

REGULATION OF THE SYNTHESIS OF RIBOSOMES AND RIBOSOMAL COMPONENTS

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PERSPECTIVES AND SUMMARY

The protein-synthesizing machinery of *Escherichia coli*, the ribosome, consists of some 52 ribosomal proteins (r-proteins), and three ribosomal RNAs (rRNAs). Under the most favorable laboratory growth conditions, as much as 40% of the total dried cellular mass of this bacterium is ribosomes and almost all the ribosomes are actively engaged in protein synthesis. With so much of the cell's energy devoted to constructing this elaborate apparatus, it is essential that the multiple ribosomal components be synthesized and utilized efficiently so that energy is not wasted. The control of ribosomal synthesis can be considered in relation to three basic problems:

The first problem is the coordination of the production of 52 r-proteins with each other and with the three rRNAs. Except for r-protein L7/L12, which is present in four copies, each r-protein and each of the three rRNAs is present in one copy per ribosome (1). Free r-proteins and rRNAs are found in only very small quantities in growing cells (2–4), and degradation of these components is insignificant except for the degradation of rRNA during slow growth (3, 5). Thus, their rates of synthesis reflect the amounts they represent in the ribosome. The coordinate and equimolar synthesis of the more than 50 different components could be the result of independent regulation of all the r-proteins and rRNA, or of direct primary regulation of either r-protein or rRNA synthesis coupled with the subsequent regulation of the remaining species as a consequence of the primary regulation. The major facets of the mechanism of this coordination have been worked out since the most recent reviews of ribosome regulation in this series (6, 7). In summary, it has been demonstrated that certain r-proteins function as inhibitors of protein synthesis from their own mRNAs. This translational feedback mechanism plays a crucial role in the balanced and coordinate synthesis of the 52 r-proteins, which is in turn coordinated with the synthesis of rRNA. In this review we discuss in detail the essential features of the current model for this translational regulation including the identification of the specific repressor r-proteins and their units of regulation, the determination of the target sites for this inhibition, probable structural features of the target sites for repressor r-proteins, and the mechanism by which multiple cistrons are regulated by the action of a single repressor protein. Translational coupling of several cistrons on a polycistronic mRNA is most likely the basis for this mechanism, and its existence is now firmly established for some r-protein operons.

The second problem is how cells attain the number of ribosomes appropriate for the growth rate achieved under a particular set of nutritional conditions. It is known that, under most growth conditions, the cellular concentration of ribosomes is proportional to the growth rate (i.e. to the total protein synthesis rate). Under these conditions, cells utilize all but a small fraction of the ribosomes for protein synthesis, and the level of total protein synthesis required for growth is achieved by controlling the number of ribosomes rather than modulating the activity of individual ribosomes (for reviews see 8–10). An important feature of the mechanism of this control, termed “growth rate-dependent control,” has recently become clear. It appears that ribosomes synthesized in excess of the amount needed for protein synthesis under a given set of growth conditions act directly or indirectly to inhibit, by a feedback mechanism, the transcription of rRNA genes and other related genes such as tRNA genes. This model and its alternatives are discussed in this review.

The third problem is the response of ribosome synthesis to conditions of limiting aminoacyl tRNA availability (amino acid starvation) and has been termed "stringent control". *E. coli* cells have the ability to respond to a decrease in the degree of charging of tRNA with a rapid decrease in the synthesis of ribosomal components and tRNA and a concomitant large accumulation of guanosine-tetraphosphate (ppGpp) and pentaphosphate (pppGpp). While some advances have been made since the last review on the subject (11), the definitive answers are yet to come. In particular, it has not been determined whether a common mechanism is involved in both stringent control and growth rate-dependent control. In the following pages we discuss recent advances in this area, including possible roles for ppGpp and models for the mechanism of stringent control.

Since the regulation of ribosome biosynthesis in *E. coli* has been extensively studied and is now fairly well defined, we can attempt to put this knowledge into some sort of perspective with regard to the regulatory mechanisms utilized by other organisms. An understanding of the process of ribosome biosynthesis in eukaryotic cells is still in the early stages. We shall not attempt to cover the subject rigorously, but will make a brief note on some recent advances in this area and consider the possibility of the existence of feedback regulatory mechanisms, similar to those identified in *E. coli*, in higher organisms.

Finally, the genes for several tRNAs, RNA polymerase subunits, translation elongation factors, and even some for proteins involved in DNA synthesis and the protein secretory apparatus are linked genetically to r-protein or rRNA genes. This interspersed gene arrangement allows speculation on the possible coordination of the control of gene expression for these cellular components with ribosome biosynthesis. A detailed analysis of the regulation of these various components is beyond the scope of this review and only some of them are touched upon. Recent reviews on these subjects (12–14), on ribosome structure (15–17), and on ribosome genetics and regulation (6, 7, 10, 11, 18–22) can also be consulted.

REGULATION OF RIBOSOMAL PROTEIN SYNTHESIS

Regulation of r-protein synthesis is obviously related to the regulation of rRNA synthesis, but for the sake of convenience they are described separately in this review. Inevitably, our discussion focuses almost exclusively on the *E. coli* ribosome system, which most of the studies on regulation have centered on. The genetics and regulation of ribosome biosynthesis of another bacterium, *Bacillus subtilis*, have recently been reviewed (23).

Organization of Ribosomal Protein Genes

The existence of some mutations involving r-proteins allowed the early identification of a few r-protein genes and implicated two regions of the *E. coli* chromosome where r-protein genes and other related genes might be clustered. Subsequently, λ transducing phages carrying these DNA regions, the “*str-spc*” region at 72 min (24, 25), and the “*rif*” region at 89 min (26), were isolated and used for identification and mapping of r-protein genes (for reviews, see 7, 27), as well as for regulation experiments discussed later in this article.

The “*rif*” region was found to encode four ribosomal protein genes, organized into two transcriptional units (“operons”), and the “*str-spc*” region 27 ribosomal protein genes subdivided into four operons. Figure 1 shows the exact arrangement of these genes. In addition, it was found that some of these r-protein operons contained genes for translation/transcription factors, such as elongation factors EF-Tu and EF-G, the RNA polymerase subunits α , β , and β' , and a gene involved in protein secretion (37, 38) (see Figure 1 and legend). Further studies employing saturation mutagenesis to create temperature-sensitive mutants carrying r-protein electrophoretic mobility alterations (see 20) and recently available recombinant DNA techniques have resulted in the identification of all remaining r-protein gene locations with the exception of L20 (Figure 1). The transcriptional organization for a few of these operons is known and indicated on the figure. As in the *str-spc* and *rif* regions, several of the newly mapped genes for r-proteins are transcriptionally linked with genes important in transcription or translation. The S21 gene (*rpsU*) is cotranscribed with the gene for DNA primase (*dnaG*) and the σ subunit of RNA polymerase (*rpoD*) (31, 32); the genes for S16 (*rpsP*) and L19 (*rplS*) are cotranscribed with the gene for tRNA (m^1G) methyltransferase (*trmD*) (30). The significance of these gene arrangements is still unclear. Finally, it should be noted that while the direction of transcription of the majority of the r-protein genes is the same as the direction of DNA replication, as in the case of the transcription of rRNA operons (see below), more recent studies have shown that this is not always true (e.g. for the S21 gene, see 31).

Translational Feedback Regulation

DNA SEQUENCE ANALYSIS OF R-PROTEIN PROMOTERS As just described, the genes responsible for the synthesis of the 52 r-proteins are scattered throughout the *E. coli* chromosome and organized into at least 20 transcriptional units containing from 1 to 11 r-protein genes. Yet the synthesis of proteins from all these genes is balanced and coordinately regulated (5, 10). A simplistic solution to this complex regulation problem

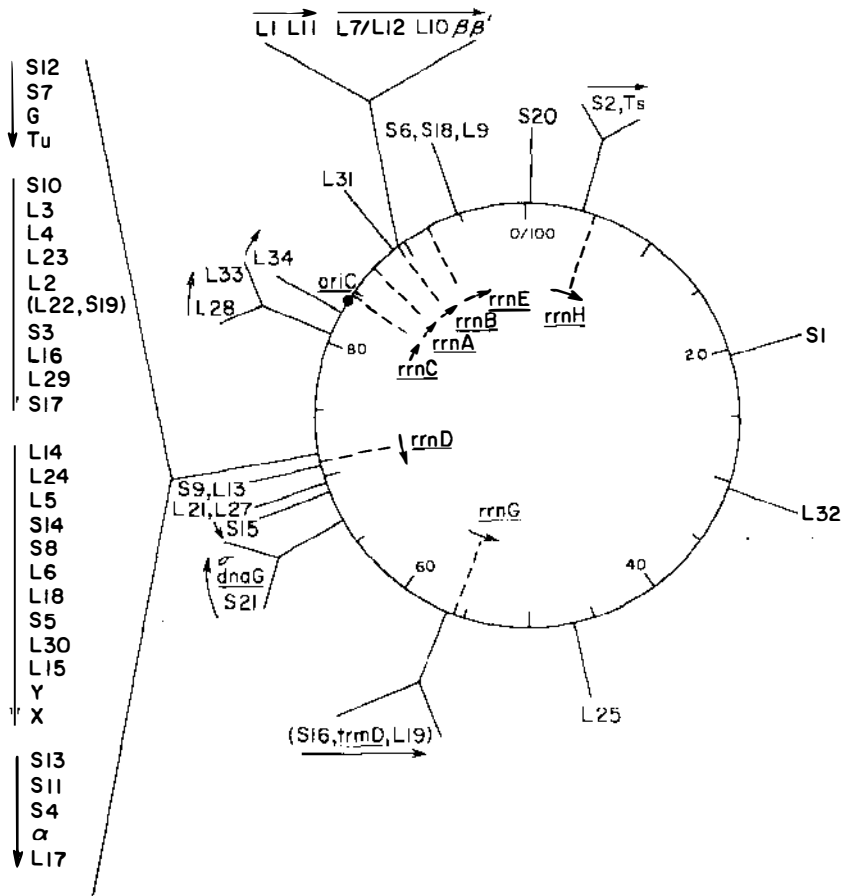


Figure 1 Genetic map of *E. coli* showing the locations of r-protein and rRNA genes. Genes for r-proteins are represented by the protein product. The map positions of many of the r-protein genes were taken from (20, 28, 29). Additional information on the map location and transcriptional orientations of the S16/L19 operon (30), the S21 operon (31, 32), and the S9 operon (33) is included in the figure. The genes for RNA polymerase subunits α , σ , β , and β' ; elongation factors EF-Tu, EF-Ts, and EF-G; DNA primase (*dnaG*), tRNA (m^1G) methyltransferase (*trmD*); and a gene, Y, for a component of the protein secretion apparatus (*secY* or *prlA*) cotranscribed with r-protein genes are also shown. X is a protein of unknown function (34). The gene order of L22 and S19 is unknown. The map positions of the rRNA operons are indicated by their respective genetic nomenclatures, and were taken from (29, 35). To avoid confusion, it should be noted that *rrnF* was originally mapped incorrectly, and so is not included on the map. The seventh rRNA operon was subsequently named *rrnH* (see 35, 36 for discussion). The directions of transcription of the rRNA operons and r-protein genes, when known, are indicated by the arrows.

would be to postulate that all of these various r-protein promoters possess identical transcriptional strengths (activities) and respond similarly to regulatory "signals" present in the cell. In this manner mRNA synthesis would be coordinated and, with the further assumptions of equal translational efficiencies and stabilities of all the messages, would result in equimolar synthesis of the protein products. Thus, similar to other gene systems controlled by a common effector, such as the *E. coli* operons of the SOS system (39), the nucleotide sequences of r-protein promoter regions would be expected to have common features uniquely involved in this regulation. To examine this possibility the DNA sequence of the promoter regions of five r-protein operons was determined (40–42). Although the sequence analysis revealed (40, 43) a G-C-rich region between the "Pribnow box" (44) and the transcription start site as a common feature of the promoter region, the degree of homology was not as striking as would be expected from the simple transcriptional model described above (reviewed and discussed in 28). The subsequent determination of ribosomal protein promoter sequences for additional operons (30–32, 45–47) has also failed to reveal any persuasive evidence for common features.

