

Diverse Mechanisms for Regulating Ribosomal Protein Synthesis in *Escherichia coli*

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Because proteins are the most abundant cellular macromolecules, cell growth requires that a large fraction of the cell mass be devoted to the protein synthesis machinery, namely, the ribosomes and their ancillary factors. For example, in rapidly growing bacteria, ribosomes account for as much as 50% of cellular dry mass. To avoid unnecessary investments in ribosome formation, mechanisms have evolved to reduce the synthesis of ribosomes during slower growth, when maximum rates of protein synthesis are not required (reviewed in 1). Because ribosome formation commands such a large fraction of cellular resources, even minor adjustments in the rate of ribosome synthesis can have a major impact on the economy of the cell.

Ribosomes are complex multicomponent organelles, containing three or

more different RNA molecules and at least 50 different proteins. Therefore, the regulation of ribosomal synthesis presents two problems: (1) coordinating the synthesis of individual ribosomal proteins (r-proteins) and rRNA molecules, and (2) balancing the overall rate of ribosome formation against the rate of synthesis of total biomass. In *Escherichia coli*, there is little turnover of ribosomal components under most growth conditions, indicating that the regulation of formation of ribosomal components is predominantly at the level of synthesis.

Even though the regulation of ribosome synthesis in many organisms has been investigated [for example, *Bacillus subtilis* (2), *Saccharomyces cerevisiae* (3, 4), *Dictyostelium* (5), plants (6), and *Xenopus* (7, 8)], this review is focused on *E. coli*. Earlier work on the regulation of *E. coli* ribosome synthesis (reviewed in 1, 9–11) was directed mainly toward elucidating the organization of ribosomal genes and the basic principles of their regulation. In recent years, the focus has shifted toward the analysis of detailed molecular mechanisms. Here we summarize current knowledge of the organization and regulation of ribosomal genes, emphasizing recent work elucidating the molecular mechanisms regulating ribosomal protein synthesis and illustrating the diversity of these mechanisms. The regulation of rRNA synthesis has been reviewed recently (12).

I. Organization of Ribosomal Protein Genes in *Escherichia coli*

In *E. coli*, the small ribosomal subunit contains one rRNA molecule (16 S) and 21 proteins. The large subunit harbors two rRNA molecules (23 S and 5 S) and 34 proteins (Table I). All of the genes for these ribosomal components have been mapped on the *E. coli* chromosome. Furthermore, all but three of the r-protein genes have been sequenced. The organization of the transcription units (here called operons) encoding r-proteins is summarized in Fig. 1. Note that several r-protein operons contain genes for non-ribosomal proteins, usually other components of the transcription and translation apparatus, such as the subunits of RNA polymerase.

A number of the genes for r-proteins and RNA polymerase subunits can mutate to generate resistance to various antibiotics. Historically, these antibiotics were used to name the genes. For example, the genes for r-proteins S12 and S5 and the β subunit of RNA polymerase are referred to as the *str* (streptomycin-resistance), *spc* (spectinomycin-resistance), and *rif* (rifampicin-resistance) genes, respectively (Table I). Similarly, the operon containing the S12 gene is referred to as the *str* operon and the S5-encoding operon is called the *spc* operon.

TABLE I
RIBOSOMAL PROTEIN AND RIBOSOMAL RNA GENES OF *Escherichia coli*^a

Protein	Gene	Map position ^b	Operon	Protein	Gene	Map position ^b	Operon
30-S proteins				L7/L12 ^c	<i>rplL</i>	90	L10
S1	<i>rpsA</i>	21	S1	L13	<i>rplM</i>	69	L13
S2	<i>rpsB</i>	4	S2	L14	<i>rplN</i>	73	<i>spc</i>
S3	<i>rpsC</i>	73	S10	L15	<i>rplO</i>	73	<i>spc</i>
S4	<i>rpsD</i>	73	Alpha	L16	<i>rplP</i>	73	S10
S5	<i>rpsE</i>	73	<i>spc</i>	L17	<i>rplQ</i>	73	Alpha
S6	<i>rpsF</i>	95	S6	L18	<i>rplR</i>	73	<i>spc</i>
S7	<i>rpsG</i>	73	<i>str</i>	L19	<i>rplS</i>	57	<i>trmD</i>
S8	<i>rpsH</i>	73	<i>spc</i>	L20	<i>rplT</i>	37	L20
S9	<i>rpsI</i>	69	L13	L21	<i>rplU</i>	69	Not sequenced
S10	<i>rpsJ</i>	73	S10	L22	<i>rplV</i>	73	S10
S11	<i>rpsK</i>	73	Alpha	L23	<i>rplW</i>	73	S10
S12	<i>rpsL</i>	73	<i>str</i>	L24	<i>rplX</i>	73	<i>spc</i>
S13	<i>rpsM</i>	73	Alpha	L25	<i>rplY</i>	48	L25
S14	<i>rpsN</i>	73	<i>spc</i>	L27	<i>rpmA</i>	69	Not sequenced
S15	<i>rpsO</i>	69	S15	L28	<i>rpmB</i>	82	L28
S16	<i>rpsP</i>	57	<i>trmD</i>	L29	<i>rpmC</i>	73	S10
S17	<i>rpsQ</i>	73	S10	L30	<i>rpmD</i>	73	<i>spc</i>
S18	<i>rpsR</i>	96	S6	L31	<i>rpmE</i>	89	Not sequenced
S19	<i>rpsS</i>	73	S10	L32	<i>rpmF</i>	23	L32
S20	<i>rpsT</i>	0	S20	L33	<i>rpmG</i>	82	L28
S21	<i>rpsU</i>	67	S21	L34	<i>rpmH</i>	83	L34
				L35	<i>rpmI</i>	37	L20
				L36	<i>rpmJ</i>	73	<i>spc</i>
50-S proteins				rRNA transcription units			
L1	<i>rplA</i>	90	L11	—	<i>rrnA</i>	87	—
L2	<i>rplB</i>	73	S10	—	<i>rrnB</i>	90	—
L3	<i>rplC</i>	73	S10	—	<i>rrnC</i>	85	—
L4	<i>rplD</i>	73	S10	—	<i>rrnD</i>	72	—
L5	<i>rplE</i>	73	<i>spc</i>	—	<i>rrnE</i>	90	—
L6	<i>rplF</i>	73	<i>spc</i>	—	<i>rrnF</i>	57	—
L9	<i>rplI</i>	96	S6	—	<i>rrnG</i>	5	—
L10	<i>rplJ</i>	90	L10	—			
L11	<i>rplK</i>	90	L11	—			

^aData in this table are based on studies of *E. coli* K12 (see the legend to Fig. 1 for references). Even though the gene organization is probably very conserved among different strains, there are some differences. For example, Southern blot analysis shows that some *E. coli* strains lack the approximately 15-kb DNA segment that separates the *str* and S10 operons in *E. coli* K12 and *E. coli* B (N. Smith, J. M. Zengel, and L. Lindahl, unpublished).

