined in one protein in *B. subtilis*, even though two distinct synthetase enzymes have been identified in other *Bacillus* species (77, 315). Finally, *relC* mutations mapping in the large ribosomal subunit protein L11 have been isolated in *B. subtilis* (352), *Bacillus megaterium* (307), and many *Streptomyces* species (233), suggesting that the interaction of RelA with the ribosome is also well conserved across species barriers.

In the yeast *Saccharomyces cerevisiae*, amino acid starvation also leads to a rapid shutdown of both rRNA and ribosomal protein synthesis (349, 350). However, unlike in the bacterial system, the production of tRNA appears to be exempt from stringent control. The signal for stringent control in *S. cerevisiae* is also thought to be the accumulation of uncharged tRNA in the cell (236), but there is no evidence of either guanosine tetra- or pentaphosphate synthesis by the cytoplasmic ribosomes (169, 245, 268). Indeed, this is true for a host of other eukaryotic organisms (cited in references 32 and 245). A single relaxed mutant of *S. cerevisiae* has been isolated (308, 347) but has yet to yield much information about rRNA control during amino acid starvation. In other systems, nutrient starvation often leads to inactivation of the rRNA-specific RNA polymerase I (PolI) by covalent modification and a subsequent shutdown of rRNA synthesis. In *Acanthomoeba castellanii*, this modification has been mapped to the α-subunit homolog of PolI, AC40, although the nature of the modifying group is not yet known (249). An analogous mechanism may be present in the bacterial system; Ishihama has found growth phase-dependent phosphorylation of *E. coli* RNAP on entry into stationary phase, and preliminary data suggest that this covalent modification may lead to an inactivation of the polymerase on *rrn* templates in vitro (139).

GROWTH RATE-DEPENDENT REGULATION

Beginning with the observation of Schaechter et al. in 1958 that *S. typhimurium* cells grown on rich medium are larger and contain more RNA than those grown on poor medium (284), the relationship of rRNA synthesis to conditions of growth has been an important topic of interest for microbial physiologists. Further studies have shown that over a broad range of growth rates, the number of ribosomes per unit amount of cellular protein in *E. coli* is proportional to the growth rate (μ) and the rate of ribosome synthesis is proportional to μ^2 (95). This phenomenon has been termed growth rate-dependent regulation of ribosome synthesis, and its function is to ensure a sufficient supply of ribosomes to meet the cell's demand for protein synthesis. The exact mechanism by which this control is achieved has been the subject of much debate.

The focus of attention in studying the growth rate regulation of ribosome synthesis has been rRNA expression rather than ribosomal protein (r-protein) synthesis. This is because although the regulation of r-protein genes is diverse, most r-protein genes occur in large operons that are regulated by a feedback mechanism. In this control, one of the operon-encoded r-proteins that binds to rRNA also binds to its own mRNA, blocking further transcription or translation (for reviews, see references 147 and 363). Thus, there is direct competition between the mRNA and rRNA for the binding of these regulatory r-proteins in vivo. In general, an excess of r-protein relative to rRNA leads to feedback inhibition of r-protein expression, whereas a deficit is predicted to allow r-protein expression. In this way, the synthesis of selected r-proteins is coupled to the production of rRNA, and it was proposed that ribosomal growth rate-dependent control could take place at the level of *rrn* expression. This prediction was subsequently shown to be accurate (106, 201, 281), and the regulation of rRNA synthesis as a function of the growth rate has become the focus of laboratories interested in this phenomenon.

Many tRNA genes located outside of the *rrn* operons are also regulated by growth rate. These are predominantly major codon tRNAs, that is, tRNAs whose codons occur frequently in *E. coli* genes (73, 74). Since these tRNA genes display promoter structures similar to those of the *rrn* operon *P1* promoters, and since the expression of these tRNAs is affected similarly by perturbations that alter rRNA synthesis (below), it is assumed the same mechanism governs both tRNA and rRNA operons.

Although many models have been proposed to explain the growth rate-dependent regulation of rRNA and tRNA production, they can basically be categorized into two groups: ppGpp models and ribosome feedback models. The ppGpp models propose that the intracellular concentration of ppGpp directly regulates the level of rRNA synthesis by restricting the number

of RNAP molecules available to initiate transcription at stable RNA promoters. According to these models, the stringent response simply represents an extreme example of growth rate-dependent control. The ribosome feedback models, on the other hand, propose that the rate of rRNA synthesis is governed by a feedback mechanism sensitive to the translational capacity of the cell. Each of the models has its merits, and although they are often presented as such, they are not necessarily mutually exclusive. It is entirely possible that these systems coexist and complement each other. It has also been suggested that ppGpp levels are in fact the sensor determined by the translational capacity of the cell, making ppGpp a candidate effector of the ribosome feedback mechanism (130, 147), a combination of the ppGpp and feedback models.

ppGpp Models

An inverse linear correlation between growth rate and the intracellular concentration of ppGpp, under most experimental conditions tested, led to the model in which ppGpp is the effector of growth rate-dependent control of ribosome synthesis in *E. coli* (11, 12, 130, 144, 172, 173, 178, 278, 283). This idea is also supported by genetic evidence which relates increased activity of *rrnB P1-lacZ* fusions to decreased steady-state ppGpp levels (131). The growth rate control of ppGpp synthesis is independent of the *relA* gene product responsible for mediating the stringent response described above (8, 12, 172, 173, 278), and the sole function of RelA appears to be the synthesis of the transiently high levels of ppGpp required to shut off *rrn* transcription in response to amino acid starvation. The second enzyme for producing ppGpp, the *spoT* gene product, is therefore thought to be the growth rate-regulated activity in *E. coli* (131, 355). Initial support for the involvement of SpoT in the growth rate-dependent control of rRNA synthesis came from the observation that the SpoT hydrolase activity decreases dramatically upon carbon source deprivation (92) and that the PSII activity decreases in response to nutritional shift-up (85) or to any inhibitor of translation, such as chloramphenicol (92) or starvation for an amino acid (172, 173), which leads to a temporary internal shift-up because of a lack of substrate utilization. Further evidence consistent with a role for SpoT in the growth rate phenomenon comes from the experiments of Sarubbi et al. (283) with a set of strains carrying mutant *spoT* alleles that have increasingly impaired hydrolase activity. These strains accumulate increasingly high levels of ppGpp under steady-state conditions, coupled with decreasing expression from an *rrnA* promoter fusion and decreasing growth rate. The empirical relationship between the ppGpp levels in these strains and their growth rates is a precise inverse linear function, consistent with the original observation by Ryals et al. of this type of correlation (278). Thus, the steady-state levels of ppGpp in the cell are believed to be primarily determined by the balance of the synthetase and hydrolase activities of SpoT, which somehow respond to the internal amino acid and energy source pools in the cell (130, 131). By this theory, ppGpp levels are adjusted depending on whether translating ribosomes under- or overutilize the intracellular supply of amino acids or energy-dependent substrates for translation, such as charged tRNA, GTP-activated EF-Tu, and the charged tRNA-EF-Tu complex. It is the assembly or delivery of these substrates to the ribosome that is thought to be sensitive to the energy supply. However, the mechanism by which the SpoT enzyme monitors the pools of the various substrates for translation remains to be elucidated, and the theory would appear to be complicated by fact that SpoT is not associated with the ribosome (98).

Two theories of how ppGpp might regulate stable RNA synthesis have been proposed: the RNAP partitioning model and the RNAP sequestering model, both of which assume a limited concentration of RNAP in the cell.