GENE DOSAGE EXPERIMENTS Early evidence that r-protein synthesis is regulated at a post-transcriptional level came from measurements of r-protein and mRNA synthesis rates in *E. coli* strains carrying extra copies for given r-protein genes (gene dosage experiments). In these experiments, the gene copy number of given r-protein genes in the cell was elevated with respect to the remaining haploid r-protein genes by the introduction of specialized transducing phages, hybrid plasmids constructed in vitro, or F factors carrying the given r-protein genes (48–56). Measurements of the r-protein mRNA synthesis rates in these strains demonstrated that the transcription of the r-protein genes present in elevated amounts increased in proportion to the gene dosage. The synthesis rates of the corresponding r-proteins, however, was not proportional to the gene dosage; i.e. their synthesis did not significantly increase relative to the synthesis rates of r-proteins whose genes were present in a single copy. Thus the cells were able to maintain the balanced synthesis of all the r-proteins despite the unbalanced synthesis of some r-protein mRNAs, indicating the presence of a post-transcriptional regulatory mechanism. In addition, it was found that the oversynthesized mRNA was preferentially degraded so that the steady-state amount of mRNA increased only to a lesser extent (48, 49, 54, 56).

The presence of post-transcriptional regulation revealed in this manner has been substantiated for the *spc* and α -operon genes (48, 49, 54), the L10-L7/L12 genes (50), the S1 gene (55), the S15 gene (52, 53), and the S20 gene (56). However, failure to observe such regulation was reported for the S21,

L21, and L27 genes (52). The failure may indicate that they are regulated by a different mechanism (see a later section), or that the previous conclusion on the regulation of r-protein synthesis is not completely correct and the synthesis of some r-proteins including S21, L21, and L27 is not regulated or is only "loosely" regulated. Further experiments are necessary to clarify the regulatory behavior of these "exceptional" r-proteins.

On the basis of the gene dosage experiments, a model for the translational feedback regulation of r-protein gene expression was proposed (49). In this model, it is proposed that certain free r-proteins act as feedback inhibitors of the translation of their own mRNAs, so that the synthesis of r-proteins is coupled with the process of ribosome assembly; as long as the process of ribosome assembly removes r-proteins, the corresponding mRNAs escape translational inhibition. Such a mechanism would ensure the balanced and coordinate synthesis of all r-proteins with respect to one another and with the synthesis of rRNA. The essential features of the model have been previously reviewed (22, 57) and a summary of these features and their implications will be described below.

R-PROTEINS AS TRANSLATIONAL REPRESSORS When the feedback regulation model was originally proposed it was not known whether every r-protein or only certain selected r-proteins could act as translational inhibitors ("repressors"). A DNA-dependent *in vitro* protein-synthesizing system was used to test the model directly as well as to identify the postulated r-protein repressors. Purified DNA templates carrying r-protein genes were used to direct the synthesis of r-proteins *in vitro*, and the effects of the addition of individual purified r-proteins on the synthesis of r-proteins were monitored. The results demonstrated a specific inhibitory effect on r-protein synthesis by the addition of certain, not all, r-proteins. This inhibitory effect was shown to be operon specific; i.e. the identified inhibitory r-proteins (repressor r-proteins) specifically inhibited r-protein synthesis from genes within their own operons, but not from other operons examined. The following r-proteins were identified in this manner to have repressor activity: S4 (58), S7 (59), S8 (58, 60), S20 (61), L1 (58, 62), L4 (63), and L10 (64-67). [Inhibition of *in vitro* synthesis of S1 by S1 was recently reported (68), but because of some observed differences in the regulatory pattern *in vivo*, it was suggested that S1 regulation might be different from the standard translational regulation observed with other r-proteins (68).] By uncoupling the transcription step from the translation step in this system it was shown that the inhibitory effect of the repressor r-protein takes place at the level of translation (58, 59, 63, 64). The results of these *in vitro* experiments are shown in Figure 2, which indicates the identified repressor protein for each operon and the genes regulated by repressor action.

Consistent with the demonstration that the inhibition occurs post-transcriptionally are the following observations. First, the unit of regulation is not always equal to the transcriptional unit. For example, in the *spc* operon the S8 repressor protein does not inhibit the synthesis of the two promoter proximal proteins, L14 and L24, while synthesis of the third and fourth gene products, L5 and S14, show marked inhibition (60). This is also true with the *str* operon (59). Second, it has been demonstrated that the specific translational inhibition in vitro of S7, L7/L12, and of L11 and L1 synthesis can occur when templates lacking the genuine promoters are used to direct the synthesis of these gene products (59, 66, 71).

L11 OPERON	P	L11	L1										
IN VITRO		+	+										
IN VIVO		+	(+)										
RIF OPERON	P	L10	L7/12	β	β'								
IN VITRO		+	+	-	-								
IN VIVO		(+)	+	-	-								
STR OPERON	P	S12	S7	EF-G	EF-Tu								
IN VITRO		-	+	+	-								
IN VIVO		-	(+)	+	-								
S10 OPERON	P	S10	L3	L4	L23	L2	(L22, S19)	S3	L16	L29	S17		
IN VITRO		+	+	+	+	±	-	-	-	-	-		
IN VIVO		+	+	(+)	+	+	+	+	+	+	+		
SPC OPERON	P	L14	L24	L5	S14	S8	L6	L18	S5	L30	L15	Y	X
IN VITRO		-	-	+	+	-	-	-	-	ND	ND	ND	ND
IN VIVO		-	-	+	+	(+)	+	+	+	+	+	ND	ND
α OPERON	P	S13	S11	S4	α	L17							
IN VITRO		+	+	+	-	-							
IN VIVO		+	+	(+)	±	+							
S20 OPERON	P	S20											
IN VITRO		+											
IN VIVO		(+)											

Figure 2 Regulation of gene expression in vivo and in vitro, by the identified repressor r-protein. Genes are represented by the protein product, and the direction of transcription from the promoter (P) of each operon is indicated by the arrow. It should be noted that in vivo, the primary *rif* (formerly called the β operon; see e.g. 41, 69) and α -operon transcripts are the result of read-through from the upstream L11 and *spc* operons, respectively (70, 34). Regulatory r-proteins are indicated by the boxes, and the effects of the boxed proteins on the in vitro or in vivo synthesis of proteins in the same operon are indicated. Results obtained in vivo are those from experiments using hybrid plasmids to achieve overproduction of the regulatory protein. Symbols: +, specific inhibition of synthesis; -, no significant effect on synthesis; ±, weak inhibition of synthesis; (+), inhibition presumed to occur in vivo; ND, not determined. It has not been established how L14, L24, or S12 is regulated, but the results of gene dosage experiments suggest that L14 and L24 each regulates the translation of its own mRNA, respectively (P. Singer, D. Dean, M. Nomura, unpublished).

The assignment of repressor function to these select r-proteins was verified *in vivo* by the use of gene fusion plasmids. As with the addition of exogenous purified r-proteins to the *in vitro* protein-synthesizing system, the *in vivo* overproduction of repressor r-proteins should result in the decreased synthesis of r-proteins contained within the regulatory unit. Gene fusion techniques were used to place the genes for given r-proteins under the control of inducible promoters (*lac* or *ara* operon promoters) on multicopy plasmids. Measurements of r-protein synthesis *in vivo* subsequent to induction of the fused genes indicated that overproduction of the repressor r-proteins identified *in vitro* resulted in the decreased synthesis of r-proteins within the regulatory unit (59, 60, 66, 72–74; see Figure 2). Similar experiments for the S20 repressor protein could not be performed as there are no coregulated proteins in this monocistronic operon. Control experiments indicated that the overproduction of the following proteins had no effect on the synthesis of other r-proteins: S3, S5, S10, S11, S12, S17, S19, L2, L3, L6, L7/L12, L14, L15, L16, L22, L23, L24, L29, L30 (73; D. Dean, M. Nomura, unpublished). As with the S20 operon, however, the results of this type of experiment do not exclude the possibility that any of these proteins participates in the regulation of its own synthesis.

Although the results of these two types of experiments show perfect correlation with regard to the assignment of repressor activity to certain r-proteins, there are some inconsistencies in the definition of the “regulatory unit” defined by the experiments for each repressor protein. In the S10 and *spc* operons, for example, synthesis of the distal r-proteins is not inhibited by the designated repressor protein (L4 and S8, respectively) *in vitro*, but does show decreased synthesis as a result of repressor overproduction *in vivo*. Although we do not have formal proof, we believe that the *in vivo* results define the actual unit of translational regulation. Possible explanations for the apparent escape of the distal cistrons from the regulation *in vitro* will be discussed below.

With the exception of EF-G, the synthesis of nonribosomal protein gene products (such as, EF-Tu, α , β , β') encoded on these regulated operons appears to be insensitive to translational feedback inhibition by repressor r-proteins. The synthesis of β and β' appears to be modulated by mechanisms involving attenuation (75–77), mRNA processing (77), and a feedback inhibition by RNA polymerase (see reviews 12, 14, 78).

MECHANISM OF TRANSLATIONAL FEEDBACK REGULATION

Single target site mechanism The results of both the *in vitro* and *in vivo* experiments indicate that a particular repressor r-protein acts to regulate the synthesis of multiple r-proteins from a polycistronic mRNA molecule. The inhibition of translation of several cistrons by a given repressor

r-protein could occur by interaction of repressor r-protein molecules at each sensitive cistron on the polycistronic mRNA, or by the action of a repressor protein at a single mRNA target site that affects the translation of all the regulated cistrons as a direct consequence of this single interaction. The results of experiments conducted in vitro and in vivo demonstrate that the latter mechanism is correct. In the L11 operon, L1 inhibits the synthesis of both L11 and L1 (see Figure 2). When a template containing the carboxyl portion of the L11 gene and the intact L1 gene was used in the in vitro system, synthesis of L1 was not inhibited by exogenously added L1 protein. On the other hand, when a template contained the promoter for the L11 gene and a small portion of the N-terminal sequence of the L11 gene, this template directed the synthesis of a small polypeptide consisting of the first approximately 30 amino acids of L11, and this synthesis was inhibited by L1 (79). More recently it has been demonstrated that deletions in the leader mRNA region of this operon result in unregulated synthesis of both L11 and L1 in vivo and in vitro (71). The results of this experiment localized the target site near the ribosome binding site. Similarly, while L10 regulates the synthesis of both L10 and L7/L12 in vitro from intact DNA templates, synthesis of L7/L12 as directed by a template lacking the promoter region as well as the preceding L10 gene is not inhibited by exogenously added L10 (66). The target site for the L10 repressor has been inferred from mutational analysis (80) and localized by direct in vitro experiments (67). [Mutants isolated earlier and thought to carry alterations in the target site for L10 (81) were found to have reduced translation initiation efficiencies for the L10 cistron rather than alterations in the target site (80).] Thus the repressor r-proteins act to inhibit translation of multiple cistrons by interaction with a single target site located near, or at, the translational initiation region of the first regulated cistron. Inhibition of the synthesis of the first dipeptide of the first cistrons by repressor r-proteins was also demonstrated (82).