^bMap position is in minutes of the *E. coli* chromosome (172).

^cL7 and L12 are encoded by the same gene; L7 is the acetylated form of the r-protein.

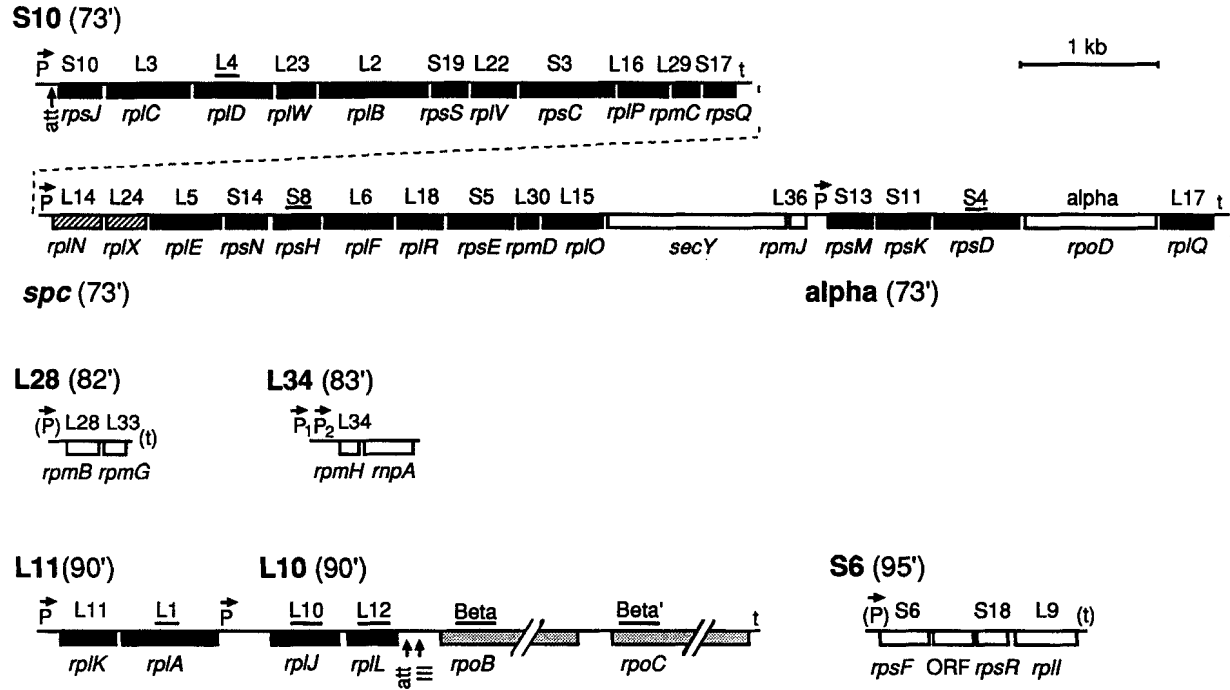


FIG. 1. Organization of ribosomal protein genes of *Escherichia coli*. Transcription units containing r-protein genes are drawn to scale according to their nucleotide sequences. Gene names (below the gene) and their products (above the gene) are shown. The chromosomal map positions are given after the operon names. Confirmed promoters and transcription terminators are indicated as P and t, respectively. Tentative promoters and terminators are shown as (P) and (t). Attenuator sites (att) and RNase III processing sites (III) are indicated. Gene products demonstrated to be autogenous regulators are underlined. The genes under their autogenous control are shown as solid black boxes; the genes reported to be under retroregulation are indicated as hatched boxes. Genes encoding proteins for which there are more tentative data are shown as stippled boxes. References for most of the information summarized in the figure are given in our previous reviews (9, 173). Other data are from 174 (L32 operon); 21, 22, 141, 147, 175–178 (*thrS*/L20/*pheS* operons); 179 (L25 operon); 180–182 (S21 operon); 18, 91, 93, 183 (S15 operon); 14, 184 (S10, *spc*, and *alpha* operons); 20 (*str* operon); and 185 (S6 operon).

About half of the r-protein genes are located in the classical *str-spc* region at about 73 minutes on the *E. coli* chromosome. These genes are organized into four operons containing from 4 to 12 genes (Fig. 1). The remaining genes are scattered around the chromosome in transcription units encoding one to four r-proteins (Fig. 1).

The boundaries of r-protein operons are often ambiguous, because of multiple promoters and transcription read-through between adjacent operons. For example, because there is no efficient transcription terminator between the *spc* and alpha operons (Fig. 1), the alpha operon is transcribed from both the *spc* and the alpha promoters (13, 14). Similarly, there is no transcription terminator at the end of the L11 operon in the *rif* region (Fig. 1), so the downstream L10 operon is transcribed from both its own promoter and the L11 promoter (15–17). In addition, some transcription terminators are “leaky.” For example, about 20% of the RNA polymerase molecules transcribing the S10 operon continue into the *spc* operon (14), and half of the polymerases transcribing the S15 gene proceed through the terminator after this gene (18). Several of the r-protein operons, including the S21 (19), *str* (20), and L20 operons (21, 22), contain internal secondary promoters (Fig. 1). Because many r-protein genes are expressed from several different classes of transcripts, we have defined the r-protein operons based on their regulatory patterns, rather than on transcriptional parameters.

II. Overview of the Control of Ribosomal Protein Synthesis in *Escherichia coli*

A. A Variety of Molecular Mechanisms for Autogenous Control

In the late 1970s, several research groups independently proposed that r-protein synthesis in *E. coli* is subject to “autogenous control” (23–25). That is, one gene in an operon encodes an r-protein, which serves both as a structural component of the ribosome and as a regulatory protein controlling the expression of itself and other genes in the operon (Fig. 2). (The term “feedback” regulation has also been used, although to biochemists this term refers specifically to inhibition of enzyme activity, not synthesis.)