RNAP partitioning model. Ryals et al. (278) have adapted the stringent-control RNAP partitioning model of Travers et al. (333) to explain growth rate-dependent control. Baracchini and Bremer suggested that the distinction between stringent control and growth rate-dependent regulation is largely semantic and that ppGpp is the sole regulator of *rrn* transcription in response to the nutrient environment (12). To briefly summarize this model, two forms of RNAP are thought to exist, a (p)ppGpp-bound form and a free form, and only the free form is able to initiate stable RNA transcription. At slow growth rates, the intracellular concentration of (p)ppGpp is high, and thus the fraction of unbound RNAPs available to synthesize rRNA is low. Credence for the partitioning model (also known as the promoter selectivity theory) comes from the demonstration that RNAPs with different template specificities and different ppGpp sensitivities can be isolated from block gradients (333) and the fact that ppGpp preferentially inhibits transcription initiation from rRNA promoters in vitro (157, 328, 336). In addition, ppGpp has been shown to restrict RNAP binding to rRNA promoters in both filter-binding and electron micrographic studies (115, 162). Also, Bremer's group has found that *rrnB P1-lacZ* fusions are under normal growth rate-dependent control in wild-type cells but that this control is abolished in Δ*relA* Δ*spoT* mutants (ppGpp^0 strains) (132, 365), which are incapable of synthesizing (p)ppGpp (131, 355). This result has been contradicted by the work of Gourse and coworkers, however, who have also measured the expression of similar *rrnB P1-lacZ* fusions in Δ*relA* Δ*spoT* strains (14, 87). In those experiments, *rrnB P1-lacZ* expression at different growth rates was unaffected by the Δ*relA* Δ*spoT* mutations, suggesting that growth rate-dependent regulation of rRNA synthesis can occur in the absence of ppGpp. Various possible explanations have been put forth by both groups to explain their lack of experimental accord. These include the possibility that chromosomal location, orientation with respect to the direction of chromosome replication, and translational effects of the different *rrnB P1-lacZ* fusions might influence the two systems differently (14, 132, 365). The experiments of Gourse and coworkers have the advantage that they also used negative controls (non-growth rate-regulated *rrn* promoter mutations in the same fusion system at the same chromosomal location) which do not display growth rate-dependent regulation in either the wild-type or the Δ*relA* Δ*spoT* strain (14, 87).

RNAP sequestering model. Jensen and Pedersen (144) have adapted their RNAP sequestering model from the passive regulation model proposed originally by Maaløe (191). In its current form, the Jensen-Pedersen model suggests that ppGpp, by slowing down the transcription elongation rate, sequesters RNAP in the elongation phase and that stable RNA promoters, because they are assumed to be difficult to saturate with RNAP, are relatively more sensitive to the decrease in the limited free RNAP pool. Evidence in support of this model comes from a direct measurement of a decrease in the transcription elongation rate of mRNA in response to amino acid starvation and growth rate (341, 343) and measurements of the saturation parameters of *E. coli* promoters with RNAP (103, 142, 157). However, the relatively small decrease in mRNA elongation rate may not be sufficient to account for the effect on rRNA synthesis over the range of growth rates examined (341). Furthermore, data from the same group have shown that transcription elongation rate varies by only a few percent over

the range of ppGpp concentrations normally found under steady-state growth conditions (302).

From in vitro measurements at ppGpp concentrations similar to in vivo steady-state pools, the inhibitory effects of ppGpp on mRNA elongation rate were initially assumed to also apply to rRNA transcription (104, 161, 163). It is now apparent, however, that in vivo, assembly of the *rrn* antitermination complex protects *rrn* transcripts from ppGpp-induced pausing (339, 340).

A variation of the sequestering theory has recently been proposed by Bremer and Ehrenberg to explain data obtained with $ppGpp^0$ strains which showed that ppGpp inhibits the synthesis of mRNA at fast growth rates (30). This model proposes that ppGpp-dependent pause sites exist downstream of most mRNA promoters. Even at low concentrations of ppGpp, RNAPs are thought to form queues back from the paused polymerases to eventually occlude the promoter, shutting off further mRNA transcription. Furthermore, it is suggested that the blocking of mRNA promoters in this fashion diverts RNAPs to the rRNA operons. Unlike the Jensen and Pedersen model, this theory argues that the mRNA promoters are not in themselves difficult to saturate but appear to be so because of promoter occlusion at high growth rates. In $ppGpp^0$ strains, no such occlusion occurs, thus possibly explaining how mRNA levels increase with growth rate in these strains. Unfortunately, we are a long way from knowing how frequently promoter-proximal pause sites actually occur in *E. coli*, and there is, as yet, little experimental evidence to support the Bremer-Ehrenberg model.

There are several general problems which apply to both ppGpp models for growth rate-dependent regulation. First, a major assumption of both models is that the concentration of RNAP is limiting in the cell. (For this reason, Bremer and coworkers have argued that the most valid parameter in studying the regulation of stable RNA synthesis is not the absolute rate of synthesis but rather the rate of stable RNA synthesis relative to total instantaneous rate of RNA synthesis [*rs*/*rt*] [132, 278].) However, indirect evidence suggests that RNAP is not limiting, at least not for *rrn* transcription. A twofold overproduction or underproduction of RNAP subunit genes under inducible control of the *lac* promoter led to similar changes in the level of mRNA production, but no change in rRNA synthesis was observed (232). Furthermore, *rrn* gene dosage experiments with defective *rrn* operons also indicate that free RNAP concentrations cannot be limiting for rRNA transcription (49, 108, 146). These defective operons have intact *rrn* promoters and similar RNAP packing density to intact operons throughout their lengths (48); yet, despite this very significant source of RNAP sequestration, there are apparently enough RNAPs to afford a 2.3-fold increase in expression of the intact operons (49). Finally, it should be noted that the synthesis of RNAP is autoregulated (16, 62, 247), and should its concentration become transiently limiting for transcription, the cell will presumably respond by making more. According to the ppGpp models, this would eventually restore the synthesis of the preshift levels of rRNA despite the increased presence of ppGpp, a situation which clearly does not arise.

Second, there are many ways in which *E. coli* can achieve a particular growth rate; besides the nutrient environment, temperature, mutations in many genes, and the simple presence of plasmids can all affect the growth rate. It is difficult to imagine how all of these processes could be coordinated to establish a particular level of ppGpp in the cell for a given growth rate. Indeed, a recent study has shown that the intracellular levels of ppGpp for a given growth rate were dependent on how that growth rate was achieved; i.e., whether by changing the carbon source or by overexpressing the *relA* gene from an isopropylthiogalactopyranoside (IPTG)-inducible promoter (152).

Third, both models predict that for a given concentration of ppGpp, there should be a characteristic level of rRNA synthesis, and at least in the case of the RNAP partitioning model, this should hold under non-steady-state as well as steady-state conditions. However, this relationship breaks down under a variety of circumstances. For example, we have reported that expression of an *rrnA-cat* fusion can vary more than twofold at a single ppGpp concentration (49). Workers have also reported stringent-like control in the absence of ppGpp accumulation (243, 303), and in addition, the relationship between ppGpp levels and rRNA synthesis breaks down during both α-methylglucoside (117) and pyrimidine (342) shifts. Some but not all of these aberrancies might be explained by the fact that rRNA accumulation was measured by using labeled RNA precursors, such as uracil, whose uptake is inhibited by ppGpp.

In conclusion, the two ppGpp models described above show how ppGpp might play a role in the distribution of the RNAP between stable and mRNA synthesis. However, whether this distribution is important for the growth rate regulation of ribosome synthesis and whether ppGpp is the sole mediator of the growth rate phenomenon seem much less certain. Definitive evaluation of these alternatives relies heavily on whether *rrn* promoters are saturated with RNAP holoenzyme (i.e., all subunits) under conditions of balanced growth.

Ribosome Feedback Models

The ribosome feedback model, first elaborated by Jinks-Robertson et al. (146), proposes that cells have an inherent capacity to synthesize excess amounts of all ribosomal components but that feedback regulation of rRNA expression occurs to prevent the production of more ribosomes than are needed for protein synthesis. One of the most attractive aspects of the ribosome feedback inhibition model is that it explains how the appropriate concentration of ribosomes is ensured for a particular growth rate independently of how that growth rate is achieved. Thus, in the view of the feedback model, *rrn* expression is not regulated in direct response to the nutritional environment but rather by a powerful homeostatic mechanism that somehow monitors the amount of functional rRNA in the cell. The original model proposed that the effector of feedback regulation was free, nontranslating ribosomes. While the pertinent evidence for and against this assignment will be examined in detail below, suffice it to say here that the effector is now believed to be excess translational capacity.