A possible exception to the single target site mechanism for repressor protein action might be the regulation of the synthesis of proteins from the α -operon. Experiments suggested that S13, S11, S4, and L17 synthesis is regulated by S4, yet the regulation of the synthesis of α , whose gene is located between the S4 and L17 genes, appears to be different from that of the surrounding r-protein genes under some conditions, e.g. during amino acid starvation or rifampicin treatment (83–85). Further experiments are required to determine if the S4 repressor protein has two target sites—one at or near the beginning of the S13 cistron and the other at or near the beginning of the L17 cistron—or if some other mechanism is involved.

Homologies in binding sites between r-protein mRNA and rRNA It has been noted earlier that most of the identified regulatory r-proteins are

r-proteins known to bind to rRNA, strongly and specifically, early during the process of ribosome assembly (86, 87). Consequently it was possible to speculate that the repressor r-proteins recognize the same general structural features on their mRNAs that they recognize on rRNA during ribosome assembly (57). Regulation of r-protein synthesis would be the result of a competition between similar binding sites on rRNA and mRNA for repressor r-protein binding. Presumably the affinity of repressor r-protein for rRNA is higher than that for mRNA and thus ribosome assembly is favored over feedback inhibition. In addition, it should be noted that ribosome assembly is a highly cooperative process; a r-protein that has a rather low affinity for rRNA and participates in ribosome assembly mainly through protein-protein interactions could still have a sufficiently strong interaction with its mRNA for feedback inhibition to occur and thus serve a regulatory function (88). In addition, the cooperativity in the assembly reaction is expected to improve the discriminatory power of the protein binding to rRNA vs mRNA so that inhibition of mRNA *in vivo* begins at free repressor concentrations where most rRNA is in the assembled particles (89).

The localization of the target site for translational regulation to a small region on the mRNA near (or at) the initiation site for the first cistron in the regulatory unit allowed the inspection of this mRNA sequence for features homologous to the known rRNA binding sites for these proteins. Homologies in nucleotide sequence and/or potential secondary structure were recognized for r-proteins S4, S7, S8, L4, L1, and L10 (57, 67, 90–92). In all cases the homologous regions of the target mRNA include the ribosome binding site (Shine-Dalgarno sequence and/or the translation initiation codon), suggesting that the repressor function involves inhibition of ribosome binding and/or translation initiation at the first sensitive cistron.

There is experimental evidence supporting the suggested competition between rRNA and the target mRNA for repressor r-protein binding. Translational repression of *in vitro* r-protein synthesis by L1 (79) or L10 (67) is relieved by the addition of purified 23S rRNA to the assay mixture. Similarly, the addition of 16S rRNA to a DNA-directed *in vitro* protein-synthesizing system containing the S20 operon causes a specific stimulation in S20 synthesis (93, 94). More directly, the L10 protein has recently been shown to bind to the L10 leader sequence (the presumed target site), and 23S rRNA competes for this binding (67).

Recent studies of L11 operon regulation have provided preliminary evidence that the repressor r-protein L1 utilizes similar structural features in the target mRNA and rRNA for its specific recognition and binding. A survey of L1 rRNA binding sites from various eukaryotic and prokaryotic species (91, 92) identified a conserved stem-loop structural feature involving at least 3 G-C pairs for the stem formulation. This stem structure is present

in all L1 rRNA binding sites (*Dictyostelium* and *Xenopus*, *E. coli*, and other bacteria) and also in the proposed homologous L1 mRNA target site. The disruption of this postulated stem structure by alteration of two of these G residues by site-specific mutagenesis using a synthetic oligonucleotide relieved L1 inhibition (and presumably L1 binding) in vitro (G. Baughman, M. Nomura, J. Rossi, unpublished).

The above discussion on structural homologies between rRNA and r-protein mRNA implies that the same region of repressor r-proteins is involved in the interaction with both rRNA and r-protein mRNA. Consistent with this inference is the finding that some mutational alterations in S4 affect both its ability to function as a repressor and its ability to bind to rRNA (95–97; for further discussion see 88).

Under conditions of steady-state growth, the competition for repressor r-protein binding between the rRNA and target mRNA sites could occur by either of two mechanisms: One possibility is that the newly synthesized repressor r-proteins immediately interact with their own mRNAs and that prevention of such an interaction requires competition for repressor r-proteins by ribosome assembly (i.e. repressor function takes place only in *cis*). The alternative possibility is that the newly synthesized repressor r-protein is released from the translated mRNA (i.e. enters a “free pool”) and then interacts with either rRNA or an mRNA target site, according to its affinity for these RNAs. In this case, repressor r-proteins function not only in *cis*, but also in *trans* on any homologous mRNA with the proper target site. Results of experiments in vivo examining the ability of S4 protein synthesized from an operon carried on a transducing phage to regulate chromosomal synthesis of a mutant S4 protein demonstrates that S4, and probably all translational repressor r-proteins, function in *trans* (95).

Mechanisms for regulation from a single target site and translational coupling It is possible to envision several mechanisms to explain the translational inhibition of several cistrons as the result of an interaction at a single target site. These possibilities include messenger RNA degradation, transcriptional polarity, and translational coupling. These are discussed below.

The post-transcriptional regulation of multiple cistrons within a regulatory unit could result from the recognition of repressor r-protein target mRNA complexes by specific nucleases, leading to selective degradation of the multicistronic message and inhibition of distal gene expression. It was found in the gene dosage experiments using strains diploid for r-protein genes (49) that the excess synthesized mRNA in the diploid strains was preferentially degraded. Specific inhibition of r-protein synthesis by repressor r-proteins in vitro, however, can take place without mRNA

degradation (64, 79). It is probable that the selective inactivation of mRNA observed *in vivo* is a secondary consequence of translational repression and that this mRNA degradation is not the primary mechanism responsible for the regulation of multiple cistrons within a regulatory unit.

A second possibility is that inhibition of translation of the first cistron in a regulatory unit by the repressor r-protein causes termination of transcription, thus preventing the expression of all the downstream cistrons in the operon. In support of this mechanism it has been reported that the overproduction of L4 *in vivo* results in a marked decrease in the synthesis of mRNA from the S10 operon (73). Although inhibition of transcription could be considered as a secondary consequence of inhibition of translation by the repressor r-protein (22, 98), recent experiments by Lindahl et al (99) demonstrated that inhibition of transcription is due to transcription termination at a site preceding the translation initiation site of the first gene. Thus they suggested an additional independent "attenuation" mechanism superimposed on the previously observed L4-mediated translational repression mechanism demonstrated *in vitro* (63). This attenuation mechanism, however, does not appear to operate in other r-protein operons. The early gene dosage experiments mentioned above clearly demonstrate that translational regulation occurs without concomitant transcriptional inhibition in the *spc*, α , and *rif* operons (48–50, 54). Gene dosage experiments on single gene operons, the S1, S15, and S20 operons (53, 55, 56), do not indicate the presence of the attenuation mechanism either. In addition, comparison of L11 operon messenger RNA synthesis from hybrid plasmids in which the L1 translational regulation site is intact or deleted to various extents indicated that inhibition of translation of the proximal cistron either by translational inhibition by L1 or by inactivation of the translation initiation site does not cause significant inhibition of distal message synthesis as measured both *in vivo* and *in vitro* (71). Finally, the inhibition of r-protein synthesis *in vitro*—not only from the first but also from at least some of the distal genes—was found to occur in the α , L11, *spc*, and S10 operons in the absence of transcription (58, 60, 63). Clearly, such *in vitro* effects cannot be explained by a transcription termination mechanism. It is not clear why two apparently independent regulatory mechanisms, translational repression and attenuation, exist in the S10 operon but not in any other r-protein operons studied so far.

Recent evidence suggests that the translation of downstream cistrons in a regulatory unit is dependent upon the translation of the first cistron in that unit; that is, they are translationally coupled. By this mechanism inhibition of translation of the first cistron through the direct interaction of the repressor r-protein with the target mRNA results in translational inhibition of distal cistrons. Hybrid plasmids were constructed that contained the L11

operon genes under the transcriptional control of the *lac* promoter, so that a large stimulation of the synthesis of L11 and L1 mRNA could be observed upon induction with a lactose operon inducer, isopropyl thiogalactoside (IPTG). Deletions that disrupt translation initiation at the L11 gene (the first structural gene in this hybrid operon) were shown to abolish the synthesis of the distal L1 protein without significant inhibition of the synthesis of the message for L1 (71). Thus the synthesis of L1 is translationally coupled with the synthesis of the preceding L11 protein, and this must be the basis for the translational regulation of L1 synthesis by L1.

It has been suggested for this and other operons demonstrating translational coupling (100, 101) that independent initiation of translation is prevented at the distal cistrons. The exact mechanism by which this coupling occurs remains obscure. One possibility is that the initiation codon for the regulated downstream gene is normally sequestered in an RNA secondary (or tertiary) structure and unavailable for recognition by ribosomes. Translation of the upstream gene would disrupt this structure and allow ribosomes to bind to the initiation site of the coupled distal gene. Then two possibilities can be considered. First, independent ribosomes can initiate at the newly opened initiation site, or second, only the same translating ribosome (or a ribosomal subunit, e.g. the 30S subunit) can initiate and continue on to translate the downstream cistrons. This second mechanism is called "sequential translation" as a special case of translational coupling (79). In either case, coregulation is guaranteed, but in the second possibility equimolar synthesis would be assured. [There is a precedent for the translational coupling mechanism proposed above: In the RNA phage R17 system the translation initiation site of the replicase gene is usually masked by a secondary structure and becomes available for ribosome binding only upon translation of the preceding coat cistron (102). Translation reinitiation as studied in the T4 phage rII system (102, 103) and the *lacI* gene system (104) also indicates that some internal AUG codons are normally masked. When translating ribosomes stop at a mutationally created chain termination codon located close to these AUG codons, these codons become available for initiation by ribosomes.]

In the case of translational coupling of the L10 and L7/L12 cistrons (66), it is clear that new ribosomes must bind to the opened initiation site of the distal L7/L12 cistron because the synthesis of L7/L12 protein is at least four times higher than that of the proximal protein L10. It is known that the distance between the L10 and the L7/L12 cistrons is large [66 nucleotides (41)], while in all known cases where equimolar synthesis is observed, the intercistronic distance between the two coupled cistrons is relatively small (105). In the case of L11-L1 the two cistrons are separated only by three nucleotides (41). Thus, physical proximity of the translation termination

sites and the subsequent initiation sites might guarantee reinitiation by the same ribosome with a frequency of one; that is, sequential translation [see also (106) for a discussion of reinitiation after premature termination of translation].