The idea for autogenous control arose from three types of experiments. First, gene-dosage experiments showed that an increase in the copy number of ribosomal protein genes usually led to little or no increase in the rate of synthesis of the encoded proteins (23, 25). Second, conditional oversynthesis of individual r-proteins demonstrated that excessive accumulation of a single

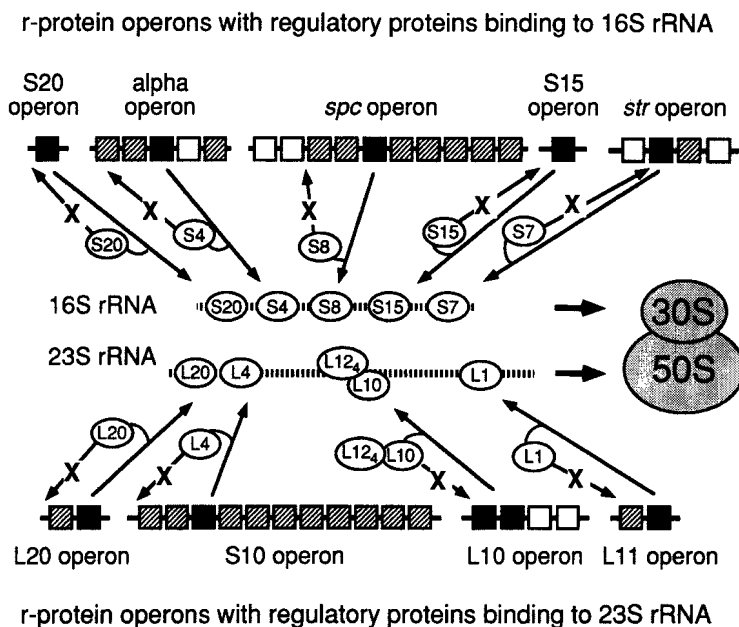


FIG. 2. Linkage of autogenous control of r-protein synthesis to ribosome assembly. Operons whose regulatory r-proteins have defined binding sites on 16-S or 23-S rRNA are shown. Genes encoding regulatory proteins are represented by solid black boxes; other genes whose transcription or translation is under their autogenous control are indicated by hatched boxes; unregulated or retroregulated genes are represented by empty boxes. The approximate locations of the regulatory targets on the mRNA are indicated by the feedback arrows.

key r-protein from an r-protein operon repressed expression of all genes in that operon (24, 26). These induction experiments also provided the first direct evidence that autogenous regulation is operon specific. Third, *in vitro* synthesis of r-proteins from a given operon could be inhibited by addition of a specific purified r-protein from the operon under study (27–29).

The dual function of the regulatory r-proteins suggests a mechanism for linking gene expression to the ribosome-assembly process (26, 30). During balanced growth, newly synthesized r-proteins bind to rRNA and are assembled into mature ribosomes. If the production of a given regulatory r-protein exceeds the production of other ribosomal components, the accumulated free protein represses the synthesis of most or all proteins encoded by its own operon to restore the balanced synthesis of r-proteins and rRNA (Fig. 2). The autogenous regulation is operon specific; that is, a regulatory r-protein represses only its own operon and has no direct effect on synthesis of proteins from other operons. This specificity, in conjunction with the con-

sumption of equimolar amounts of r-proteins during the ribosomal assembly process, ensures the coordinate expression of the various r-protein operons.

Regulatory r-proteins have now been identified for about half of the r-protein operons, and include both 30-S and 50-S components (summarized in Figs. 1 and 2). Although autogenous control is a recurrent theme for regulating expression of r-protein operons, there are several different mechanisms by which the autogenous control is achieved: (1) control of translation initiation, (2) control of mRNA elongation, and (3) control of mRNA degradation. Even within a single operon, different genes may be subject to different control mechanisms or to more than one type of regulation (for examples, see discussions of the S10, *spc*, alpha, and L10 operons in Section III).

B. Interaction of Regulatory r-Proteins with mRNA

Almost all the regulatory r-proteins are rRNA "primary" binding proteins, i.e., proteins that can bind directly and specifically to naked rRNA *in vitro* and therefore associate with the ribosomal particle during the early stages of ribosomal assembly (26, 30). Two inferences have been drawn from this point. One is that the rate of r-protein synthesis is directly tied to the rate of rRNA synthesis, such that a fluctuation in the amount of rRNA immediately induces a parallel change in the amount of r-proteins (26) (Fig. 2). The other is that, because the regulatory r-proteins have the ability to recognize specific RNA targets, autogenous control is likely to involve direct binding of a regulatory protein to a region of its own messenger RNA that is structurally related to its rRNA binding site (30).

The canonical model of direct binding of regulatory proteins to their respective mRNAs has been verified experimentally for several operons (although it is interesting to note that many of the originally proposed secondary structures of rRNA and mRNA that inspired the model turned out to be incorrect). Direct binding of a complex of L10 and L12 to the L10 operon mRNA (31), of r-protein S4 to the alpha operon mRNA (32), and of S8 to *spc* operon mRNA (33) has been demonstrated by filter-binding assays. The r-proteins S15 (34) and S7 (M. Nomura, personal communication) have been shown by "footprinting" experiments to bind to their respective mRNAs. However, not all attempts to show binding of a regulatory r-protein to its own mRNA have succeeded. No binding of S20 (35) or L4 (36) to their respective transcripts has been observed.

In some cases, a similarity between secondary structures of the binding sites on mRNA and rRNA for a given protein is obvious (e.g., see later, Figs. 8 and 9). In other cases such similarities are less clear. Perhaps the relationship between binding sites will become more apparent when the three-dimensional structures of the RNAs become known. It is also important to note that RNA sequence similarities can be deceiving. In several cases,

experimental analysis has failed to confirm the involvement of mRNA segments that display obvious sequence similarity to rRNA (see discussions of S10, S15, *spc*, and S20 operons in Sections III,A, III,C, III,D, and III,F, respectively).

There are a number of r-protein operons that do not contain genes for an rRNA primary binding protein. For example, the S1 operon is under autogenous control (37, 38), yet the regulatory protein, S1 is apparently not a primary rRNA binding protein (39). Also, r-protein S2 is not thought to bind directly to rRNA, yet its operon appears to be under autogenous control (40). Studies of such operons could be very interesting, because they apparently do not conform to the paradigm illustrated in Fig. 2. On the other hand, neither of the two r-proteins encoded by the *trmD* operon is an rRNA primary binding protein, nor does this operon appear to be autogenously regulated (41). Thus, in this case the absence of rRNA-binding r-proteins may reflect the absence of an autogenous regulatory mechanism.

The binding of regulatory r-proteins to their own mRNAs implies competition between rRNA and mRNA for the r-proteins (Fig. 2). It is obviously important that the proteins bind preferentially to rRNA so that r-protein synthesis is repressed only when there is a shortage of nascent rRNA molecules. Yet, in the few cases where comparative measurements of the affinity for rRNA and mRNA have been made, modest or no differences in the binding constants for the two RNA targets have been observed (32, 33). The preferential incorporation of regulatory r-proteins into ribosomes is apparently achieved, not by a "tighter" binding to rRNA, but rather by the cooperativity of the ribosome assembly process and the fact that proteins bound to rRNA will become trapped in mature ribosomal particles (42).

C. Mechanisms for Repressing Translation Initiation

Early studies showed that most autogenously controlled r-protein operons are inhibited at the level of translation (9, 11). For the L10 operon (43, 44) and the S10 operon (N. Brot, personal communication), *in vitro* dipeptide synthesis assays showed that the translation inhibition is at a step prior to the formation of the first peptide bond.