The first evidence in support of the feedback regulation model came from rRNA gene dosage experiments with strains containing multicopy plasmids carrying intact *rrn* operons (*rrnB* or *rrnD*) (146). The extra copies of *rrn* operons did not lead to increased expression of rRNA, but rather the synthesis of rRNA and associated tRNAs from individual operons was decreased, keeping total rRNA production the same. On the other hand, when the plasmid contained a defective *rrn* operon that had been inactivated by an internal deletion, the total amount of *rrn* transcription (i.e., from both intact and defective operons) became gene dosage dependent, suggesting that *rrn* expression is regulated directly by the amount of functional rRNA in the cell. In a logical extension of these experiments, the *rrnB* operon was expressed under the control of the heat-inducible lambda P_L promoter (108). rRNA synthesis was then measured before and immediately following a shift to high temperature. As was observed under steady-state conditions, expression of chromosomal *rrn* operons is reduced upon overexpression of an intact operon but not when the operon is

defective. The fact that plasmids containing defective *rrn* operons are expressed in a gene dosage-dependent manner would also appear to indicate that the number of RNAPs available for rRNA transcription is not limiting under balanced growth conditions, as already mentioned above.

The converse of these experiments has also been shown to be true: the inactivation of *rrn* operons on the chromosome leads to increased expression of the remaining intact copies, consistent with a feedback mechanism sensitive to the amount of functional rRNA produced in the cell (49). The increase in expression occurs at the level of promoter activity and, surprisingly, is accompanied by a 50% increase in the RNAP elongation rate. Whether the observed increase in elongation rate contributes to the overall increase in rate of rRNA synthesis remains uncertain. When cells are grown in rich medium, there is still sufficient space on the *rrn* operons to accommodate about twice the number of RNAPs. This suggests that RNAPs are "gated" at or near the 5′ end of the operon and that the polymerase elongation rate is faster than the rate at which the gate is cleared. Obviously, if the transcription initiation step is rate limiting, an increased elongation rate could have no influence on the overall rate of rRNA synthesis. However, if initiation is immediately followed by a region that is normally transcribed slowly, a mechanism that increases elongation rate could, in turn, increase the frequency of initiation. Thus, increased elongation rate might indirectly increase the rate of rRNA synthesis by more rapidly clearing the promoter region of RNAPs.

Additional evidence in support of the feedback inhibition model has come from the study of the *fis* and *nusB* genes. The NusB protein is required for *rrn* antitermination in vitro (305), and mutations in this gene lead to premature termination of *rrn* transcription within the coding region (292). A feedback mechanism that senses the amount of functional rRNA would be predicted to derepress synthesis of rRNA to compensate for this defect. Indeed, *rrn* synthesis is increased in *nusB* mutants, supporting the predictions of the feedback model (292). Deletion of the Fis binding sites reduces in vivo expression of an *rrnB P1* promoter fusion to 8% of the wild-type activity. However, rRNA synthesis does not decrease substantially in a *fis* null mutant (275). This result is also explained by derepression of a feedback system to compensate for the loss of Fis-mediated activation of transcription. (The roles of the *fis* and *nusB* genes in activation and antitermination will be discussed in greater detail later.)

The existence of a feedback system to monitor functional rRNA synthesis does not necessarily mean that this method is used to control *rrn* expression as a function of growth rate. The link between these two observed regulatory mechanisms was provided by Gourse et al. (106), who showed that the promoter determinants for feedback inhibition and growth rate-dependent control were the same. First, both control mechanisms were effective on *rrn P1* rather than *P2* promoters (see also reference 281), but more significantly, deletions around the *rrnB P1* promoter that abolished feedback inhibition also caused the loss of growth rate-dependent control. A saturation mutagenesis study of the *rrnB P1* promoter revealed that mutations which changed the wild-type −35 or −10 sequences or the 16-bp spacing between these two elements disrupt growth rate-dependent regulation regardless of whether these mutations increase or decrease the strength of the promoter (66). (Whether these mutations also affect feedback inhibition has not yet been reported.) In addition, the GC-rich discriminator element downstream of the −10 region plays a significant role in growth rate-dependent control (66, 150). Most of the *rrnB P1* mutants with altered growth rate-dependent control also had a defective stringent response. However, mutations have been isolated which uncoupled the two regulatory mechanisms; that is, some of the growth rate-dependent control mutants have a relatively normal stringent response (150). Stringent and growth rate-dependent controls have also been separated by a similar mutational analysis of the *leuV* tRNA promoter (15). Notably, a *leuV* promoter mutated to consensus in the −35 region retains stringent control but loses growth rate regulation in vivo (135). Although these data have been cited as evidence that ppGpp-dependent stringent control and growth rate-dependent regulation of rRNA synthesis involve distinct mechanisms, the possibility remains that the mutations have simply altered the sensitivity of the promoter to ppGpp, i.e., these promoters are insensitive to the relatively low levels of ppGpp that occur during steady-state conditions but remain sensitive to the high levels that occur during the stringent response. It is interesting in this regard that promoter mutants which retain growth rate control but lose stringent regulation have yet to be reported. Consistent with the idea of threshold levels of transcriptional sensitivity to ppGpp is a recent report demonstrating resistance to the antibiotic mecillinam (a specific inhibitor of penicillin-binding protein 2) in cells containing more than 140 pmol of ppGpp per A_{600} unit but sensitivity to the drug if ppGpp levels fall below this value (152).

As stated above, the first proposed effector for the feedback mechanism was free nontranslating ribosomes. While a significant amount of evidence has accumulated that this is probably not the case, it is still pertinent to review the experiments that originally led to that conclusion. First, prevention of ribosome assembly in conditional assembly-defective mutants leads to a stimulation of stable RNA synthesis (33, 192, 319). Since the rRNA transcripts are normal in these mutants, this indicates that it is not the rRNA per se that is the feedback effector; rather, the cell senses the deficiency of functional ribosomes. Consistent with this idea, Takebe et al. (320) observed that overproduction of the translational repressor r-protein S4, which inhibits ribosome assembly in vivo, causes a preferential stimulation of rRNA synthesis. Second, slowing down the translation rate with the chain elongation inhibitor fusidic acid (21) or by using streptomycin-resistant mutants, which have slower peptide chain elongation rates (364), resulted in an elevation of rRNA synthesis. A similar increase in *rrn* expression occurs with the chain elongation inhibitor chloramphenicol (190, 295). Since slowing down the chain elongation rate would be predicted to deplete the pool of free ribosomes, it was postulated that free, nontranslating ribosomes were the effectors of feedback inhibition and growth rate-dependent regulation.

Subsequent experiments by Nomura and colleagues, however, have forced a reevaluation of this model. First, an attempt to show direct inhibition of rRNA synthesis by ribosomes in vitro was not successful (146). Also, the induction of a plasmid-borne *rrnB* operon carrying a mutant anti-Shine-Dalgarno sequence in the *16S* gene led to the accumulation of many free ribosomes that were incapable of translation but did not cause feedback regulation of rRNA synthesis (357). Finally, strains that are incapable of effective translation because of a limitation in the availability of translation initiation factor IF2 accumulate high concentrations of free ribosomes but also do not repress *rrn* expression (45). These data do not support a model in which free ribosomes are the effector of feedback inhibition but rather suggest that ribosomes must be translating for regulation to occur. It is not yet known whether the translation process per se activates the signal molecule or whether this pathway relies on the translation of a signaling mRNA. The proponents of the ppGpp models argue that

ppGpp is, in fact, the effector generated by such an excess translational capacity.

Concluding Remarks for *E. coli* Regulation

All of the current evidence suggests that some event linked to the translation process is responsible for triggering feedback regulation of rRNA synthesis in *E. coli* and that this is one possible mechanism for growth rate-dependent control. There is also a significant amount of data consistent with a role for ppGpp in growth rate-dependent regulation. The questions remaining are whether (i) ppGpp is the sole effector of feedback control, (ii) the ppGpp and feedback systems exist independently and operate in parallel to complement each other, and (iii) ppGpp is simply a by-product of the translation process and has no regulatory role in the growth rate-dependent control of rRNA synthesis. In the last case, the function of ppGpp might be to couple the elongation rates of mRNA transcription and translation (76, 144), important in avoiding transcriptional polarity and in the regulation by attenuation of many genes, or to indirectly ensure continued translational fidelity by making mRNA limiting for translation during amino acid starvation (302).