As mentioned above, the genes regulated by certain translational repressor r-proteins, such as L4 and S8, are not unambiguously defined; there are discrepancies between the *in vitro* results and the *in vivo* results (see Figure 2). According to the translational coupling model the apparent escape of the distal cistrons from inhibition by the repressor protein *in vitro* is perhaps due to physical separation of distal cistrons from the target site, for example, by nonspecific nucleolytic cleavage of the mRNA in the *in vitro* system; such fragmentation of mRNA would uncouple translation and allow independent ribosome initiation, with reduced efficiencies, at the normally sequestered initiation sites of downstream cistrons. Alternatively, the apparent escape could be due to independent ribosome initiation at the distal cistrons under *in vitro* conditions as opposed to *in vivo* conditions.

PHYSIOLOGICAL SIGNIFICANCE OF TRANSLATIONAL REGULATION Several lines of experimental evidence indicate that translational feedback regulation as described in the preceding sections plays a significant role in the control of r-protein synthesis *in vivo*. For example, strains that have mutational alterations in repressor r-proteins specifically overproduce the r-proteins in the corresponding regulatory unit (95, 97, 107, 108). Such observations indicate that the capacity for r-protein synthesis in the cell exceeds the actual synthesis under a given set of conditions and that the overproduction of r-proteins is normally prevented by repressor r-proteins by use of the translational feedback mechanism. We shall discuss this point in more detail in the section on transcription of r-protein genes.

TRANSLATIONAL REGULATION IN OTHER SYSTEMS Feedback regulation of gene expression, as exemplified by the translational feedback regulation involved in the regulation of r-protein synthesis, may turn out to be a common mechanism to regulate synthesis of proteins that are components of supramolecular structures or nucleic acid-protein complexes. Use of free, uncomplexed subunit proteins as repressors would prevent unnecessary synthesis of these component proteins. When proteins are nucleic acid-binding proteins, their ability to bind nucleic acid could be used for their regulatory function as in the case of r-protein. Several examples of translational regulation are known (for review see 109) and are briefly discussed below.

Autogenous translational regulation was first discovered in the phage T4 gene 32 system (110–112). The gene 32 protein has a strong affinity for single-stranded DNA and, to a lesser extent, for single-stranded RNA (113).

Gold and co-workers (111, 112) have shown that the gene 32 protein can reversibly inhibit translation of its own mRNA, and that the inhibition takes place only after the titration of the gene 32 protein onto available intracellular single-stranded DNA. Thus during infection, gene 32 protein is synthesized and, by preferentially binding to newly synthesized single-stranded phage DNA, participates in the replication of this DNA. When the phage DNA is saturated with this protein, the concentration of free gene 32 protein increases, resulting in specific binding to a target region on its own mRNA at the translation start site, thereby preventing further synthesis of gene 32 protein. The target site on the mRNA was recently identified and demonstrated to be strikingly AU rich, indicating the lack of secondary structures as suspected (114). Quantitative treatments of the system have also been carried out successfully by von Hippel and co-workers, and similarities to the r-protein system were discussed (89, 113).

The *regA* protein of phage T4 has also been demonstrated to act as a translational repressor on the synthesis of the rIIB cistron (and probably other proteins including the *regA* protein itself) (for review see 109). The *regA* protein regulates the synthesis of a variety of T4 gene products including genes involved in deoxyribonucleotide metabolism (115, 116), DNA replication (117, 118), and the *regA* gene product itself (119). Analysis of mutants altered in the regulation of one *regA*-sensitive gene, the rIIB gene, has identified a nucleotide sequence (AUGUACAAU) located at the translation initiation site for this gene that is probably involved in the recognition and binding of *regA* protein (120, 121). It should be noted that gene 32 protein feedback regulates only its own gene expression; some repressor r-proteins (such as S20) are similar to gene 32 protein in this respect, but others inhibit by a feedback mechanism the translation of their own messages together with other neighboring cistrons on the same polycistronic mRNA molecules; in contrast, the *regA* protein translationally regulates the expression of not only its own gene, but also other *unlinked* genes. Thus the possibility exists that some (unidentified) repressor r-proteins translationally regulate not only their own genes and neighboring genes, but also some unlinked genes, as does the *regA* protein. In this case, one would expect that an increase in copy number of the regulated unlinked gene without an increase in the gene copies for the (hypothetical) repressor r-protein genes would lead to a gene dosage-dependent increase in the unlinked r-proteins, and this could be an explanation for the apparent absence of regulation in the synthesis of S21, L21, and L27 in diploid strains, as mentioned in a previous section (52). In this connection, we note that products of rRNA operons, presumably ribosomes, feed back to regulate (transcriptionally) not only the expression of rRNA operons, but also the expression of unlinked tRNA genes. In experiments to be discussed

in a later section, a gene dosage-dependent increase of gene expression was observed with unlinked tRNA genes, even though rRNA gene expression was gene dosage independent.

Finally, preliminary evidence exists indicating that elongation factor EF-Tu in *E. coli* may act as a translational repressor of its own synthesis from the *tufB* gene (122–125). EF-Tu is synthesized from two genes, which specify nearly identical amino acid sequences: *tufA*, located in the *str-spc* region, and *tufB*, located in the *rif* region. The *tufB* gene is cotranscribed with the genes for four tRNAs (126–128). The novel cotranscription of the *tufB* gene with tRNA genes has allowed speculation on the mechanism of regulation of the *tufB* gene (129, 129a). As in other examples of translational feedback inhibition, the EF-Tu protein is a nucleic acid-binding protein that interacts specifically with aminoacyl tRNA during protein synthesis. Regulation of *tufB* gene expression may therefore be accomplished by an analogous competition between the tRNA elements of the multicistronic *tufB* mRNA and free aminoacyl tRNA for the putative translational repressor, free EF-Tu. [If the synthesis of EF-Tu from *tufB* is in fact feedback regulated, the possibility exists that the expression of the *tufA* gene is translationally regulated by S7. In the *in vivo* experiments shown in Figure 2, overproduction of S7 might have actually inhibited the synthesis of EF-Tu from the *tufA* cistron, but the decreased synthesis from *tufA* was possibly compensated by increased synthesis from the *tufB* gene as a result of this postulated feedback mechanism.]

Transcription of Ribosomal Protein Genes and Its Regulation

IN VIVO MEASUREMENTS OF THE AMOUNTS AND THE SYNTHESIS RATES OF R-PROTEIN mRNA The presence of a translational feedback regulatory mechanism in the regulation of r-protein synthesis does not imply that there is no transcriptional regulation of r-protein gene expression. The rate of transcription of r-protein genes varies in response to environmental changes, and it is certainly possible that under some conditions transcription becomes rate limiting in r-protein synthesis, and thus plays a dominant role in the regulation of r-protein synthesis.

Measurements of transcriptional activities of r-protein genes were carried out mainly using, as hybridization probes, transducing phage DNA carrying the r-protein genes in the *str-spc* region. The amounts (3, 130) as well as the synthesis rates (3, 75) of r-protein mRNA were determined under a variety of steady-state growth conditions. In one case, the data were obtained for the growth-rate range covering between approximately $\mu = 0.1$ and 1.4 (μ is growth rate expressed as doublings per hour) (3), and in the other, between 0.7 and 2.1 (75, 130). Basically, the same conclusions can be drawn from these two sets of data. As the work of Gausing (3) involved

more comprehensive measurements, we use them to discuss the subject of transcription in this section. Figure 3 shows some pertinent data and calculated values. As mentioned above, the cellular concentration of ribosomes (often expressed as α_r , the ratio of the amount of total r-protein to that of total cellular protein) is proportional to μ in the growth-rate range above approximately $\mu = 0.7$ (Figure 3, graph (a), curve 1). Consequently the rate of accumulation of ribosomes is approximately proportional to μ^2 in this range. The synthesis rates of rRNA and r-proteins are both approximately proportional to μ^2 (Figure 3, graph (b), curves 1 and 3, respectively), and discrepancies between the two become noticeable only in a very slow growth-rate range, presumably reflecting the excess production of rRNA followed by degradation, as suggested previously (3, 131). However, the synthesis rate of r-protein mRNA does not change in proportion to μ^2 . It increases only slightly faster than a linear function of μ with increasing growth rate (Figure 3, graph (b), curve 2). Since the synthesis rates of r-protein mRNA were measured simultaneously with those of rRNA, the difference in growth-rate dependency between the two was clear-cut (3; see also 75). On the other hand, the *amounts* of r-protein mRNA (per total protein) were found to be approximately proportional to μ^2 (3, 130;

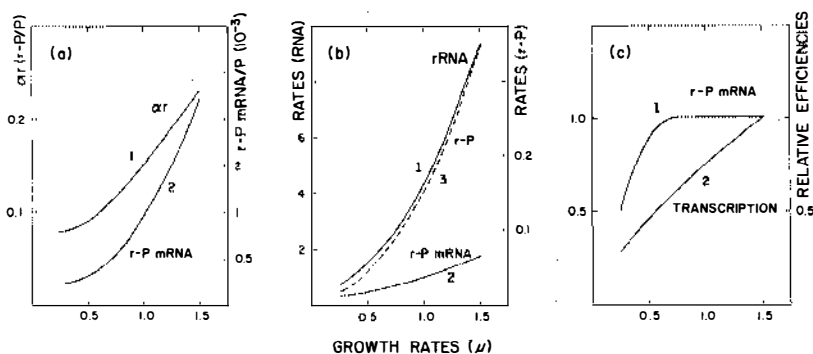


Figure 3 Growth rate-dependent regulation of the synthesis of ribosomes and ribosomal components. Data were taken from Gausing (3) and some additional calculations. Growth rates, μ , are given in doublings per hour. Graph (a): The amounts of ribosomes [expressed as the ratio (w/w) of r-proteins to that of total protein (α_r); curve 1] and r-protein mRNA [expressed as the ratio (w/w) to total protein; curve 2]. Graph (b): Synthesis rates of rRNA (curve 1), r-protein mRNA (curve 2), and r-proteins (curve 3). Curves 1 and 2 are shown as weight RNA/weight total protein per min $\times 10^3$ (or $\times 10^4$ for curve 2). Curve 3 was calculated by multiplying α_r by μ so that the values correspond to weight r-protein/weight total protein $\times 60/\ln 2$. Graph (c): "Relative efficiencies" of translation of r-protein mRNA (curve 1) and of transcription (curve 2) were calculated by dividing r-protein synthesis rates by the amounts of r-protein mRNA (for curve 1), or by the r-protein mRNA synthesis rates (for curve 2) and then by normalizing the values to the ones at $\mu = 1.5$.

see Figure 3, graph (a), curve 2). Thus growth rate-dependent changes in r-protein synthesis rates closely follow the pattern observed for the amounts, but *not* the synthesis rates, of r-protein mRNA. This can be clearly seen when one compares the ratios of r-protein synthesis rate to the amount of r-protein mRNA (Figure 3, graph (c), curve 1) with the ratios of the r-protein synthesis rate to the r-protein mRNA synthesis rate (Figure 3, graph (c), curve 2). Thus it is clear that the number of r-proteins synthesized from a given mRNA molecule during its lifetime decreases with decreasing growth rate, and yet the rate of r-protein synthesis is approximately proportional to the cellular concentration of r-protein mRNA except in a very slow growth-rate range [$\mu \leq 0.5$; Figure 3, graph (c)]. These results are entirely consistent with the translational feedback regulation model described in the previous sections; the transcription of r-protein genes takes place in excess of that actually required for r-protein synthesis, the final synthesis rates of r-proteins are determined by translational feedback regulation, and the extent of the translational repression becomes larger with decreasing growth rate. The data also indicate that the physical stability of the r-protein mRNA decreases with decreasing growth rate, presumably reflecting the higher degree of translational repression, as observed in the gene dosage experiments (49).