One can envision several ways by which translation initiation could be blocked. First, the protein could bind to a site on the mRNA that overlaps the ribosome-binding region, thereby inhibiting translation by steric blocking. This model has been proposed for a number of nonribosomal operons (45, 46). A second possibility is that binding of the regulatory protein induces a conformational change in the mRNA that makes the ribosomal binding region inaccessible, e.g., by involving the Shine-Dalgarno region and/or the initiation codon in intramolecular base-pairing. Even though the regula-

tory protein and the ribosome would not have overlapping binding sites, the protein would indirectly prevent binding of the ribosome to the translation initiation region. Third, the regulatory r-protein might block a step in the translation initiation pathway subsequent to binding of the 30-S ribosomal subunit to the mRNA but before formation of the first peptide bond. That such a pathway exists is suggested by the recent observation that the 30-S ribosomal subunit interacts with the Shine-Dalgarno region prior to formation of a canonical initiation complex (47).

The mechanism of translation repression is now being investigated for several r-protein operons. So far, none of the mechanisms seem to involve simple direct competition. Rather, the available evidence is compatible with the second and third possibilities.

In most r-protein operons in which the regulatory protein inhibits translation of a string of two or more genes, only the most proximal gene in the series is directly inhibited. The downstream genes are regulated by translational coupling, in which a given gene is translated efficiently only when ribosomes traverse the upstream mRNA. Such coupling has been demonstrated in the L11, L10, S10, *spc*, L20, and alpha operons.

D. Regulation of mRNA Elongation

In the S10 operon, the same r-protein, L4, autogenously regulates both transcription and translation (26, 48, 49). The transcription regulation is the result of premature termination of transcription at a terminator (attenuator) in the leader sequence about 30 bases upstream from the first gene of the operon (48, 50). The attenuation process is independent of the translation control by L4 (49, 50), and appears to be unique to the S10 operon (51).

E. Regulation of mRNA Stability

In two r-protein operons in which more than one gene is translationally inhibited, the gene directly repressed by the regulatory protein is not the most proximal gene of the operon (Figs. 1 and 2). For the *str* operon, *in vitro* experiments showed that regulatory protein S7 inhibits translation of the second and third genes, coding for S7 and EF-G, but not the proximal S12 gene (52). Similarly, for the *spc* operon, regulation by S8 *in vitro* starts at the third gene of the operon, with little or no effect on the upstream L14 and L24 genes (27). Nevertheless, the upstream genes in both operons are at least partially regulated *in vivo* (52, 53). The solution to this puzzle appears to be due to a specific destabilization of the upstream part of the mRNA (54; M. Nomura, personal communication).

Less specific destabilization of mRNA has been observed when translation of the L11 (55) and alpha operons (56) is inhibited by their respective autogenous regulators. For the L11 operon, the decreased stability of the

mRNA is an indirect effect of reduced translation, presumably resulting from the decreased ribosome density on the message (55). How much such mRNA destabilization contributes to autogenous regulation is not yet clear.

III. Review of Individual Operons

A. S10 Operon

1. TWO L4-MEDIATED REGULATORY PROCESSES

L4 regulates both transcription and translation of the 11-gene S10 operon (24, 26, 28, 49, 50). Transcription control was first detected in pulse-labeling experiments showing that synthesis of structural gene message from the entire S10 operon is reduced four- to fivefold in response to L4 oversynthesis (26). Nuclease mapping of pulse-labeled RNA subsequently revealed that the L4 inhibition of transcription is due to the protein's stimulation of premature transcription termination (attenuation) about 140 bases from the transcription start, or about 30 bases upstream from the initiation codon for the most proximal structure gene (48, 57, 58). Transcription control has also been demonstrated by *in vitro* studies showing that L4 induces premature termination in the S10 leader in a transcription system that contains only purified RNA polymerase and, as discussed below, transcription factor NusA (58).

Repression of translation of S10 operon mRNA mediated by L4 was first seen in *in vitro* experiments (28). Subsequently, translation control was also shown *in vivo* by comparing transcription and translation rates: oversynthesis of L4 reduced protein synthesis to about 5%, whereas the rate of mRNA synthesis was reduced to 20–25% (49). These numbers imply that excess L4 reduces mRNA synthesis 75 to 80%, and translation of the residual mRNA transcripts by an additional 80%. This interpretation was confirmed by genetic analysis of the S10 leader: certain mutations eliminated translation control by L4 without affecting inhibition of transcription, whereas other mutations eliminated transcription control but left translation control intact (49). Together, these genetic and physiological studies suggest that the two L4-mediated regulatory processes work independently, but additively, to control synthesis of the 11 r-proteins encoded by the S10 operon.

2. REQUIREMENTS FOR LEADER SEQUENCES IN L4-MEDIATED REGULATION

Structure-probing studies showed that the S10 leader RNA can fold into a series of hairpin structures that are illustrated in Fig. 3 (59). Transcription termination occurs around base 140, on the descending side of the large

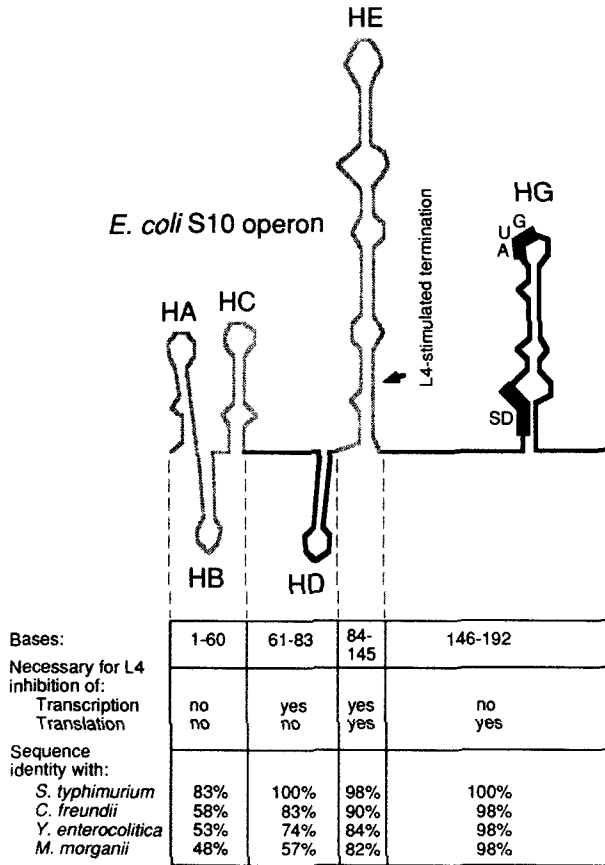


FIG. 3. Functional domains of the S10 leader. The secondary structure of the S10 leader was determined by Shen *et al.* (59). The stem-loop structures are identified as HA, HB, HC, HD, HE, and HG. The positions of the Shine-Dalgarno sequence (SD) and the initiation codon for the S10 gene are also shown. Domains needed for L4-mediated transcription and translation control are indicated (49, 50, 58; J. M. Zengel and L. Lindahl, unpublished). Data comparing the S10 leader sequence from *E. coli* with other enterobacteria are also summarized (60).