There is a significant amount of indirect evidence in support of the idea that ppGpp is an effector of growth rate-dependent control. Intracellular levels of ppGpp are in inverse proportion to growth rate and appear to be controlled by SpoT in response to fluctuations in the amino acid and energy source pools. Thus, SpoT could certainly be a "growth rate" sensor of the translational capacity of the cell. However, the examples of an uncoupling between ppGpp and growth rate-dependent regulation cited above, in particular, the observations for Δ*relA* Δ*spoT* strains, suggest the presence of a second mechanism. Despite numerous efforts, however, no other effector of growth rate regulation or feedback inhibition has been identified. Schemes to identify such an effector have turned up mutations in the β-subunit of RNAP (322), sigma factor (159), SpoT (131), and the gene for fructose-1,6-diphosphate aldolase, *fda* (297), none of which point to an obvious ppGpp-independent pathway. Furthermore, there has been a report that is contradictory of the interpretation of the early feedback inhibition experiments of Nomura and coworkers (11). However, if, as the general picture appears to suggest, two mechanisms do exist, they may operate under slightly different growth conditions or at different levels of sensitivity, and an ability to knock out either pathway without totally abolishing regulation would explain much of the controversy surrounding this issue.

Growth Rate-Dependent rRNA Control in Other Organisms

While the original observations pertaining to growth rate control of rRNA synthesis were made in *S. typhimurium* (284), there has been little research of this phenomenon in bacteria other than *E. coli*. However, growth rate-dependent control of *B. subtilis rrn-cat* fusions in *E. coli* has been reported (61). Thus, the *B. subtilis* growth rate effector(s) and promoter determinants appear to be similar to those of *E. coli*. Interestingly, unlike the *E. coli rrn* operons, in *B. subtilis* it is the downstream of the two promoters, *P2*, which is regulated as a function of the growth rate (277).

In eukaryotes, the synthesis of rRNA is also regulated in accordance with the cellular growth rate (for reviews, see references 250, 299, and 300). At low growth rates and in quiescent cells, rRNA expression is low and is stimulated by conditions which induce cell proliferation (36, 251). In general, the activity of cellular extracts on rDNA templates in vitro reflects the growth rate of the cells from which they were prepared (36, 114, 251). Despite the usefulness of the system, only recently has some progress been made in the dissection of the yeast PolI transcriptional apparatus in vitro (160, 271). Schnapp et al. have identified TIF-IA as a protein that is, at least in part, responsible for the growth rate-dependent activity of cellular extracts in mice (287). TIF-IA is 75 kDa in size and is thought to be the mammalian homolog of the prokaryotic sigma factor (288). Interestingly, in a scheme designed to identify mutations in the growth rate-dependent pathway of rRNA synthesis in *E. coli*, Keener and Nomura (159) isolated a mutation in sigma factor. In light of these data, it may be worth reassessing the role of sigma factor in prokaryotic growth rate-dependent control.

Two reports have suggested that phosphorylation of some component of the transcription machinery is important for rDNA activation under slow-growth conditions. Addition of protein kinase NII to nuclei isolated from quiescent cells stimulates rRNA transcription fivefold (17), and dephosphorylation of partially purified fractions of PolI renders it inactive for rDNA transcription in vitro (287). However, since these studies were not done with purified proteins, it was not possible to distinguish between phosphorylation of PolI or some other necessary transcription factor(s). It is now believed that phosphorylation of UBF is responsible for the activation of rRNA transcription under active growth conditions (238, 345) and that the PolI transcription complex is actually repressed by phosphorylation (170, 171). Grummt's group has shown that phosphorylation of a component of the PolI transcription complex, probably TIF-IB, by DNA-dependent protein kinase bound in *cis* inhibits rRNA transcription in vitro by as much as 50-fold. As far as growth rate-dependent control is concerned, it appears that it is the phosphorylation status of UBF (upstream binding Factor) rather than PolI that is important. Phosphopeptide mapping has shown that qualitative changes in UBF phosphorylation (i.e., the particular amino acids that are phosphorylated) are probably more important than quantitative changes in explaining the difference in UBF activity between growing and serum-starved cells (344).

In summary, the growth rate-dependent control of eukaryotic rRNA transcription appears to occur at the level of both a limiting regulatory factor (TIF-IA) and a posttranscriptional modification of UBF. These two mechanisms, although as yet poorly understood, combine to allow the cell to adapt rapidly to a variety of extracellular signals.

UPSTREAM ACTIVATION

rRNA transcription is significantly enhanced by *cis*-acting sequences upstream of the *rrn P1* promoters, a phenomenon known as upstream activation. Upstream activation has been studied extensively by Gourse and coworkers, using fusions of the *rrnB P1* promoter to *lacZ*. A set of upstream deletions, extending towards the promoter from a position 600 bp before the transcription start site, showed a dramatic reduction in β-galactosidase activity as the endpoint of the fusion was moved towards the promoter (106). In addition, restriction fragments containing the upstream region ran anomalously on acrylamide gels, suggesting a bent DNA conformation (106), although this bend is not thought to be necessary for activation (88). An intense mutational analysis of this region resulted in mutations giving as much as a 100-fold decrease in promoter activity (86). However, no mutations which significantly increase promoter activity have been found, suggesting that the upstream activation region (UAR) has evolved towards maximal activity. The UAR possesses sites that bind a protein factor, Fis, and a factor-independent activation region, called

the UP element, that binds the α-subunit of RNAP. Both Fis, acting at its binding region, and the UP element increase the activity of the *rrn P1* promoter manyfold. In this section, we will examine these properties of the UAR of *E. coli rrn* operons and then assess the role of upstream activation in other organisms.

Fis—Factor for Inversion Stimulation

Fis is a homodimeric protein of 11.2-kDa subunits that binds to specific DNA sites. In addition to the activation of *rrn* operon transcription, it also plays several other roles in the cell. Fis was first identified for its role in the Mu phage *gin* and *Salmonella hin* site-specific inversion reactions (148, 149, 154) and is involved in bacteriophage λ excision (10, 324), initiation of DNA synthesis at *oriC* (79, 101), and expression of many tRNAs (15, 28, 75, 176, 213, 225, 226). The crystal structure of Fis protein has been determined (166, 167, 360), and it has been shown that Fis uses a C-terminal helix-turn-helix motif of each subunit (165, 240) to bind a 15-bp degenerate DNA consensus sequence (80, 81, 137). Fis induces 40° to 90° bends in DNA (80, 101, 323) and is thought to wrap the DNA around itself (242). The extent of the wrapping is dependent on the flanking sequences, often AT-rich, of the Fis recognition site.

All seven *E. coli rrn* operons possess three potential Fis binding sites in their UAR sequences (49, 337). In vitro, the factor-dependent domain of the *rrnB P1* UAR specifically binds Fis protein at the three predicted binding sites (Fig. 3) (275). Several lines of evidence suggest that Fis site I, closest to the *rrnB P1* promoter, is sufficient for most of the activation (275). First, in vivo, deletion of sites III and II results in only a modest reduction in *rrnB P1* promoter activity (to 71% of the original activity), while deletion of all three sites causes reduction to 8% of the original activity. Second, in vitro, Fis fails to activate transcription when all three binding sites are absent but activates transcription as much as 20-fold when site I is present. Finally, there is a correlation between the effect of single base deletions or substitutions away from consensus at Fis site I, reduced promoter activity, and the inability of DNA fragments to bind Fis in gel retardation assays (275).

The participation of Fis in *rrn* promoter activation in vitro might lead to the prediction of a dramatic decrease in *rrn* expression in *fis* null strains in vivo. However, a *fis* null mutation caused only a 2.0- to 2.5-fold decrease in expression of fusions to each of the seven *rrn P1P2* promoter regions (51), consistent with the effect of *fis* measured in other studies in vivo (275, 279, 362) and suggesting that all *rrn* operons are indeed subject to Fis-mediated activation. The 2.5-fold in vivo reduction of *rrn* expression is much less than the in vitro effect of Fis at the UAR, described above, and is also less than the 5- to 10-fold in vivo reduction in the expression of fusions lacking the Fis binding sites (275, 362). These results suggest that the full effect of the *fis* mutation may be masked by derepression of the feedback inhibition system described earlier. That is, if *rrn* transcription is decreased because of a *fis* mutation, the cell somehow senses this and responds by increasing *rrn* transcription via another (unknown) control mechanism.