Although, in retrospect, the data on the r-protein mRNA synthesis rates hinted at the existence of translational regulation as discussed above, Gausing (3) emphasized the role of transcriptional control in determining the final rate of r-protein synthesis by arguing that the ratio of the rate of r-protein mRNA synthesis to the rate of total mRNA synthesis (the latter calculated as a difference between the total RNA synthesis rate and the stable RNA synthesis rate) is proportional to α_r . We believe that this observation does not identify the correct causative mechanism. First, the results of the gene dosage experiments as well as those obtained with hybrid plasmids containing r-protein genes fused to the *lac* promoter (71) discussed above, indicate that this ratio (r-protein mRNA synthesis rate/total mRNA synthesis rate) can be elevated to a large degree without significantly influencing the relative synthesis rate of r-proteins encoded by genes present in increased dose. Second, the results of analysis of the expression of the *galK* gene (or *lacZ* gene) fused to and under the control of one of the r-protein promoters were different from what one would expect from the argument made by Gausing. The synthesis rate of galactokinase or β -galactosidase increased approximately in proportion to μ , rather than μ^2 , as expected from the transcriptional activities of r-protein promoters [Figure 3, graph (b), curve 2] in the absence of translational regulation (132). Third, as mentioned in a previous section, the data obtained with mutants with altered repressor r-proteins (95, 107) indicate that transcrip-

tion of r-protein genes usually takes place in excess of that required for the level of r-protein synthesis that the cell is actually carrying out in exponentially growing conditions. Thus we feel it is not correct that the relative r-protein synthesis rate is determined by the ratio of the r-protein mRNA synthesis rate to the total mRNA synthesis rate.

The above discussion is also pertinent to the passive regulation model proposed by Maaløe (133, 134) to explain the growth rate-dependent regulation of r-protein synthesis. In this model, it was proposed that r-protein promoters are essentially constitutive and that changes in the transcription rates of these promoters take place as a result of competition with other regulated promoters that are not involved in ribosome biosynthesis; transcription of r-protein promoters increases with increasing growth rate because many operons for catabolic and biosynthetic enzymes are repressed in rich media. Inherent in the model is an assumption that the relative r-protein synthesis rate is determined by the ratio of the rate of r-protein mRNA synthesis to the rate of total mRNA synthesis [(133, 134; see also Chapter 8 in (135)]. Thus, regardless of the validity of the hypothesis that r-protein promoters are constitutive, it would be difficult to accept this assumption in light of the observations discussed above.

REGULATION OF R-PROTEIN mRNA SYNTHESIS Although we have emphasized that the final rate of r-protein synthesis is not *determined* by the rate of r-protein mRNA synthesis, the rate of r-protein mRNA synthesis does change with growth rate. There are two possible ways to think about this variation. One is the passive regulation model suggested by Maaløe (see above), and another is that the transcription rate actually changes either as a result of specific (or nonspecific) inhibition or activation of promoter activity. Since the total rate of RNA transcription is probably not limited by the number of RNA polymerase molecules (10, 12, 13; see a later section), the explanation based on passive regulation, even though it is ingenious, is unlikely. Regarding the second possibility, we can suggest two possible candidates as specific inhibitors of r-protein mRNA synthesis: One is guanosine tetraphosphate (ppGpp) or related compounds, and the other is free nontranslating ribosomes. These possibilities are discussed later in conjunction with the growth rate-dependent regulation of rRNA synthesis.

Another condition in which transcription of r-protein genes is altered is during the stringent response where amino acid starvation results in the specific decrease in the synthesis rates of rRNA, tRNA, and r-proteins, accompanied by a dramatic increase in the concentration of ppGpp (and pppGpp; a more general discussion of the stringent response is made later). It was initially established that the amount of r-protein mRNA is regulated by the stringent control system (136), and subsequent pulse-labeling

experiments have indicated that stringent control probably occurs at the level of transcription of r-protein genes (137; see also discussion in 22). Whether ppGpp is the direct negative effector acting on the transcription is considered in a later section.

As mentioned previously, transcriptional regulation has also been observed in the S10 operon after overproduction of the repressor r-protein L4. However, the observed decrease in distal mRNA synthesis was shown to be the result of an attenuation-type mechanism and not of effects at the promoter (99). Similar attenuator-like structures may be drawn for other r-protein operons (40). The observed structures could serve as transcriptional termination sites *in vitro* (40), but no transcriptional termination or regulation has been observed so far *in vivo*, except in the S10 operon. The significance of these putative attenuator-like structures is yet to be determined.

Finally, in considering possible transcriptional regulatory mechanisms active in r-protein operons, we note that DNA sequence analysis of several r-protein operons, in particular S20 (138, 139), L34 (46), S21 (32), and S1 (68, 140), has revealed the presence of tandem or multiple promoters. Possible transcriptional regulation of r-protein synthesis based on the selective use of one or more of these promoters has not been established.

REGULATION OF rRNA AND RIBOSOME SYNTHESIS

As discussed earlier, the exact coordination and balancing of the synthesis of rRNA with r-protein synthesis are achieved through the translational feedback mechanism. It has been known for some time that the 16S, 23S, and 5S rRNA are cotranscribed as a single 30S RNA precursor (see below), and processing of the precursor results in equimolar amounts of the three RNAs (concerning a possible small excess production of 5S RNA, see below). Thus, the question of coordination and balancing of the synthesis of all the ribosomal components can now be understood. The major question left is how the amount of ribosomes is regulated in response to changes in environmental conditions. Since the synthesis rate of rRNA is approximately the same as the accumulation rate of rRNA in ribosomes (e.g. 3) and transcription of r-protein genes is not rate limiting under normal conditions except perhaps during very slow growth conditions (see above), the control of transcription of rRNA genes is of primary importance in the regulation of ribosome biosynthesis. After a brief survey of some recent studies on rRNA genes, we discuss the regulation of rRNA synthesis with respect to the stringent response and growth rate-dependent control, and then models to account for the overall regulation of ribosome synthesis.

Organization of *rRNA Genes and Their Transcription*

CHROMOSOMAL LOCATIONS OF rRNA GENES The number of rRNA gene sets ("operons") per haploid chromosome of *E. coli* K12 has now been established (seven operons) by restriction enzyme analysis of total *E. coli* DNA using the Southern blot technique (141) and by analyses of isolated recombinant DNA clones and transducing phages (35, 142). All the operons have now been isolated on transducing phages, plasmids, or both, and their chromosomal origins determined (see Figure 1; for review see 19).

The completion of mapping of the rRNA operons in *E. coli* and the determination of their transcriptional orientations allows consideration of several important points: The initiation of DNA replication occurs more frequently at higher growth rates. Therefore, the clustering of rRNA operons near the origin of replication, as was found in *B. subtilis* (23, 143), could contribute to the increased rRNA synthesis at high growth rates by increasing the gene dosage. However, in *E. coli*, calculations indicate that the dosage of rRNA genes per unit amount of DNA (or protein) increases only approximately 20% as growth rate varies from 0.9 to 2.7 doublings per hour (35). Further, as discussed below, it is now clear that rRNA synthesis is gene dosage independent (144) and thus the regulation of rRNA synthesis cannot be explained in this way. However, the rRNA operon arrangement with respect to the origin of replication does result in the elevated gene dosage of origin-proximal operons (such as *rrnC*) with respect to origin distal operons (e.g. *rrnG* or *H*) as a function of growth rate. Thus the relative contribution to total rRNA synthesis of the individual operons varies with growth rate. The physiological significance of this variation is unclear.

Finally, it should be noted that the direction of transcription of all the rRNA operons is identical to that of DNA replication. It is possible, as previously speculated (7), that this striking chromosomal organization has evolved to prevent "collisions" of the DNA replication machinery with transcribing RNA polymerase in these active rRNA operons.

OPERON STRUCTURE All seven rRNA operons are transcribed in the order Promoter-16S-23S-5S rRNA. In addition, it has been known for some time that each operon has at least one and sometimes as many as three tRNA genes in the same transcription unit as the rRNA genes, either between the 16 and 23S genes ("spacer tRNAs") or distal to the 5S RNA gene ("distal tRNAs") (see 19, 35). Recently, it has been shown that *rrnD* contains a second 5S RNA gene (145), with the tRNA gene for tRNA^{Thr} sandwiched between the two 5S RNA genes. Since the distal regions of *rrnA*, *rrnE*, and *rrnG* have not been sequenced, we do not know the total number of 5S RNA

genes, and hence the significance of the extra 5S RNA gene in *rrnD* is not clear.

rRNA PROMOTERS AND TRANSCRIPTION At the time of this writing, six of the seven rRNA promoter regions have been analyzed by DNA sequencing with only *rrnC* remaining. Each operon contains tandem rRNA promoters, called P1 and P2, separated by about 120 bases (146–151). Easily identifiable – 10 and – 35 sequences common to *E. coli* promoters (44) are present for both P1 and P2. A striking sequence conservation among rRNA operons was noted including and surrounding the – 10 sequence of P1 (19, 148), and this common sequence may play an important role in the regulation of rRNA gene expression (see also 43).

Transcripts from P2 start with CTP, rather than with ATP or GTP as is normal for *E. coli* transcription starts (151, 152). It seems likely that the differences between P1 and P2 promoter structures result in differential regulation of their transcription initiation. Early studies indicated that P1 is the major promoter used in vivo, and the ratio of P1 to P2 starts was estimated to be about three to five (153) or five to ten (154) to one under the growth conditions used. Recent studies carried out both in vivo and in vitro (155–158) suggest that transcription from the two promoters is subject to different control (see below). This differential control may be reflected by differences in the rates of open complex formation at equilibrium for the two promoters of *rrnE* (159).

Additionally, Boros et al (160, 161) have identified two promoters more than one kilobase upstream of P1 and P2 in *rrnB* and called them P3 and P4. Although evidence was presented to indicate that these promoters direct transcription through the *rrnB* region in vitro and in vivo, it is unclear what fraction of total *rrnB* transcription is directed from the P3 and P4 promoters. In addition, such promoters were looked for but not found by the same workers in the regions preceding *rrnD* or *rrnH* (161). DNA sequence work revealed an open reading frame following the P3/P4 promoters and preceding the P1/P2 promoters, and the synthesis of a protein from this open reading frame was demonstrated in minicells (162). Similarly, an open reading frame was found upstream of the *rrnG* operon (150). The significance of these two additional promoters (P3, P4) as well as the proteins encoded by the open reading frames in rRNA regulation remains unclear.