"attenuator" hairpin structure HE (58). The leader can be divided into several functional domains according to effects of mutations on L4-mediated regulation (Fig. 3). The proximal three hairpins (HA, HB, and HC) can be deleted individually or *en bloc* without any detectable effect on the L4-mediated inhibition of either transcription or translation (J. M. Zengel and L. Lindahl, unpublished). Hairpin HD is absolutely required for transcription control by L4 (*ibid.*), but is dispensable for translation control (49). The HE hairpin region is essential for both transcription and translation control (49).

Finally, the leader downstream from the attenuation site is essential only for translation control (50, 58; J. M. Zengel and L. Lindahl, unpublished).

This division into functional domains is consistent with our phylogenetic studies of the S10 leader sequences of the closely related enterobacteria *Salmonella typhimurium*, *Citrobacter freundii*, *Morganella morganii*, and *Yersinia enterocolitica* (Fig. 3). For example, the region upstream from hairpin HD, which is dispensable for both levels of L4 control, has as little as 48% sequence identity between *E. coli* and the other enterobacteria, whereas the region from HE to the initiation codon of the S10 gene has greater than 80% identity (60). Interestingly, sequences in the HA, HB, and HC regions of *M. morganii* and *Yersinia enterocolitica*, which show the least homology to *E. coli*, can still be organized (at least theoretically) into three hairpins, but the lengths and number of bulges and internal loops on the stems vary considerably from the *E. coli* leader. The HD hairpin structure, involved in L4-mediated transcription control in *E. coli*, is conserved in the other four species, with several compensatory changes in the stem and variations in the sequence, but not length, of the loop. Similarly, base changes in hairpin HE are all in loops or bulges, or are nondisruptive (e.g., G·C → G·U). These phylogenetic studies are consistent with our conclusions from genetic studies that the leader sequence and/or secondary structure from HD to the beginning of the S10 gene are critical for the L4-mediated regulation. In fact, these "foreign" S10 leaders, when placed upstream from the *E. coli* S10 gene, display L4-mediated regulation (60).

3. BINDING OF L4 TO RNA

As discussed in Section II, B, several regulatory r-proteins have been shown to bind directly to their cognate mRNA. However, attempts to show binding of L4 to the S10 leader have failed (P. Shen, J. M. Zengel, and L. Lindahl, unpublished). One possible explanation is that the L4 binding site includes protein determinants contributed by components of the transcription and/or translation system. Another possibility is that the leader RNA undergoes structural changes during its synthesis. L4 might, for example, bind to a region that is only transiently single-stranded in nascent RNA, but that becomes double-stranded once transcription of the leader region is completed (Fig. 4; see also Section III,A,5). The RNA we have used for binding studies is full-length leader RNA that may have assumed the "wrong" conformation for L4 binding. In any case, the L4 target is likely to include the S10 leader RNA, because it is otherwise difficult to explain how L4 specifically regulates the S10 operon.

If a single target for L4 is involved in both transcription and translation regulation, then it must be within hairpin HE, because this is the only part of the leader required for both types of regulation (see Section III,A,2 and

Fig. 3). An attractive, but untested, possibility is that L4 binds around base 90 of the leader (Fig. 4). This region is likely to be single-stranded during the transcription pause observed at the attenuator (see Section III,A,4). Furthermore, binding of L4 to this region might also serve to affect the equilibrium between the two isomers of the mRNA hypothesized to be involved in translation control (Section III,A,5). Finally, this region would be sequestered in a double-stranded structure in full-length mRNA and thus explain why our attempts to bind L4 to the leader transcript have failed.

The generic model for autogenous control of r-protein synthesis is based on the idea of structurally related targets on rRNA and mRNA for binding of the regulatory r-protein. We have recently mapped the L4 binding site on 23-S rRNA to a region about 300 bases from the 5' end, i.e., within domain I, by showing that specific fragments of 23-S rRNA added to *in vitro* transcription reactions can bind L4 and eliminate the regulatory protein's stimulation of attenuation (36, 61). This region of the 23-S RNA has no striking similarity with the primary or secondary structure of the S10 leader (Fig. 4), although analysis of tertiary interactions in each of the two RNA molecules may reveal similarities not yet obvious.

In intact 50-S ribosomes, L4 has been cross-linked to bases in both domain I (62) and domain II (63) of 23-S rRNA. Since the domain-II site was found much sooner than the domain-I site, it was long believed that the L4 binding site was within domain II. This hypothesis was appealing because of scattered primary sequence identities between the S10 leader in the attenuator region and the region of domain II in 23-S rRNA that includes the L4 cross-linking site (64). However, subsequent genetic studies showed that the bases of the S10 leader involved could be mutated or deleted and still leave intact one or both forms of L4-mediated autogenous control (49, 50, 58). Thus, it now seems likely that the similarity between the S10 leader and domain II of 23-S rRNA is irrelevant for the autogenous control.

4. MECHANISM OF L4-MEDIATED TRANSCRIPTION REGULATION

In vitro transcription studies have shown that L4 stimulates termination of transcription only in the presence of the protein NusA (58, 61), a transcription factor that generally increases the ability of RNA polymerase to correctly "interpret" termination and antitermination signals (65). A possible pathway for transcription control, based on these *in vitro* experiments, is summarized in Fig. 5. The first step is a NusA-dependent pause by RNA polymerase at the site where transcription termination will take place (61, 66). L4 is not required for pausing, but strongly prolongs the pause, presumably accounting for its stimulation of termination. Interestingly, L4 can stabilize the paused complex even if the r-protein is added to the reaction *after* the NusA-modified RNA polymerase has already reached the pause site (66).

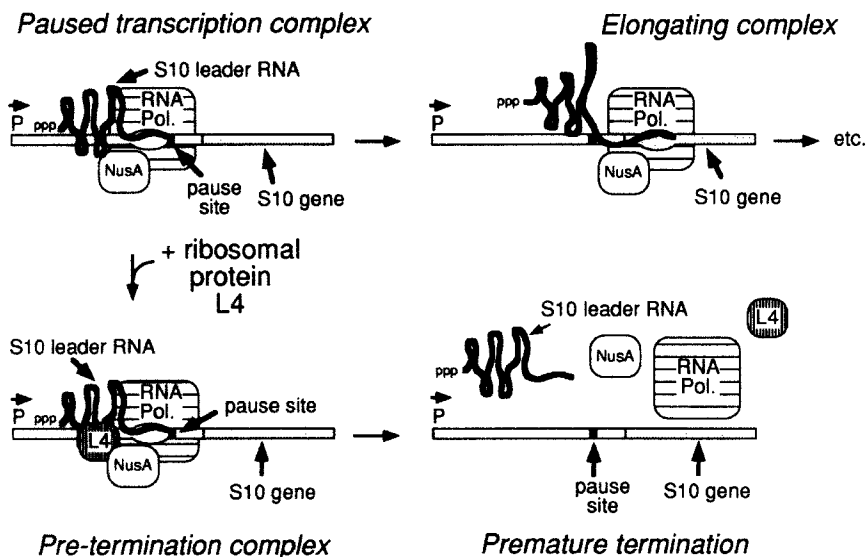


FIG. 5. Model for L4-mediated termination of transcription. In the presence of NusA, most of the RNA polymerases pause briefly at the attenuation site before continuing elongation through the S10 and downstream genes (66). The addition of r-protein stabilizes the pause, thereby promoting premature termination of transcription (66). In the absence of NusA, RNA polymerase ignores the pause signal (not shown).