Evidence from several laboratories has suggested that factor-dependent upstream activation of rRNA transcription occurs via direct interaction between Fis and RNAP. First, Fis activates *rrn* transcription in vitro in the absence of any added factors (275). Second, UAR-mediated activation is abolished by a 3-bp deletion (−38 to −40) between Fis site I and *P1* and is restored by insertion of a random sequence that reestablishes the correct spacing. This result implies that activation by Fis is face-of-the-helix dependent and requires the correct orientation of Fis relative to RNAP (151). Other experiments showing that only fusions which maintain an integral number of helical turns between the promoter and Fis sites possess UAR-mediated activity also support this suggestion (213, 223, 362). Third, mutant Fis proteins which are defective for Hin-mediated recombination fall into three classes with respect to their ability to activate *rrn* transcription: those that fail to bind to the upstream activation region, those that bind but fail to bend the DNA normally, and those that bind and bend but fail to activate (105). The function of Fis cannot be simply to bend the DNA so that an upstream factor is brought in proximity to RNAP, since no sequences upstream of Fis site III are required for activation. The mutant Fis protein that binds to and bends the DNA but fails to activate transcription provides particularly compelling evidence that Fis must interact with RNAP to accomplish activation. A further observation, made by Kahmann's group, suggests that Fis and RNAP may cooperate in binding at the *tyrT* promoter, although the apparent nonspecificity of DNA binding by Fis and RNAP requires some caution in interpretation of the results (213). While there appear to be some differences between the *rrn* and *tyrT* systems in terms of the number of Fis sites required for maximal stimulation and whether Fis binds cooperatively to its three recognition sites (25, 213, 275, 337), it is likely that Fis activates the two systems in a similar way. The binding of Fis to site I of the *rrnB P1* promoter is mutually cooperative with promoter binding of RNAP (25), which is consistent with the observed Fis and RNAP cooperativity in the *tyrT* system (213). Thus, Fis may act by increasing the RNAP promoter-binding constant. However, in a transcription system involving whole-cell extracts with the *rrnD P1* promoter, Mueller's group reported that Fis activation occurs later in the initiation process, at the conversion of open complexes to transcribing complexes (175, 279). It thus remains to be seen whether Fis acts at RNAP binding, at open complex formation, or at both steps, or whether the differences observed with these two promoters can be ascribed simply to the reaction conditions used.

Other proteins in addition to Fis may also bind to *rrn* promoter regions and influence their expression. Tippner et al. have recently reported that the *E. coli* histone-like protein H-NS binds specifically to the *rrnA*, *rrnB*, and *rrnD* promoter regions. H-NS bound to three sites around the *rrnB P1* promoter (between −18 and −89) and caused in vitro repression of *rrnB P1* transcription (325). They found that one of these sites overlapped the major Fis activation site, site I, and another overlapped the −35 region of the promoter. Repression appeared to take place without Fis displacement and, indeed, was most pronounced in the presence of Fis, suggesting an antagonistic effect of H-NS on upstream activation of rRNA expression by the Fis protein. Wagner has proposed that H-NS caused this antagonism by binding to the strand opposite Fis and bending the DNA in the contrary direction to that required for Fis activation (346). The concentration of H-NS used in these in vitro experiments was quite high, and the in vivo significance of these observations is not yet known, although a role during stress conditions has been proposed (325).

Does Fis Have a Regulatory Role?

Levels of Fis vary dramatically throughout the growth cycle in *E. coli* (10, 227, 228, 324). Since the interaction between Fis and the UAR influences *rrn* expression substantially, Fis regulation should be considered one of the several mechanisms that influence *rrn* transcription control. There is a 1,000-fold induction of Fis upon subculturing of stationary-phase cells

into rich medium, and this induction occurs at the level of *fis* mRNA (10, 228). As cells enter exponential growth, *fis* transcription is essentially turned off and intracellular Fis levels are diluted out by cell division. This is accomplished in part by negative autoregulation, because Fis protein binds to sites that overlap the −35 and −10 regions of the *fis* promoter and shuts down transcription (10). When exponentially growing cells are shifted to a richer medium, synthesis of Fis is transiently induced, and the magnitude of the shift appears to determine the amount of Fis that is made.

Fis is transcribed as part of an operon, downstream of an open reading frame of unknown function (10, 228). The *fis* promoter is similar to *rrn* promoters in many ways: nonconsensus −35 region, 16-bp suboptimal spacing between the −35 and −10 regions, and a GC-rich discriminator sequence. Therefore, it is highly possible that the *fis* gene responds to the same controls as the *rrn* operons. Indeed, by using promoter-*lacZ* fusions, Ninnemann et al. (228) have shown that the promoter of the *fis* operon and *rrnB P1* behave very similarly during a nutritional shift-up. Furthermore, this group has shown that the *fis* promoter is subject to stringent control, and Ball and Johnson (10) have preliminary evidence which suggests that steady-state levels of Fis vary as a function of growth rate.

Despite the fact that it is both stringently controlled and growth rate regulated, Fis does not appear to affect these two control mechanisms as they pertain to *rrn* transcription. In *fis* null strains, both stringent control and growth rate-dependent regulation of the *rrnB P1* promoter are virtually indistinguishable from those in wild-type strains (275). In addition, an *rrnB P1* promoter lacking the three Fis binding sites has unaltered growth rate-dependent regulation (106). This is apparently in contrast to the situation in many of the tRNA genes. The *thrT-tufB* operon, which encodes several tRNA genes as well as the gene for elongation factor EF-Tu, shows impaired growth rate-dependent regulation in the absence of the Fis binding sites, suggesting that Fis plays some role in this phenomenon for the *thrT-tufB* operon (227). In two recent studies (75, 225), it was shown that while Fis activates the expression of many tRNA isoacceptors with increasing growth rate, some tRNA genes are unaffected by a *fis* null mutation, and still others actually show a negative effect by Fis. Thus, it has been suggested that the role of Fis in tRNA expression is to regulate the composition of the tRNA pool. However, for the *rrn* operons, Fis appears simply to fine tune the mechanism controlling nutritional shift-up, perhaps priming rRNA synthesis for a more rapid adaptation to the new growth conditions.

Factor-Independent Activation—UP Element

In the absence of Fis, the UAR was originally thought to afford an additional two- to fourfold factor-independent activation of *rrn* transcription in vivo (275) and in vitro (179). This turned out to be only part of the *cis* activation of the UAR, because the endpoint of the fusions used (from −154 to −50 with respect to the start site of transcription from *rrnB P1*) cut the *cis*-activating sequence in half. A closer examination of this region identified a 20-bp (from −60 to −40) sequence, termed the UP element, capable of a 30-fold activation of the *rrnB P1* promoter both in vivo and in vitro (Fig. 3) (274). The UP element binds the α-subunit of RNAP and is thus considered an extension of the core promoter region; a DNase I or hydroxyl radical footprint of RNAP on this promoter shows protection around −50 in addition to protection of the −35 and −10 regions (224, 274). The *rrnB P1* UP element still binds RNAP and retains activity when placed one (but no more) helical turn away from the promoter (223), suggesting both face-of-the-helix and distance constraints on the mechanism. This element is a detachable promoter module and can activate transcription from other, unrelated promoters. Although it is rich in A and T, the UP element appears to be only slightly curved, and the previously detected pronounced bend in the *rrnB P1* promoter region lies upstream (88, 346). The UP element component of the UAR is not thought to play any regulatory role but to simply contribute to the high level of activity demonstrated by *rrn* promoters. It increases the rate of promoter recognition or closed-complex formation by RNAP (179, 263) and possibly also the rate of isomerization to the open complex in the absence of added factors in vitro (263).

The specific binding of α-subunit dimers to the UP element can occur in the absence of the other subunits of RNAP and has been mapped to the C-terminal tail of alpha (23, 263). Interestingly, α-subunits containing an Arg-265 to Cys mutation, which show defective transcriptional activation by the catabolite gene activator protein CAP (368) and the oxidative stress regulator OxyR (321) and which are no longer ADP-ribosylated in this position during T4 infection, an event thought to shut off host RNA synthesis (97), are also defective in binding the UP element (274). This suggests a similarity between the mechanisms of promoter activation by upstream DNA sequences and by class I transcription factors, which contact the C-terminal tail of alpha and generally have binding sites immediately upstream of but not overlapping the core promoter region (140, 141). RNAPs containing α-subunit C-terminal truncations have no effect on Fis activation in vitro (274).

UP elements are found upstream of many other naturally occurring *E. coli* promoters, including the *rrnB P2* promoter (274). Indeed, this region had been shown to activate *P2* transcription in vivo long before the element was characterized (189). Besides the A-T richness, no obvious sequence consensus in the UP elements has been identified. A tracts are common, however, upstream of many strong promoters (63, 90, 257). It remains to be seen how many of these sequences can be assigned UP element-type properties.