As the promoter region has been isolated and identified, and the function of the promoters can now be studied both in vivo and in vitro, attempts to define exact regions required for promoter function and/or regulation have been initiated using various approaches in conjunction with recombinant

DNA techniques. For example, promoter regions were fused to the *lacZ* or *galK* gene and growth rate-dependent expression of these genes (as opposed to the expression from their genuine promoters) was demonstrated (132, 163, 164; see also 165). Similarly, the elucidation of the target region for stringent control is under current study (166; see below). Using similar approaches, Travers and co-workers (167) recently obtained evidence indicating the importance of a region upstream of the -35 sequence for promoter function. In addition, using a foot-printing technique (168), they obtained evidence suggesting that RNA polymerase interacts with this region perhaps as a dimer (169), the proposed form active in transcription of rRNA and (tRNA) genes, according to Travers (170). In view of powerful modern recombinant DNA techniques, we certainly expect rapid progress in research along these lines.

Numerous in vitro studies on the transcription of rRNA genes have been carried out with two primary goals: The first was to explain why in vitro transcription of rRNA genes is generally not as efficient as one might expect. Factors that might be responsible for the very high transcription rate in vivo have been sought, and various crude protein preparations were obtained as stimulatory factors (171–173). The presence of such factors was also suggested from the isolation of temperature-sensitive mutants that affect rRNA synthesis preferentially (174, 175). A higher efficiency of transcription was also observed in vitro through the use of supercoiled rather than linear DNA templates (157, 176); some in vivo analyses that used inhibitors of DNA gyrase appear to support the in vitro results (177, 178; but see 179). The second goal was to demonstrate selectivity in transcription of rRNA genes; many in vitro conditions and the presence of effectors, such as ppGpp (reviewed in 180), EF-Tu (181), fMet-tRNA (182), and IF-2 (183) were claimed to have selective inhibitory or stimulatory effects on rRNA synthesis in vitro. However, it is unclear at this time whether any of these conditions and/or factors play important roles in rRNA transcription and its regulation in vivo except for the possible role of ppGpp, which is discussed below. For details of these in vitro studies, previous reviews can be consulted (7, 19, 21, 184).

rRNA TERMINATORS The probable transcription termination signals of several rRNA operons have been sequenced (145, 146, 185, 186; reviewed in 19). All have a stem-loop structure followed by several U residues characteristic of rho-independent termination signals, and at least one of them has been shown to function in vitro in the absence of rho (186). Several investigators have reported that plasmids containing rRNA promoters can be constructed without difficulty only in the presence of rRNA terminators. Several other terminators did not appear as effective as the rRNA

terminators in this respect (155, 160). A speculation is that transcription from rRNA promoters *in vivo* is special, perhaps able to read through ordinary terminators, and that the rRNA terminators are unique in preventing such read-through (see below).

Stringent Control

As already mentioned, during aminoacyl tRNA deprivation, ppGpp (and pppGpp) accumulates rapidly concurrent with the drop in stable RNA (rRNA and tRNA) synthesis. The product of the *relA* gene (stringent factor) was shown to be involved in ppGpp synthesis. Mutants defective in *relA* gene function continue to synthesize stable RNAs after amino acid starvation and are therefore called "relaxed" for the stringent phenotype. Several recent reviews discuss the metabolism of ppGpp and the vast literature on attempts to identify its role in the stringent control mechanism (6, 11, 19, 21, 184, 187, 188). We attempt only to clarify a few points from the recent literature.

The role of ppGpp in the inhibition of rRNA synthesis has been a subject of debate over the past 15 years. The debate has centered on whether or not ppGpp is a direct effector involved in the inhibition of stable RNA synthesis, whether its effects on transcription are exerted indirectly, or whether the appearance of ppGpp merely correlates with the reduction in stable RNA synthesis.

Many investigators have reported that ppGpp preferentially inhibits the synthesis of rRNA in *in vitro* transcription systems, while others have observed no specific effects (reviewed in 11). Most recently, Glaser et al (157) and Kajitani & Ishihama (158) found that ppGpp at low concentrations (0.3 mM) decreased rRNA transcription 75%–90% *in vitro*, an effect approaching the reduction that is found *in vivo*. Transcription from the rRNA promoter P1 was affected more than that from P2 or the *lac* promoter. Selective inhibition of *in vitro* transcription from tRNA gene promoters by ppGpp was also reported (189).

The selectivity of the stringent response for transcription from P1 vs P2 promoters was borne out *in vivo* as well. Transcription from P1 was severely reduced after amino acid starvation and this reduction was found only in *relA*⁺ strains (155). On the other hand, transcription from P2 was reduced in both a *relA*[−] and *relA*⁺ background. Based on these results, the authors proposed that P1 but not P2 is a stringently controlled promoter. Travers and colleagues (170, 180, 181, 183, 190, 191) have carried out many *in vitro* experiments and proposed that ppGpp influences the selectivity of RNA polymerase for stable RNA relative to other mRNA promoters. According to this model, only a particular form (most likely a dimer) of RNA polymerase is capable of transcription from stable RNA promoters

and ppGpp would inhibit the necessary conformational changes (dimerization) of RNA polymerase, thereby reducing binding of polymerase to stable RNA promoters.

Another possible regulatory role for ppGpp other than its inhibitory effect on transcription initiation was also suggested from in vitro experiments (192, 193). It was observed that RNA polymerase pauses during in vitro transcription of rRNA operons at specific sites in the regions following the P1 promoter, and that the duration of these pauses was increased by ppGpp. The effect of ppGpp on pausing at one site was seen only in the presence of *nusA* protein, a factor shown to participate in antitermination in phage λ (194). However, the pausing observed in vitro does not appear to play a major role in stringent control in vivo because deletion of the DNA regions corresponding to the pause sites in a plasmid *rrnB* gene did not affect the stringent control of transcription from its promoter (166). Of course, it is still possible that the stimulation of pausing or premature termination by ppGpp might become relevant to regulation under some other conditions.

There is no in vivo evidence that ppGpp interacts directly with RNA polymerase and thereby exerts its effects on rRNA synthesis. Recently, however, mutants have been isolated that have an alteration in RNA polymerase and exhibit a relaxed phenotype while continuing to synthesize ppGpp in response to amino acid starvation (195). If the observed in vitro effects of ppGpp described above are relevant to in vivo regulation, the RNA polymerase from this mutant should show resistance to ppGpp in vitro in contrast to the wild-type polymerase.

If inhibition of rRNA synthesis requires the production of ppGpp, the reduction of rRNA synthesis should always correlate with the appearance of ppGpp. However, there are reports (e.g. 196-198) describing instances in which amino acid starvation resulted in a stringent response with respect to RNA synthesis, but no increase in ppGpp was observed. Conversely, there is a report that in an EF-G mutant of *B. subtilis*, inhibition of rRNA synthesis during amino acid starvation was abolished by a temperature shift-up without decreasing the concentration of ppGpp (199).

It should be emphasized that inhibition of the synthesis of stable RNA is only one of many responses bacterial cells exhibit during amino acid starvation (see reviews 6, 11, 184). For example, transcription of amino acid biosynthetic operons (reviewed in 11) and protein degradation (200) are reported to be stimulated in *relA*⁺, but not *relA*⁻, strains and a direct participation of ppGpp has been suggested. Another role of ppGpp suggested by several workers is in the prevention of translational errors during starvation (201-203). In this connection, O'Farrell (203) presented experimental evidence to show that ppGpp causes general inhibition of the

initiation of translation, and explained the decrease in translational errors in *relA*⁺ strains compared to *relA*⁻ strains on that basis. In addition, he pointed out that the proposed role of ppGpp in the inhibition of translation initiation is consistent with many earlier reports that polysomes break down during amino acid starvation in *relA*⁺ cells, but not in *relA*⁻ cells (reviewed in 184, 203). We discuss this point later.

Growth Rate-Dependent Control

We have already introduced this subject in connection with the regulation of r-protein synthesis. To explain growth rate-dependent control, a model must fulfill two functions: First, it must propose a mechanism by which the cell senses the state of the nutritional environment. Second, there must be a mechanism for responding to this signal, giving the appropriate amount of rRNA transcription for that growth rate, and thereby producing the correct number of ribosomes. We consider here three different basic models to explain the growth rate-dependent control of rRNA synthesis.

PASSIVE CONTROL MODEL Although the passive control model of Maaløe (133, 134; see a previous section) was formulated for r-protein synthesis and does not include the regulation of rRNA synthesis, one could use similar reasoning to explain the growth rate-dependent changes in the rate of rRNA synthesis. Here it is assumed that the total rate of RNA transcription is limited by the number of RNA polymerase molecules, that the rRNA promoters are constitutive, and their activities are altered as a result of competition with other regulated nonribosomal promoters involved in various metabolic pathways. Thus, in this model, because the amount of RNA polymerase is limited, the polymerase itself senses the environmental conditions and determines the RNA transcription rate without any additional effectors. Although the model is interesting, there are two major conflicting experimental observations. First, as discussed below, there is experimental evidence to indicate that RNA polymerase is present in excess in the cell. Second, recent gene dosage experiments, which are discussed below, show that the transcriptional activity of rRNA operons can be changed without apparent changes in other competing nonribosomal transcriptional activities.

DIRECT ppGpp CONTROL MODEL AND SIMILAR MODELS An obvious possibility for regulating rRNA operons would be to have a positive or negative effector. This effector would somehow sense the nutritional conditions and then interact with DNA or RNA polymerase or both, and the degree of this interaction, and hence the rate of transcription of the rRNA operons, would be determined according to direct or indirect signals from the environment. In this section we briefly discuss data implicating ppGpp as an effector of

rRNA synthesis and then mention problems that we find to be inherent in direct effector models for the control of rRNA synthesis.

Because of the striking inverse correlation between ppGpp and rRNA synthesis during stringent control and the results of some in vitro experiments as already described, a favored effector is ppGpp. An inverse correlation between rRNA synthesis and ppGpp concentration has also been observed in exponentially growing cells under a wide range of nutritional conditions although this correlation is not so striking as that seen in stringent control.

It was found that ppGpp concentration decreases with increasing growth rate both in *relA*⁻ and *relA*⁺ strains (204–206; but see 207). In *relA*⁻ cells, the synthesis of basal amounts of ppGpp at high growth rates and the stimulated synthesis of ppGpp observed upon nutritional shift down indicate the presence of a second ppGpp-synthesizing system different from that used during amino acid starvation (208, 209). Although the molecular mechanism involved in this second system is unknown, there is evidence from *B. subtilis* suggesting the participation of RNA polymerase in the second system (210). Similarly, the existence of a rifampicin-resistant *E. coli* mutant, which has a tenfold reduced basal concentration of ppGpp relative to the parent, may suggest such a possibility, although a different interpretation was given in the original report (211). Thus the possibility is not entirely eliminated that the observed variation of ppGpp concentration at various growth rates represents a *result* of variation in the transcriptional activity of RNA polymerase (e.g. an “idling” reaction) at the rRNA operons, rather than the *cause* of the variation of rRNA synthesis rates.