Leader deletion studies showed that the leader region containing only the HE hairpin is sufficient for the NusA-dependent pause, but the upstream HD hairpin is essential for L4 stabilization of the paused complex (J. M. Zengel and L. Lindahl, unpublished). Analysis of base substitution mutations altering the upper stem-loop region of the HE hairpin suggests that the G·C-rich stem is part of the pause signal. The role of hairpin HD in the effect of L4 is not clear. It might represent the L4 binding site on the leader RNA. However, because the HD hairpin is dispensable for L4 inhibition of translation (Fig. 3), this would imply that translation control involves a separate L4 binding site. In any case, further studies are needed to pinpoint the L4 target (see also Section III,A,3) and to identify how a ribosomal protein influences RNA polymerase.

5. MECHANISM OF L4-MEDIATED REGULATION OF TRANSLATION

The mechanism for L4-mediated translation regulation has not yet been analyzed as rigorously as the transcription attenuation mechanism. A working model has been proposed (59), based on structure-probing data suggest-

ing that the leader exists in at least two conformations differing with respect to the length of the bottom part of HE and the formation of a hairpin (HF) between HE and HG. Surprisingly, the Shine–Dalgarno region is sequestered in both forms (59). This presumably prevents ribosomes from binding directly to the ribosome binding site of the S10 gene. We have hypothesized that a ribosome gains access to the translation initiation site of the S10 gene by first binding to the single-stranded region between HE and HG in the structure shown in Fig. 4 (59). Once associated with this “ribosome entry site,” the ribosome may compete with the distal sequence of HG for the Shine–Dalgarno region, or possibly even destabilize the HG hairpin by diffusing along the RNA molecule. Thus, we propose that the S10 mRNA is translatable when the ribosome entry site is in single-stranded form, but untranslatable when the site is sequestered in the HF hairpin.

Our working model for translation repression of the S10 gene is that r-protein L4 induces a shift in the equilibrium between the translatable and untranslatable forms (Fig. 4). Consistent with this proposal, an 8-nt deletion removing much of the putative ribosome entry site results in a 90% reduction of the translation efficiency and eliminates translation control by L4 (J. M. Zengel and L. Lindahl, unpublished).

Although this model is appealing in its relative simplicity, the process for regulating translation of the S10 gene is probably more complex. For example, this model cannot account for our observation that translation control is affected by several mutations in the upper stem-loop of hairpin HE, well upstream from the ribosome entry site and the Shine–Dalgarno sequence (49). It is interesting to note that the *in vivo* translation control of these hairpin mutations more or less correlates with the effect on NusA-dependent pausing observed in the *in vitro* transcription system (our unpublished experiments), suggesting that L4 may inhibit translation of the S10 gene only if it binds to the mRNA during a pause in transcription. For example, the L4 target may involve a region of the S10 leader on the ascending side of the HE hairpin, still single-stranded while RNA polymerase is paused. Once bound to this region, the L4 protein might prevent the lower stem of HE from forming on completion of the leader synthesis and thereby favor formation of hairpin HF, i.e., the untranslatable form (Fig. 4).

Whatever the precise molecular mechanism by which L4 inhibits translation of the S10 gene, the effect is probably propagated down the multicistronic transcription unit by translational coupling. We have analyzed translational coupling between the first two genes of the S10 operon by constructing a gene fusion between the second gene of the operon (encoding L3) and *lacZ*, and monitoring how mutations that decrease or eliminate translation of the upstream S10 gene affect expression of the downstream fusion gene (67). Such mutations severely reduced the expression of the

downstream L3'/lacZ' gene, but the reduction was partially due to premature transcription termination (polarity), as is often observed in *E. coli* when mRNA translation is blocked (68). This problem was circumvented by replacing the S10 promoter with a promoter for bacteriophage T7 RNA polymerase. Transcription by this enzyme proceeds even in the absence of translation (67, 69, 70), making possible measurements of the translation of the L3'/lacZ' fusion gene in the presence of mutations blocking S10 translation. The results showed that complete elimination of the translation of the S10 gene reduces the translation efficiency of the L3'/lacZ' gene by about 80% (67).

The incomplete coupling of the translation of the S10 and L3 genes suggests that it may be difficult to propagate translation regulation through a long operon exclusively by translational coupling: ribosomes entering at an intercistronic junction because of incomplete coupling would promote translation of genes still further downstream. Perhaps this is the reason that the S10 operon is also regulated autogenously at the transcriptional level.

B. Alpha (S4) Operon

1. TRANSLATION REGULATION BY S4

The alpha operon of *E. coli* contains genes for four ribosomal proteins and for the alpha subunit of RNA polymerase. Gene dosage experiments (53), *in vitro* translation experiments (27), and experiments with induced oversynthesis of specific r-proteins (29, 71, 72) demonstrated that the r-protein genes in the alpha operon are translationally repressed by r-protein S4, the product of the third gene in the operon. Remarkably, translation of the alpha subunit gene is only weakly inhibited by S4, even though this gene is flanked by r-protein genes that are strongly repressed by the r-protein (72). Regulation of all the r-protein genes, including the L17 gene located distal to the alpha subunit gene, is abolished by deletions in the leader of the operon (72). Thus, it appears that S4 has a single regulatory target on the alpha operon mRNA, and that the L17 gene is translationally coupled to the three upstream r-protein genes, even though the unregulated alpha gene is in between. No mechanism for this unusual coupling has been determined yet.

2. BINDING OF S4 TO RNA

Binding of S4 *in vitro* to the alpha mRNA is well-characterized. The binding site encompasses the leader and proximal 40 bases of the first gene, encoding S13 (32, 73, 74). Both S4 binding to alpha mRNA and S4-mediated repression of translation require sequences extending outside the region containing the traditional features of a ribosome binding site (72, 73), suggesting that S4-mediated repression of translation is not accomplished by a simple competition between S4 and initiating ribosomes.