Upstream Activation in Other Organisms

The Fis protein was originally identified in the inversion reactions of *S. typhimurium*, and although *Salmonella rrn* operons have not yet been sequenced, the pleiotropic effects of *fis* gene inactivation are for the most part similar in *S. typhimurium* and *E. coli* (241), suggesting conservation of Fis function between these closely related organisms. The *E. coli* consensus sequence has been used to suggest that potential Fis binding sites lie upstream of four *Clostridium perfringens rrn* operons (93). In *B. subtilis*, a UP element located upstream of the flagellar *hag* gene promoter has been shown both to activate transcription and to bind the α-subunit of RNAP (82), making it likely that this mechanism too is conserved in the bacterial kingdom.

Eukaryotic rRNA promoter regions are characteristically ~150 bp in size and are generally divided into two subdomains, a core promoter element and an upstream control element. In *S. cerevisiae*, a third subsection (domain II) has been assigned to include the region between the core and the upstream elements (256). It is tantalizing to speculate that these domains functionally correspond to the core promoter, UP element, and Fis binding sites of the prokaryotic *rrn* promoter. The eukaryotic upstream control element binds the rather sequence-nonspecific DNA-binding protein and transcriptional activator UBF (upstream binding factor). UBF is a ~97-kDa

protein which contains homology to the DNA-binding domain of the high-mobility-group protein HMG1 (143). Like Fis, UBF is thought to induce sharp bends in the DNA (259). A second activator—SL1 in humans and TIF-IB in mice—can also bind the upstream control region (19, 20). SL1 actually consists of four proteins, the TATA-binding protein TBP and three TBP-associated factors (TAFs) (47, 367), and is also the factor which imparts species specificity on the transcription of rDNA constructs from different systems in vitro (20, 200). The UBF-SL1 complex remains stably attached to the upstream control element and can activate the initiation of up to 20 rounds of transcription (129). Interestingly, Fis has been proposed to act in a similar manner in the *tyrT* operon (213). Choe et al. (42) have examined the importance of the spacing between the upstream control element (domain III in *S. cerevisiae*) and the promoter. In a further analogy with the Fis activation mechanism, changing the spacing by 5 bp drastically decreased transcription in vitro, an effect which could be partially rescued by insertion of another half turn of the helix. Spacing effects between domain II and the promoter have been studied by two groups (42, 214). As was observed for the UP element in *E. coli*, a half turn of the helix disrupted activational effects. However, in this case, activation could not be restored by insertion of a second half-helix (42). Whether the activational effects of domain II are factor independent has not been addressed.

In addition to the binding of activators close to the core promoter, *rrn* operons in eukaryotes are significantly activated by enhancer elements which occur in the spacer regions between the repeated rDNA transcription units (for a review, see reference 299). In *S. cerevisiae*, this activation is between 15- and 30-fold and is caused by a single enhancer of 170 to 190 bp that occurs ~100 bp after the 26S rRNA of the upstream operon and ~2.2 kb before the transcription initiation site of the downstream operon (70, 71). The enhancer contains a site for the ribosomal enhancer-binding protein REB1/RBP1 and also the terminator of the upstream operon, leading to an interesting looping model in which the PolI RNAP is passed directly from the terminator of the preceding operon to the promoter of the subsequent one (256). In higher eukaryotes, multiple repeated enhancers of 60 or 81 bp, in addition to 35-bp and 100-bp repeats, are found. The 60- or 81-bp repeats bind both UBF (255) and SL1 (cited in reference 299). In addition to the enhancer repeats, promoter duplications have been noticed in the spacer region between tandem rDNA transcription units. Although these promoters do not contribute much stable transcript themselves, they can apparently significantly increase the efficiency of the major operon promoter, possibly by directing transcription through the 60- and 81-bp repeats (65). However, the exact mechanism of this activation is still a mystery. Finally, perhaps in an analogy to the yeast system (above), terminator elements located at each end of the intergenic spacer region also paradoxically help stimulate transcription of the downstream operon (129).

ANTITERMINATION

Translation is coupled to the transcription of most mRNAs in *E. coli*; that is, ribosomes begin translation of mRNAs before RNAP has reached the 3′ end of the transcription unit and released its transcript. It is thought that this is not simply a matter of efficiency but that the ribosomes also play a necessary role in restricting access of the transcription termination factor rho to the mRNA, thereby preventing premature termination of transcription. Evidence in support of this idea has come from the observation that nonsense mutations which cause premature translation termination and release of ribosomes from the mRNA also result in reduced transcription of regions downstream of the mutation, a phenomenon known as polarity (for reviews, see references 1 and 258). If the untranslated rRNA transcripts were also subject to polarity, equal amounts of each subunit would not be produced. However, *rrn* operons are exempt from premature transcription termination (206, 207), even though it has been demonstrated that a rho-dependent terminator resides within the 16S gene (2). How then do the *rrn* operons avoid polarity? The first clarification of this puzzle came with the observation that transposable elements which are strongly polar when inserted into translated operons are only partially polar when inserted into *rrn* operons (31, 208). This and subsequent work suggested that there is an antitermination mechanism in *E. coli rrn* operons that counteracts transcription termination signals, preventing the occurrence of polarity throughout their 5.5-kb length (2, 134, 183). Other roles in addition to counteracting polarity have also been proposed for this system (see below), and it may be that several aspects of ribosome synthesis are governed by the antitermination mechanism.

Properties of Antitermination

Transcriptional antitermination has been well characterized in bacteriophage lambda (for reviews, see references 55, 56, and 272). In one example of antitermination in lambda transcription, RNAP is modified by the lambda N protein and several *E. coli* host proteins, NusA, NusB, NusE (r-protein S10), and NusG, to form a termination-resistant elongation complex capable of proceeding for many kilobases and through many transcription terminators without stopping. Assembly of the elongation complex requires a promoter-proximal sequence in the RNA, known as the *nut* site, and it appears that the *nut* site RNA itself is the nucleation center of the complex (196, 230). The 5′ end of the message is thought to remain attached to the RNAP as it transcribes towards the 3′ end of the template. It is not yet clear how this complex becomes resistant to either rho-dependent or rho-independent terminators.

In the *rrn* operons, sequences homologous to the lambda *nut* site, first noted by Olson et al. (237), have been shown to promote antitermination of rRNA transcription in vivo (183). These sequences occur about 10 bp downstream of *rrn P2* promoters, and in theory, transcription originating at either promoter can be antiterminated. A second *nut*-like element occurs in the spacer region between the *16S* and *23S* genes, suggesting that the system has to be recharged after transcription of the *16S* gene (22, 209). The leader and spacer elements can be divided into two subdomains, known as *boxB* and *boxA*, although the order of these subdomains is reversed in lambda. The *rrn boxA* is a conserved sequence, TGCTCTTTAACA, while *boxB* is a stem-loop structure with no apparent sequence conservation. The importance of the *rrn boxB-boxA* motif is underscored by the high level of conservation of this configuration in *rrn* operon leader and spacer regions of most members of the domain *Bacteria* and some members of the domain *Archaea* (22, 304) (see below). Deletion analysis of the *rrn nut*-like sequences by Gourse et al. (106) demonstrated that *boxA* (and perhaps *boxC*—a third conserved element between *rrn* operons and *nut* sites) was required for *rrn* antitermination in vivo but that *boxB* was dispensable. Interestingly, *boxB* is required and *boxA* appears to be dispensable for N-mediated antitermination in lambda (248, 369). Using a promoter fusion test system, Berg et al. (22) have more precisely defined the regions necessary for *rrn* antitermination in vivo by saturation

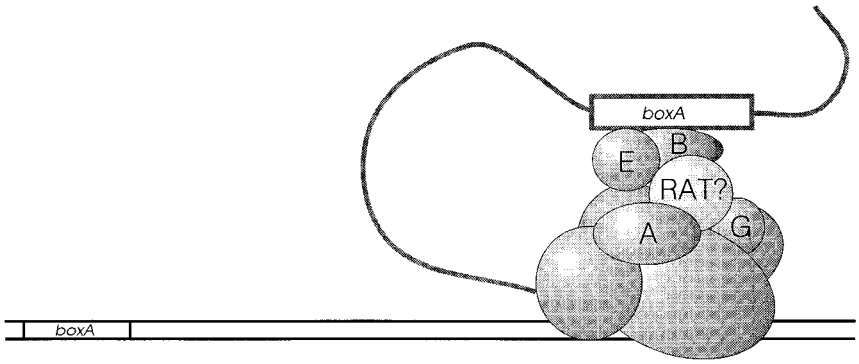

FIG. 4. Model of the *rrn* antitermination complex, showing putative tethering of the RNA *boxA* sequence to the complex. The Nus factors NusA, NusB, NusE, and NusG are represented by single-letter abbreviations. The subunits of RNA polymerase are not labeled because the interactions between specific Nus factors and RNA polymerase is not known at the subunit level. The RAT (*rrn* antitermination) factor represents the unknown host factor(s) required for *rrn* antitermination in vitro.