Bremer et al (205, 206) have proposed that ppGpp is the sole effector involved in the regulation of rRNA (and tRNA) synthesis. They compared the ppGpp concentration with the *relative* stable RNA synthesis rate [defined as the ratio of rRNA (plus tRNA) synthesis rate to the total instantaneous rate of RNA synthesis], rather than to the rRNA synthesis rate itself, and found an excellent inverse correlation between the two in both *relA*⁺ and *relA*⁻ cells. However, it seems to us that the appropriate parameter here is the *actual*, not the *relative*, stable RNA synthesis rate, since the available data suggest that RNA polymerase is present in excess (see below) and that the synthesis rate of mRNA (nonstable RNA) should be insensitive to the transcription of rRNA operons.

On the basis of their results, these authors suggested a mechanism similar to the one proposed by Travers (170, 180), and others (212); i.e. two different conformations of polymerase exist and ppGpp converts one form that can transcribe stable RNA genes to the other form that cannot. Alternatively, ppGpp could act directly on the template, either by itself or in conjunction with a hypothetical repressor, to determine the degree of repression of each

of the rRNA operon promoters, not unlike a classical operon model (e.g. the lactose operon). A prediction of these models would be that rRNA synthesis would not respond to rRNA or ribosome levels but only to the availability of the appropriate conformation of RNA polymerase or of the template (unless, of course, rRNA or ribosome levels *determines* these conformations). As in the case of the passive control model, the results of analyses of gene dosage dependence of rRNA synthesis using plasmids carrying intact or defective rRNA operons (144) makes it difficult to accept such a model, as described in a later section.

The most important feature about the growth-rate dependence of ribosome synthesis is the efficiency of the control mechanism. That is, no matter what the nutritional conditions and growth rate (except for very slow growth rates), the cell makes the appropriate number of ribosomes so that the protein-synthetic capacity is just sufficient to maintain that growth rate while the free nontranslating ribosome concentration is minimal. In order for this efficiency to be maintained, it seems to us that the mechanism that controls rRNA synthesis must also monitor the *results* of its actions; i.e. it must be able to respond not only to the nutritional environment but also to the ribosome supply, constantly fine-tuning rRNA synthesis as nutrients and ribosome concentrations shift.

A trivial explanation for the achievement of the correct ribosome concentration for every growth rate would be that ribosome concentration *determines* growth rate. As mentioned below, the presence of excess ribosomes in slow growth conditions makes it difficult to accept this premise.

The inherent weakness of direct effector models, as we see it, is that such models do not include a means for both detecting the nutritional conditions and also monitoring the result of the rRNA synthesis rate that is achieved. For example, it is difficult (although perhaps not impossible) to imagine how the cell could achieve the appropriate ppGpp concentration in response to environmental conditions and then continually adjust the ppGpp concentration as ribosome synthesis turned on and off (unless ribosome concentration determined ppGpp levels, as discussed below).

RIBOSOME FEEDBACK REGULATION MODEL This laboratory has recently proposed an alternative model called the ribosome feedback regulation model to explain the growth rate-dependent regulation of rRNA synthesis (144). In this model, it is proposed that cells always have a higher capacity for making all ribosomal components, but that the synthesis rate of rRNA (and therefore ribosomes) is feedback inhibited by free, nonfunctioning ribosomes when produced in excess of the amount needed for protein synthesis. Thus, in this model, ribosomes themselves sense environmental conditions and adjust the ribosome synthesis rate to the growth rate. To

test this idea, Jinks-Robertson et al (144) carried out a series of experiments in which the copy numbers of rRNA operons were increased by introducing plasmids containing the *rrnD* or *rrnB* operons, and the effects on the rate of transcription of rRNA operons were measured directly (or by following the accumulation of tRNAs encoded in rRNA operons). It was reasoned that if the feedback mechanism mentioned above were correct, the total rRNA synthesis rate would not significantly increase even though there were two to three times the number of rRNA templates per unit amount of cellular protein. If cells do not discriminate between the rRNA operons on the chromosome and those on the plasmid, then the synthesis from individual rRNA operons would be decreased in inverse proportion to the increase in gene dosage, leaving the total rRNA synthesis rate unchanged. The experimental results agreed with those predicted from the feedback model; the total rRNA synthesis rate did not significantly increase, and accumulations of tRNAs from individual rRNA operons decreased two to three fold in response to the two to three fold increase in gene dosage. Similar results were obtained in earlier experiments (213), but a different interpretation was made (see discussion in 144). Furthermore, it was shown that intact rRNA capable of assembling into ribosomes must be produced from the extra rRNA operons to achieve the negative feedback regulation. When deletions were made in the rRNA coding regions of the plasmid rRNA operons, the plasmid no longer had any effect on host rRNA operon transcription. The total rate of transcription of rRNA operons (chromosomally encoded *plus* defective, plasmid-encoded operons) now increased in accord with gene dosage, indicating that the regulated rRNA synthesis rates were not the result of limiting concentrations of some factor (e.g. RNA polymerase) but that a functional product from the operon, presumably the ribosome, as discussed below, is responsible for maintenance of the normal transcription rate. These results are difficult to explain by the passive control or ppGpp control models in their simplest forms. For example, the two strains used in the above experiments, the control strain and the strain with extra defective rRNA operons (containing deletions), are growing at the same growth rate in the same growth medium. Thus, the degree of repression of all the catabolic and biosynthetic operons should be the same in both strains, and according to the passive control model, the number of RNA polymerase enzymes available for the rRNA operons would remain the same. Thus, if the passive control model were correct, the same total rRNA transcription rate would be seen in the two strains, rather than the twofold difference observed in the actual experiments. Similarly, according to the ppGpp control model described above that assumes ppGpp acting on RNA polymerase, one would expect that as a result of similar growth conditions the same kind and level of signal (e.g. ppGpp) would be

produced to regulate the transcription of rRNA operons, and the same rRNA transcription rate would be predicted, contrary to the actual observations. On the other hand, classical operon-type models, which assume ppGpp acting directly on the rRNA promoters without a feedback system to monitor the amount of ribosomes produced, are in disagreement with the result that rRNA synthesis was essentially gene dosage independent when the number of *intact* rRNA operons was increased, as mentioned above (144; see also the results in 214).

In the gene dosage experiments described above, it was found not only that the synthesis of tRNAs encoded in rRNA operons was decreased in inverse proportion to rRNA gene dosage, but also that the synthesis of tRNAs *not* encoded in rRNA operons was decreased. It was concluded that tRNA promoters are also subject to this same regulatory mechanism. This finding explains the known fact that the synthesis of tRNAs is growth rate dependent and its regulation is similar to that of rRNA (6). It should be noted that cells use the products of rRNA operons, and not the product of tRNA operons, to regulate the expression of tRNA genes. Thus, one would expect that in contrast to rRNA operons the synthesis rate of tRNA would increase in proportion to gene copy numbers, and this is what actually has been observed (215–217; R. Gourse, M. Nomura, unpublished).

It is inferred from several lines of evidence that the regulatory molecule responsible for the feedback regulation of rRNA synthesis is free, non-translating ribosomes: First, some cold-sensitive, r-protein mutants that have conditional defects in ribosome assembly were shown to synthesize both rRNA and tRNA at rates higher than the parent at low temperatures (Y. Takebe, M. Tam, A. Miura, S. Jinks-Robertson, M. Nomura, unpublished). Overproduction of RNA in similar ribosome assembly-defective mutants was also observed previously (218–220). Inhibition of ribosome assembly without inhibition of macromolecular synthesis, a process that would lead to deprivation of free ribosomes, results in the stimulation of rRNA and tRNA synthesis. Second, reduction of the protein chain-elongation rate without reducing the chain initiation rate, which again would lead to deprivation of free ribosomes, causes stimulation of rRNA synthesis. Overproduction of rRNA was in fact observed in strains treated with chain-elongation inhibitors, such as fusidic acid (221) or chloramphenicol (222), as well as in a streptomycin-resistant mutant that synthesizes protein at a reduced chain-elongation rate (223). Finally, the results of analysis of rRNA synthesis rates during nutritional shift-up or shift-down (8–10) can be best explained by the ribosome feedback regulation model; for example, one can imagine that upon nutritional shift-up, protein synthesis is stimulated, and this stimulation, even to a small extent, would immediately mobilize the small amount of nontranslating

ribosomes in the pool, thereby causing a burst of derepressed synthesis of rRNA and hence ribosomes. Thus, as observed experimentally, the ribosome synthesis rate increases very quickly upon nutritional shift-up, while the total protein synthesis rate increases gradually, and a new steady state is eventually reached at which the ribosome biosynthesis is balanced with the total protein synthesis rate (i.e. with the growth rate specified by the new growth condition). The situation upon shift-down can be explained in a similar way. Thus the feedback mechanism explains how cells adjust the ribosome biosynthesis rate in accordance with cellular growth rate.

As mentioned earlier, Sarmientos and co-workers (155, 156) have shown that the P1 and P2 rRNA promoters are differentially expressed, and that P1 is responsive to growth-rate regulation while P2 is comparatively unaffected. They suggest that P2 is a constitutive promoter whose activity is significant only at very low growth rates or in recovery from stationary phase growth. It appears then that the feedback regulation by free ribosomes acts mainly on initiation at the P1 promoter and not at the P2 promoter.

Some indirect evidence suggests that the feedback mechanism by free ribosomes also acts on transcription from r-protein promoters, but to a weaker extent. Stimulation of transcription of r-protein genes was observed in cold-sensitive, ribosome assembly-defective strains at lower temperatures (Y. Takebe, A. Miura, M. Nomura, unpublished) and in cells treated with chloramphenicol (225). Analysis of r-protein mRNA synthesis rates in the strain carrying extra intact rRNA operons showed a reduction in r-protein mRNA synthesis rates, but the degree of reduction was smaller than that observed with the rRNA synthesis rate (M. Tam, M. Nomura, unpublished). It is possible that the decrease in the rate of r-protein mRNA synthesis rate with growth rate as discussed in a previous section [see Figure 3, graph (b), curve 2] is a reflection of this feedback mechanism; if the feedback mechanism acts on r-protein gene transcription, but more weakly than on rRNA gene transcription, the observed slope, which is only slightly higher than a linear increase with increasing growth rate, and the excess transcription, as discussed above, are consistent with the predictions from the transcriptional feedback model.

Of course, the degree of the negative feedback regulation of r-protein gene transcription by ribosomes should be weaker than that for rRNA transcription in order to allow the *translational* feedback regulation to operate. Such a dual mechanism, feedback regulation by free ribosomes at the transcription step as the first stage of regulation and translational feedback regulation by repressor r-proteins as the second and final stage of regulation may be a very reasonable way for cells to regulate the synthesis of r-proteins. Further studies are required to substantiate this possibility.

Although the ribosome feedback regulation model can explain various *in vivo* observations, the molecular mechanisms involved in this regulation are unknown. Efforts in this laboratory to show direct and specific regulatory effects of ribosomes on transcription from ribosomal promoters *in vitro* have been negative so far. Thus, although the model would lose the beauty of simplicity, the possibility has to be considered that the apparent feedback regulation by free ribosomes is achieved indirectly through other effectors via unknown mechanisms. Since there is substantial evidence to suggest ppGpp as the negative effector both in stringent control and growth rate-dependent control, as reviewed above, this compound is certainly a possibility. If this were the case, one would have to propose that an increase in the amount of free ribosomes somehow increases the concentration of ppGpp precisely to the concentration that would give the appropriate degree of inhibition. At the moment, no information is available to support this proposal. In this connection, we note again the many reports that describe a correlation between the breakdown of polysomes and the cessation of rRNA synthesis under amino acid starvation conditions (reviewed in 184) perhaps as a result of general inhibition of the initiation of translation by ppGpp. Thus, the converse possibility would also deserve a serious experimental test; i.e. the apparent effect of ppGpp on rRNA synthesis during amino acid starvation might be exerted through free ribosomes.