S4 mRNA

16S rRNA

FIG. 6. Targets for r-protein S4 on mRNA and 16-S rRNA. The structure of the S4 mRNA was determined by Draper and co-workers (74, 75, 80). The Shine-Dalgarno sequence and GUG initiation codon for the S13 gene are indicated by boxes. A schematic diagram of the secondary structure of the region of 16-S rRNA implicated in S4 binding is also shown (76-78). Helices shown by genetic analysis to be especially critical for S4 binding are shown as solid structures (78). The hatched region represents a domain apparently protected by bound S4 in footprinting experiments (77), but is not essential for binding as measured by other techniques (76, 78).

on the two RNA molecules could be missed if these points were widely separated in the two-dimensional models.

3. ALLOSTERIC CHANGES IN ALPHA mRNA

The mechanism for S4-mediated translation repression appears to involve an allosteric change in the mRNA induced by S4 binding (80), rather than a simple competition between S4 and initiating ribosomes for the same space on the message. This conclusion is based on the study of point mutations designed to probe the influence of each helix in the two intertwined pseudoknots on S4::mRNA binding and on translation repression. Several mutations abolished translation repression yet allowed normal S4 binding, indicating that the binding of the regulatory protein per se does not repress translation (80). Rather, it appears that S4 binding induces a conformational change in the wild-type mRNA that in turn prevents translation initiation.

The existence of different conformers of the alpha mRNA is supported by the observation of a hyperchromic shift at about 35°C, a temperature much below the general melting temperature for the mRNA secondary structure (81). The importance of this conformational transition was recently established by studies of ribosome::alpha-mRNA complexes at different temperatures. Two types of such complexes have been identified by "toeprint" experiments [mapping of mRNA-bound ribosomes by using the ribosomes as roadblocks for the progression of reverse transcriptase in primer extension (81)]. One type of complex is the conventional ternary complex consisting of a ribosome and initiator tRNA interacting with the Shine-Dalgarno region and the initiation codon of the mRNA. The other is a binary complex consisting of only a ribosome and an mRNA molecule.

Kinetic experiments suggest that conventional ternary complexes form most readily at temperatures above the conformational transition temperature, whereas the binary complexes form preferentially below the transition temperature (82). Furthermore, conversion between the two types of complexes is slow. The key to understanding translation repression seems to be that the binary complex binds S4 and prevents conversion into a conventional translation initiation complex. Thus it appears that the alpha mRNA is trapped in a complex with ribosomes and S4 and is not available for normal translation initiation (82).

These experiments illustrate that S4 regulates the translation by mRNA entrapment and not by competition with ribosomes for a common binding site on the mRNA. It should be interesting to learn whether translation initiation factors (which so far have been omitted from the *in vitro* experiments) play a role in this novel regulatory mechanism.

C. S15 Operon

1. GENETIC ORGANIZATION AND REGULATION

The S15 gene is adjacent to, and cotranscribed with, the *pnp* gene encoding polynucleotide phosphorylase. About 50% of the RNA polymerases initiating at the P₁ promoter upstream from the S15 gene (Fig. 1) terminate at the t₁ terminator between the S15 and *pnp* genes; the remainder of the polymerases continue through *pnp* (83); *pnp* is also transcribed from the weaker P₂ promoter located between the two genes of this complex transcription unit (18).

Like most other r-protein operons, the S15 gene is autogenously regulated at the level of translation (84, 85). The region of the mRNA that is required for S15 autoregulation overlaps the translation initiation site, a somewhat surprising finding because homology between a sequence at the 3' end of the S15 gene and a sequence in the S15 binding site on the 16-S rRNA had been noted previously (86). Apparently this homology was fortuitous, similar to the example of homology between the S10 leader and domain II of the 23-S rRNA (see Section III,A,2).

Chemical and enzymatic structure probing of the regulatory site (34) showed that the S15 mRNA exists in two forms that appear to be in equilibrium with each other (Fig. 7). One conformation contains two hairpins; in the other conformation, the downstream (relatively unstable) hairpin is resolved and part of the RNA is instead paired with the loop of the upstream hairpin to form a pseudoknot. Footprinting experiments have demonstrated that S15 binding to the RNA stabilizes the pseudoknot conformation (34). Neither of the two conformations of the mRNA shows obvious homology with the S15 binding site on 16-S rRNA (87–89) (see Fig. 7). However, there may be some similarity between the tertiary structures, because they both contain helices associated with unpaired adenines, either as a bulging base in 16-S rRNA or as a pair of bases spanning the groove in the pseudoknot of the mRNA (34).

2. MECHANISM OF REGULATION OF S15 SYNTHESIS

Recent analysis of S15-mediated translation repression has revealed a novel mechanism (90). The 30-S subunit apparently binds to the mRNA in its pseudoknot conformation to form a preinitiation complex that generates a toeprint at the promoter distal border of the pseudoknot, about six bases upstream from the normal toeprint. This preinitiation complex can be converted into a traditional translation initiation complex, presumably simultaneously with unwinding of the pseudoknot, to allow the Shine–Dalgarno sequence of the mRNA to base-pair with the 16-S rRNA.

These studies also provide important evidence that S15 can bind not only

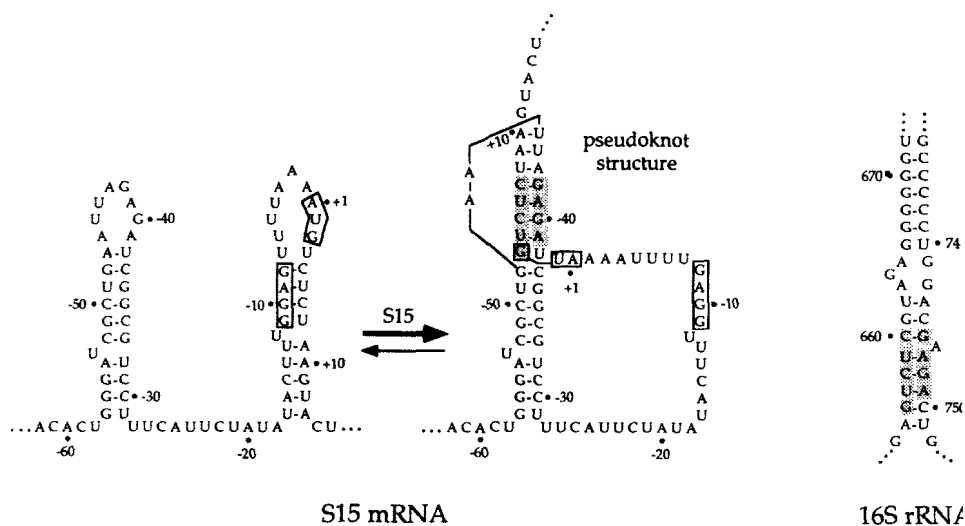


FIG. 7. Targets for S15 on mRNA and 16S rRNA. The structures of the S15 mRNA were proposed by Philippe *et al.* (34). The Shine-Dalgarno sequence and AUG initiation codon for the S15 gene are indicated by open boxes. S15 is thought to bind to the pseudoknot form (34). The region of 16S rRNA containing the S15 binding site (87–89) is shown on the right. The shaded regions indicate possible structure homologies between the pseudoknot form of S15 mRNA and the 16S rRNA (34).

to naked mRNA, but also to the preinitiation complex (i.e., the binary mRNA::30S complex). Binding of S15 to the binary complex prevents the transition to the canonical ternary initiation complex (90). It is important to note that the regulatory protein S15 and the 30S subunit can bind simultaneously to the mRNA and thus do not compete for the same or overlapping binding sites on the mRNA. Thus, the S15 gene is regulated by an “entrapment” mechanism similar to that described for the alpha operon: the regulatory protein stabilizes an unproductive ribosome::mRNA complex, making the mRNA unavailable for translation initiation.