mutagenesis of the *nut*-like region. Consistent with the evidence presented by Gourse et al. (106), a wild-type *rrn boxA* was both necessary and sufficient for readthrough of a strong rho-dependent terminator (22). No antitermination mutations were isolated in *boxC*; however, in contrast to what the data of Gourse et al. might suggest, some mutations in *boxB* decreased *rrn* antitermination in this system. Heinrich et al. have recently shown that two *boxA* mutations which show decreased antitermination in the promoter fusion system (22) also cause defective *rrn* transcription in the context of an intact *rrnB* operon on a plasmid (127). Using a reverse transcriptase assay, they were able to distinguish between rRNA derived from the wild-type chromosomal operons and the mutant *boxA*-containing operons on plasmids. The authors conclude that the *boxA* mutations both cause about a threefold decrease per operon in the amount of plasmid-derived rRNA in the cell compared with the wild-type *boxA* control. This defect occurred early in the transcription of the 16S gene, and no polarity between the 16S gene and the 23S gene was observed. They suggest that the *boxA* mutations affect either the quantity or the quality of the antitermination complexes and that all of the RNAPs that succeed in negotiating the transcription block in the 16S gene are capable of transcribing the 23S gene also, most likely by recharging the antitermination complex at the wild-type *boxA* in the spacer region between the two genes.

The sequence requirements for *rrn* antitermination suggest that this system may share some of the factors required for lambda antitermination. Consistent with this idea, overproduction of the lambda *nutL* sequence inhibits rRNA production in vivo (293), presumably by titrating out some of the factors required for *rrn* antitermination. The first demonstration that some of the Nus factors might specifically be required for *rrn* antitermination also came from an experiment by Sharrock et al. (292), which showed that premature termination occurred within *rrn* operons in *nusB5* mutants and a cold-sensitive *nusA* mutant. Another *nusA* mutation, *nusA1*, and a *nusE* mutation, *nusE71*, had no effect on *rrn* transcription. Since these mutations were selected on the basis of deficient lambda growth, the allele specificity of the *nusA* and possibly the *nusE* mutation (see below) is perhaps indicative of a qualitative difference between the lambda and *rrn* antitermination mechanisms. Further evidence in support of the participation of NusB in *rrn* antitermination comes from the observation that overproduction of NusB can partially rescue the effects of *rrn boxA* mutations in vivo (290). This role was demonstrated directly by Squires et al. (305), who showed that readdition of NusB to a NusB-depleted cell extract was required for *rrn* antitermination in vitro.

In addition to these experiments, which established *boxA* and NusB as key players in the *rrn* antitermination system, some genetic and biochemical evidence had suggested that these two components might interact (83, 136). A direct biochemical interaction between NusB and NusE was demonstrated by affinity chromatography (195), and this heterodimer was subsequently shown to be capable of binding to *rrn boxA* RNA (231). The NusB-NusE heterodimer could not bind several mutant *boxA* sequences which had previously been shown to disrupt *rrn* antitermination in vivo (22), nor interestingly, could it bind lambda *boxA* RNA, providing further evidence that the *rrn* antitermination complex is qualitatively different from that of lambda. Nodwell and Greenblatt suggested that the role of the lambda N protein may be to stabilize the NusB-NusE interaction with lambda *boxA* RNA (231). Work in that laboratory also obtained preliminary evidence that r-protein S1 binds in a sequence-specific manner to *boxA* RNA and inhibits the binding of the NusB-NusE heterodimer to *boxA* RNA (112). As such, it is a candidate for an inhibitor of *rrn* antitermination. However, S1 has not yet been shown to have a demonstrable effect on lambda (112) or *rrn* antitermination in vitro (304).

Several interactions between RNAP and components of the lambda antitermination complex that are likely to be relevant to *rrn* antitermination have been demonstrated. NusA (109) and NusE and NusG (194) can all bind core RNAP directly. NusA is thought to associate with RNAP in exchange for sigma factor, which cycles off soon after initiation of transcription (109). Both NusA (111, 348) and NusG (35, 182, 311) play important roles in transcription pausing and termination and are therefore probably also associated with RNAP engaged in non-antiterminated transcription; NusA has been shown to contact nascent RNA in transcription complexes (188). NusA also interacts with the lambda N protein (110), which can, in turn, recognize the stem and specific nucleotides in the loop of lambda *boxB* RNA (41, 177). Indeed, the *boxB*-N-NusA-RNAP series of interactions is thought to be sufficient for short-range lambda antitermination (353). The *boxA*-NusB/E-RNAP interaction, with an as yet unidentified NusG interaction, is thought to stabilize this complex to allow terminator readthrough many kilobases downstream of the *nut* site (55, 64, 194). Summaries of the known interactions in the lambda antitermination complex are given by Das (55, 56) and Roberts

(272) and have been used to assemble the preliminary model of the *rrn* antitermination complex depicted in Fig. 4.

Elongation complexes isolated from reactions containing an S100 extract and an *rrn* template have been shown to contain NusB and NusG (181). Unfortunately, these complexes were not tested for NusA or NusE. However, addition of all four Nus factors was necessary for maximum stimulation of antitermination by an S100 extract in vitro (305), which suggests that *E. coli* and lambda may share all of their Nus factor requirements. In addition to the Nus factors, *E. coli* requires additional, as yet unidentified, cellular factors for *rrn* antitermination (305, 326). These factors may perform a function analogous to the lambda N protein and stabilize interactions within the complex, such as the NusB-NusE heterodimer interaction with RNAP; however, it is unlikely they bind *boxB*, since *boxA* is sufficient to promote antitermination both in vivo and in vitro.

Mechanism of Antitermination—Antiterminated Transcription Complex

How does the antitermination mechanism cause readthrough of transcriptional terminators? Most of the experimentation on antitermination mechanisms has been done with the lambda system, in which a direct biochemical interaction between NusG and rho has been shown (182). Thus, one possible model is that the antitermination complex sequesters rho and prevents it from gaining access to the message. It is also possible that the binding of rho by NusG inhibits the ATP-dependent translocation step required for termination. However, NusG is required for rho-dependent termination of some but not all non-antiterminated transcription complexes in vivo (34, 311) and stimulates termination in vitro at earlier sites than rho alone can do (215, 216). Furthermore, since NusG binds rho in the absence of RNAP, these models cannot account for the *cis* requirement of the antitermination element. A more plausible model is that the formation of the antitermination complex speeds up the rate of transcription elongation, for example, by increasing the affinity of RNAP for nucleotides or by preventing access to RNAP by ppGpp. Such an effect would be predicted to disrupt the kinetic coupling between RNAP and rho and lead to decreased rho-dependent termination (145). Indeed, RNAP *rpoB* mutants with altered nucleoside triphosphate affinities have been shown to have defects in both rho-dependent (145) and rho-independent (197) termination in vivo. In addition, Vogel and Jensen (339–341) have shown that the high transcription elongation rate of *rrn* operons (>80 nucleotides per s versus 40 nucleotides per s for mRNA) and the resistance to ppGpp can be attributed to the antitermination mechanism. Finally, NusG has also been shown to accelerate the elongation rate of RNAP both in vivo and in vitro (35). Thus, termination and antitermination seem to be intrinsically linked to the transcriptional elongation rate.

How Is the Antitermination Complex Stopped?