RNA POLYMERASE CONTENT AND THE RATE OF TRANSCRIPTION As was evident in the previous discussion on the increased rate of transcription of rRNA operons in strains carrying extra rRNA operons with deletions, *E. coli* cells appear to have excess functional RNA polymerase under normal conditions. Since this is an important conclusion, we summarize other evidence in support of this conclusion. First, the number of functioning RNA polymerase molecules can be calculated from the total RNA synthesis rate and the RNA chain growth rate (which is independent of growth rate at a given temperature), and this value can be compared with the amount of RNA polymerase measured chemically. Since the total RNA synthesis rate increases much more rapidly than a linear function of μ (e.g. 3) and the amount of RNA polymerase probably increases more slowly than α_r (which is a linear function of μ) (see 7, 13, 226), the amount of RNA polymerase not functioning is higher at slow growth rates than at high growth rates; i.e. RNA polymerase must be present in excess at all growth rates except possibly at the highest growth rate. Gausing (10) estimated that not more than 80% of the RNA polymerase molecules are engaged in transcription at high growth rates and this fraction decreases to 25% at lower growth rates. Other measurements gave values ranging from 20% to 50% for this fraction

(13, 226, 227). Second, careful measurements of total RNA synthesis rates during nutritional shift-up indicate that the observed rapid increase could not be attributed to newly synthesized polymerases, but rather the utilization of RNA polymerases preexisting in large excess as nonfunctioning polymerase before the shift. Similarly, a large stimulation (1.3- to 3.5-fold increase) of the total RNA synthesis rate was observed after addition of chloramphenicol, and the degree of stimulation was higher during slow growth (222). Finally, the presence of RNA polymerase in DNA-less minicells implies that free, nonfunctioning RNA polymerase existed in the cytoplasm at the time of formation of the minicells (228). This fraction was estimated to be about 14%-24% of the total RNA polymerase and should represent a minimum value for the nonfunctioning RNA polymerase, since most of the nonfunctioning polymerases are probably bound to DNA in a nonspecific way (229). Thus, we conclude that *E. coli* cells have excess functional RNA polymerase under normal growth conditions.

rRNA SYNTHESIS IN SLOW GROWTH As mentioned earlier, it is known that the proportionality between ribosomes and growth rate does not hold under conditions of slow growth (230, 231). It was shown that the protein chain-elongation rate of each functioning ribosome at a particular temperature is the same regardless of the cell's growth rate (e.g. see 232), and thus the excess ribosomes in slow growth conditions are present simply as fully functional but nonfunctioning ribosomes (233). The ribosome feedback regulation model discussed above predicts that the amount of free ribosomes in the pool should be small when the transcription of rRNA operons is taking place efficiently, but the pool should increase in proportion to the degree of "repression" of rRNA operons; i.e. the amount of free ribosomes should increase with decreasing growth rate (144). The model also explains a paradoxical behavior of cells, e.g. the apparently detrimental overproduction of ribosomes that drains phosphate under conditions where cellular growth is restricted by a limiting supply of phosphate (234). Thus, while the overproduction of ribosomes at slow growth rates may be advantageous for cells that must be able to respond rapidly to better growth conditions (230), the overproduction might also be an unavoidable consequence of the feedback mechanism that bacterial cells have evolved to regulate rRNA synthesis under other ordinary growth conditions.

It has been reported that under very slow growth conditions, there is an overproduction followed by degradation of rRNA (3, 131). It appears that translation of r-protein mRNA or transcription of r-protein operons, rather than that of rRNA operons, becomes the rate-limiting step in the synthesis

of ribosomes, possibly because the "constitutive" expression of rRNA synthesis from the P2 promoter (156) is more refractory to the ribosome feedback regulation and becomes dominant under these slow growth conditions.

One important conclusion from the analysis of slow growth by Koch and co-workers (131, 230–234) is that bacterial growth is not limited by the amount of ribosomes. Rather, bacterial cells appear to have evolved the regulatory systems to adjust the ribosome synthesis rate in relation to other cellular metabolic activities so that the optimum growth rate can be attained, as discussed in previous sections.

Other Possible Modes of Regulation

Any step that could affect the efficiency of transcription initiation, elongation, or processing is a possible place for the regulation of rRNA synthesis. We have already mentioned the possible effects of supercoiling or different forms of RNA polymerase on promoter selectivity. Another possible site of regulation is a presumed antitermination function associated with rRNA synthesis that would prevent premature termination in the process of transcription of the long and untranslated rRNA operons. Morgan and colleagues (235–237) have presented evidence indicating the presence of such an antitermination function. Since antitermination processes studied in the λ phage system revealed participation of not only λ proteins but also host factors (reviewed in 238), one could speculate that some of these host factors might be involved in the process of rRNA gene transcription. Systematic examination of temperature-sensitive mutants preferentially affecting rRNA synthesis, such as those reported (174, 175), might be a useful way to study this subject. However, there is no evidence yet that antitermination is involved in the regulation of rRNA synthesis.

REGULATION OF RIBOSOME SYNTHESIS IN EUKARYOTIC CELLS

A detailed discussion of this subject is beyond the scope of the present article. We make only a few comments in relation to the two subjects discussed above: translational feedback regulation of r-protein synthesis and the ribosome feedback regulation model for rRNA synthesis and global ribosome synthesis.

As in *E. coli* cells, the synthesis of most r-proteins in eukaryotic cells is probably balanced and coordinately regulated (for review see 239). This regulatory feature has in fact been demonstrated in yeast (240). Therefore, one can ask about the mechanism responsible for the coordination,

specifically, whether any translational feedback mechanism similar to the one found in *E. coli* exists in eukaryotic cells. Recently, by the use of recombinant DNA techniques, several genes for r-proteins were cloned from yeast (241–243), *Drosophila* (244, 245), *Xenopus* (246), and mouse (247), and it has become possible to measure the amount, as well as the synthesis and the decay rates, of r-protein mRNA under various conditions (for review see 248). As a result, examples of regulation at the step of translation of r-protein mRNA have already been found, for example, in developing embryos of *Xenopus* (249) and in cultured mouse cells during the transition from resting to serum-stimulated states (250). More specifically, Pearson et al (251) carried out gene dosage experiments analogous to those done in *E. coli* by introducing into yeast cells plasmids carrying a r-protein gene; they found that transcription rates increased in proportion to gene dosages but r-protein synthesis rates increased only slightly. These results showed that r-protein synthesis is translationally regulated in *Saccharomyces cerevisiae* and that this regulation is probably responsible for balancing and coordinating r-protein synthesis. The results are consistent with what one would expect from the translational feedback regulation discovered in *E. coli*. However, Pearson et al (251) cautioned against its direct extension to eukaryotic systems because of two factors: (a) since r-protein genes in eukaryotic cells so far identified are monocistronic, most r-proteins would have to feedback regulate their own synthesis; and (b) since r-protein synthesis takes place in the cytoplasm and rRNA synthesis takes place in the nucleus, any competition between r-protein mRNA and rRNA for “repressor” r-proteins might be hindered by the presence of the nuclear membrane. Nevertheless, neither of these two factors is serious enough to exclude the possible existence of the translational feedback regulation mechanism; for example, quite a few *E. coli* r-protein genes are monocistronic and at least some of them were shown to feedback regulate their own synthesis. In addition, the possibility exists that some of these genes are regulated by r-proteins encoded by other unlinked genes, as discussed in a previous section. Similarly, since r-proteins are known to be transported from the cytoplasm to the nucleus through the nuclear envelope, one can imagine that the concentration of free r-proteins in the cytoplasm could reflect the concentration of free r-proteins in the nucleus and serve for the postulated feedback regulatory function. Experiments on *X. laevis* by Pierandrei-Amaldi et al (249) also demonstrated a striking (apparent) similarity in the regulation of r-protein mRNA translation between the *X. laevis* and the *E. coli* systems; in anucleolate mutants r-protein mRNA is synthesized, but its translation is specifically blocked as a result of the lack of rRNA synthesis, demonstrating the presence of translational regulation

resembling that found in *E. coli*. On the other hand, there are several known instances where r-protein synthesis was demonstrated in the absence of rRNA synthesis (reviewed in 239). Thus, we must await further studies to find out whether the translational regulation of r-protein synthesis found in some eukaryotic systems in fact involves a feedback mechanism similar to that found in *E. coli*.

The synthesis of mitochondrial and chloroplast ribosomes also poses several intriguing regulation questions, including ones concerning the coordination of the synthesis of r-proteins. For example, available information indicates that at least some chloroplast r-proteins in *Chlamydomonas reinhardtii* are coded for by the organelle genome and the rest are by nuclear genes. We expect large differences in gene copy numbers between organelle-coded r-proteins and nuclear-coded r-proteins, and hence the possible existence of a mechanism to coordinate these different classes of r-proteins (252). However, available information is too scarce to allow us to discuss the question at the moment.

The synthesis of rRNA (and ribosomes) in eukaryotic cells also appears to be regulated in response to changes in cellular growth rate to meet the demand for protein synthesis as is found in bacterial cells. For example, cultured cells that have ceased to grow because of serum-starvation or amino acid deprivation have lower levels of rRNA synthesis. When these cells are stimulated to grow by addition of serum, hormones, mitogens, or amino acids, stimulation of rRNA synthesis takes place, usually preceding stimulation of protein and DNA synthesis (for review see 253). In addition, at least in some experimental systems, the rRNA synthesis rates are apparently not influenced by increases or decreases in gene dosages (254–257). The results are reminiscent of those obtained in the gene dosage experiments performed in *E. coli* (144).

In eukaryotic cells, the organization of rRNA genes is different from that found in *E. coli*, and their transcription involves two distinct RNA polymerases, RNA polymerase III for the transcription of 5S rRNA genes and RNA polymerase I for the transcription of the genes for the larger rRNAs (for reviews see 258–260). In addition, in several known instances rRNA synthesis in eukaryotic cells involves unique features not found in prokaryotic cells; for example, gene amplification plays a role in the stimulation of rRNA synthesis during oogenesis in amphibians (261, 262). Thus, we expect that the regulation of rRNA synthesis in eukaryotic cells is complex and involves mechanisms not found in *E. coli* cells. Nevertheless, it is worthwhile to ask whether a feedback mechanism of the type that is postulated for *E. coli* also exists in eukaryotic cells. Several *in vitro* rRNA transcription systems are now well developed (for reviews see 253, 263, 264).

Use of these systems combined with the use of recombinant DNA techniques should provide answers to these and other questions about the regulation of ribosome biosynthesis in eukaryotes in the near future.

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¹ As noted in the preface (p. vi) to Volume 52 (1983), typesetting problems resulted in the omission of an author index from that volume. That author index appears as an extra index here in Volume 53.