One can imagine that uncontrolled transcription into downstream genes by an antiterminated RNAP complex might be detrimental to the cell. Indeed, this is thought to be the reason that the gene for the transcription termination factor rho is essential in *E. coli* (57). Studies with the *rrn* antiterminator and tandem terminators on plasmids suggest that not all RNAP molecules that have transcribed the *rrn* leader region become resistant to termination. Most likely this is because the antitermination system is overproduced by the plasmid, but cellular antitermination factors (e.g., NusB) become limiting (290). However, those polymerases which have become modified by the antitermination mechanism retain their resistance through multiple terminators (3). Thus, it is of interest to know how the antitermination complex is stopped. The answer may lie in an important distinction between the lambda and *rrn* antitermination mechanisms; that is, the major effect of *rrn* antitermination is directed against rho-dependent terminators (3), whereas lambda antitermination complexes are highly resistant to both rho-dependent and rho-independent terminators (1, 55, 99). The presence of strong rho-independent terminators at the end of *rrn* operons indicates that the *rrn* antitermination system has probably evolved to maintain this distinction in order to halt the antitermination complex. It will be interesting to learn how the difference between the *rrn* and the lambda antitermination complexes accounts for this terminator specificity.

Alternative Roles for the *rrn* Antitermination Mechanism

In addition to its function of preventing transcriptional polarity, at least two other roles have been proposed for the antitermination mechanism in *rrn* operons. One is related to the position of *boxA* sequences in *rrn* operons, while the second is based on the influence of antitermination on the rate of *rrn* transcription. The first alternative role was proposed by Morgan (209), who noted that antitermination might aid processing of the mature 16S and 23S rRNAs from the 30S precursor transcript. The idea was originally conceived because of the proximity of the leader and spacer *boxA* sequences to the processing stalks of the 16S and 23S RNAs, respectively. This model has further evolved by borrowing from the lambda paradigm, in which the 5′ end of the transcript stays attached to RNAP through the formation of the antitermination complex (230). In this way, the 5′ stem of the processing stalk could be "delivered" to the newly transcribed 3′ stem instead of having to find it by diffusion, making the processing event more efficient. This idea is consistent with the occurrence of the second *boxA* sequence in the spacer region. Since the 16S RNA is cleaved from the precursor RNA as soon as it is completed, the ability to pick up the new 5′ end is required to facilitate processing of the 23S RNA. While such a delivery mechanism seems quite reasonable, no evidence has accumulated to support the idea that this phenomenon is physiologically important.

The second proposed role for the *rrn* antitermination system is the modulation of the transcription elongation rate to aid proper folding of the rRNA (305). We have noted above that establishing a faster transcription elongation rate may help prevent the type of premature termination seen in *nus* mutants (292). RNAPs that are not antiterminated may pause more frequently and would thus be more subject to rho-dependent termination (145). Could the transcription rate also be important in allowing newly transcribed domains to fold properly, for example, by controlling whether the polymerase pauses or not at crucial sites within the coding sequence? Since the free energy of intermediate structures and the final folded structures is not necessarily the minimum energy state of the entire RNA molecule, failure to pause or prolonged pausing at particular sites may result in the formation and "locking in" of incorrect secondary and tertiary interactions. Implicit in these observations is that a "correct" rate of transcription, neither too slow nor too fast, is required for proper transcription, folding, and subsequent assembly of functional ribosomes. Consistent with this, Lewicki et al. (180) have shown that transcription of an *rrn* operon by T7 RNAP, which transcribes mRNAs at rates up to five times that of *E. coli* RNAP, leads to largely defective ribosomes. These ribosomes can subsequently

be dissociated and reassociated into functional ribosomes in vitro, indicating that misfolding is the most likely cause of the defect. Lewicki et al. (180) have further noted that if the rate of T7 RNAP transcription is slowed by lowering the temperature to 30°C, functional ribosomes are produced. Although the elongation rate of T7 RNAP is clearly too high at 37°C, there is apparently some flexibility in the system, because a 50% increase over the normal elongation rate is tolerated in the derepression experiments described by Condon et al. (49). The observation (above) that antiterminated complexes are resistant to the slowing effects of ppGpp during the stringent response (339) is consistent with the idea that it is also in the cell's interest to preserve a particular minimum rate of rRNA elongation even when it is in the process of shutting down transcription initiation. The suggested alternative roles for antitermination in *rrn* expression provide a broader framework for exploring this phenomenon.

Is the Antitermination Mechanism Regulatory?

Since the DNA determinants for stringent control and growth rate-dependent control of rRNA have been mapped to the *rrn P1* promoters by deletion analysis, antitermination is not thought to play a role in either of these processes. However, the involvement of r-protein S10 (NusE) in the antitermination process raises some intriguing possibilities about the coordinate regulation of r-proteins and rRNA (231). A deficit of rRNA relative to r-protein would be predicted to lead to an increase in *rrn* expression caused by excess S10. Conversely, cells having excess rRNA would not contain free S10, which would lead to premature transcription termination early in rRNA synthesis, perhaps at the putative ppGpp-dependent pause site t_L. The role of this system may be to complement the well-characterized translational feedback mechanism of r-protein synthesis, where the flow of information is in the opposite direction, that is, from rRNA to r-protein (147). Finally, the observation that another r-protein, S1, is an inhibitor of the interaction between NusB/E and *boxA* (above) may provide a further link between the antitermination system and balanced synthesis of ribosomal components.

Antitermination in Other Organisms

boxA-like sequences are obvious in the leader and spacer regions of *rrn* operons from most members of the domain *Bacteria* and some members of the domain ***Archaea*** (22, 304), suggesting that the antitermination process is functionally very important. *boxA* sequence conservation is coupled with an equally impressive identification of the Nus factor genes from many bacterial species. NusA has been identified in *S. typhimurium* (52) and *B. subtilis* (294), and the *nusG* gene is present in at least three *Streptomyces* species (168, 203, 235, 260, 261), a *Synechocystis* sp. (286), *Thermatoga maritima* (184), *Thermus thermophilus* (128), a citrus greening disease-associated bacterium-like organism (338), and *Staphylococcus carnosus* (198). The wide range of bacteria in which these genes have been identified may speak to their importance as general transcription and termination factors as much as to the conservation of the antitermination mechanism. In spite of such striking conservation of the antitermination machinery, there have been no studies demonstrating *rrn* antitermination in organisms other than *E. coli.*

The *boxA* motif is conspicuously absent from *rrn* operons of *Eucarya*. Although specific antitermination mechanisms have been identified in eukaryotes, in particular retroviruses (53, 54, 158) and oncogenes (69, 220, 221), antitermination of rRNA transcription in eukaryotes has not been reported. However, a PolII transcription factor, SIII, which is capable of increasing the overall rate of transcription elongation by reducing transient pausing of RNAP has recently been purified from mammalian cells (7, 94). One of the subunits of this heterotrimeric protein shows significant homology to both the rho and NusB proteins of *E. coli*, suggesting that SIII may be evolutionarily related to bacterial proteins involved in the control of termination and antitermination.

CONCLUDING REMARKS

The control of rRNA synthesis is a highly complex process, fine-tuned to the cellular requirement for ribosomes and the balanced synthesis of ribosomal components. While most of this control occurs at the level of transcription initiation, the completion of functional transcripts is also dependent on the maintenance of a high elongation rate, about twice that of mRNA. Most of the regulation of transcription initiation takes place at the more upstream of the tandem *rrn* promoters, *P1*. Here, at least four different mechanisms of regulation and activation interplay over a relatively short region of DNA. Indeed, it is this congestion that has made it difficult to dissect two of the controlling processes, stringent regulation and growth rate-dependent control. Although much has been accomplished in the understanding of *rrn* expression, there are still many unresolved issues. First, although ppGpp is the acknowledged effector of the stringent response, it is still not clear how it functions, that is, how it alters the promoter specificity of RNAP and how this is related to the effects seen in the elongation phase of mRNA. Second, it has not yet been resolved exactly how growth rate-dependent regulation is achieved, whether more than one mechanism is involved, what role is played by ppGpp, and by what mechanism SpoT responds to the intracellular carbon and energy source pools to regulate either its ppGpp synthetase or hydrolase activity. Third, how interactions between RNAP and both Fis and the UP element lead to such high levels of activation remains to be elucidated. Finally, in the area of *rrn* antitermination, the host factor(s) required to complete the antitermination complex has yet to be identified, as has the mechanism by which this large complex is resistant to termination signals and the effect of ppGpp in the elongation phase of transcription. With the resolution of some of these problems will come a clearer picture of a delicately poised network of regulatory mechanisms that is exquisitely sensitive to the growth requirements of the bacterial cell and that will hopefully serve as a paradigm for other complex regulatory networks.

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