3. PROCESSING OF S15 OPERON mRNA

Genetic evidence suggests that expression of the S15 and *pnp* genes may also be regulated by RNA processing. RNA transcribed from the two genes is cleaved by two nucleases, RNase III and RNase E. RNase III cleaves at a site downstream from the P_2 promoter, resulting in marked destabilization of the *pnp* transcripts. Consistent with this, polynucleotide phosphorylase is overproduced in RNase III-defective mutants (83, 91, 92). Experiments with an RNase E mutant suggest that this nuclease cleaves at two sites flanking the

t_1 terminator; this cleavage destabilizes the S15 transcript (93). However, the effects of RNase III and RNase E processing on translation regulation of S15 is not clear.

D. *spc* Operon

1. REGULATION OF TRANSLATION

The *spc* operon contains 11 r-protein genes and *secY*, a gene coding for a protein involved in protein secretion (Fig. 1). Early experiments *in vitro* and *in vivo* indicated that r-protein S8 regulates translation of eight contiguous genes within the operon, beginning at the third gene encoding L5; the two proximal genes, encoding L14 and L24, were reportedly not under S8 control (27, 94). Recently, a more detailed analysis of the regulation of the *spc* operon indicated that overproduction of regulatory protein S8 does temporarily repress the expression of L14 and L24, but within a few minutes this regulation fades, apparently overridden by other mechanisms (54). (There are no studies directly addressing the regulation of the two most distal genes of the operon, *secY* and *rpmJ*.) The S8-mediated regulation originates from a site located between the second gene coding for L24 and the third gene coding for L5 (95). Genes distal to this regulatory site are repressed by S8 at the level of translation (27, 95), whereas the regulation of the two upstream genes appears to be due to "retroregulation" (54; see Section III,D,3).

2. BINDING OF S8 TO RNA

The S8 binding site on *spc* mRNA (Fig. 8) was identified by isolating RNA fragments protected from nuclease digestion by r-protein S8 and re-binding of the protein to the purified RNA fragments (33). Interestingly, the rebinding requires two noncontiguous but complementary fragments of the RNA, suggesting that S8 binds to a helical segment of the mRNA (33) (Fig. 8). This position agrees well with the locations, in the region around the start of the L5 gene, of mutations that abolish the S8-dependent repression of translation (95). Secondary structure probing and phylogenetic studies (95) indicate that the structure of the S8 regulatory target on the mRNA is similar to the structure of its binding site on 16-S rRNA (Fig. 8), a homology that was also proposed after visual inspection of the sequence of the mRNA region protected from nuclease by S8 (33). The S8 regulatory protein binds to the *spc* mRNA with an association constant about one-fifth that of the constant for S8 binding to 16-S rRNA (33; see also Section II,B).

The S8 binding site on 16-S rRNA is among the best studied r-protein binding sites on rRNA (see, e.g., 33, 88, 96, 97). It is a much more compact site than, for example, the S4 binding site (see Section III,B): binding of S8 requires only a phylogenetically conserved helix with a bulge region (33, 97).

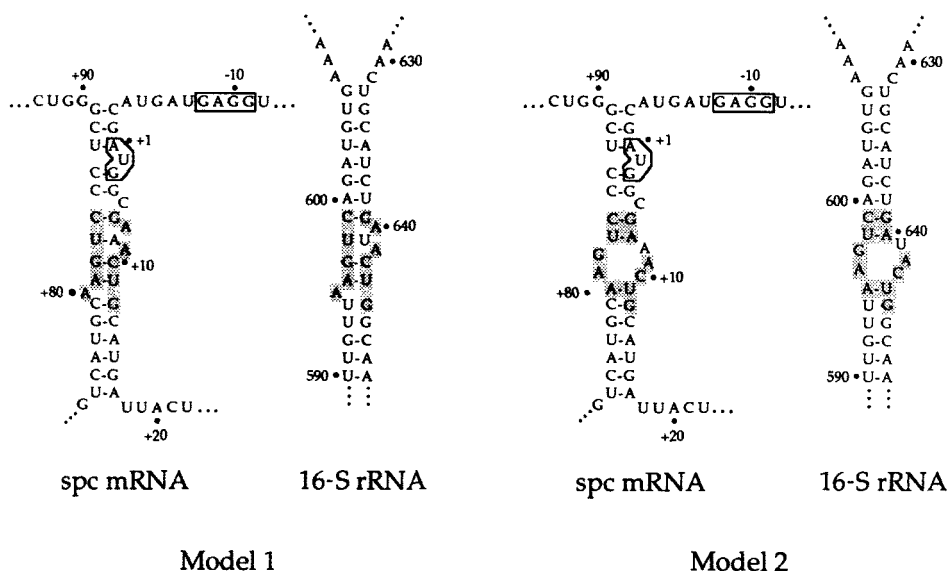


FIG. 8. Targets for S4 on *spc* mRNA and 16-S rRNA. Model 1 shows the secondary structure of the S4 target on *spc* mRNA proposed by Cerretti *et al.* (95), and the S4 target region on 16-S rRNA proposed by Mougél *et al.* (97). Model 2 shows a slightly different structure for mRNA and 16-S rRNA proposed by Gregory *et al.* (33). The AUG initiation codon and Shine-Dalgarno sequence of the L5 gene are indicated by open boxes. The regions of possible homology between mRNA and 16-S rRNA are indicated by shading.

Studies of S8 binding to mutant variants of 16-S rRNA indicate that the features required for binding to the 16-S rRNA are similar to the structural features required in the *spc* mRNA for S8-dependent repression of translation (33, 95). The interaction of S8 with the *spc* operon mRNA at a 16-S rRNA-like target thus fits the generic model for autogenous regulation (30, 98). Ironically, this model originated from *spc* mRNA secondary structures derived by computer analysis of limited segments of the RNA (98), later shown by experimental testing to be incorrect (33, 95); nevertheless, the principle of the original model was correct.

3. MECHANISM OF TRANSLATION REPRESSION

The binding of S8 to *spc* mRNA is believed to inhibit directly the translation of the L5 gene. The molecular basis for this repression is not yet clear, but the proximity of the S8 binding site to the L5 initiation codon (Fig. 8) suggests that binding of S8 to the helix that includes the initiation codon may stabilize this structure, thereby either directly preventing binding of ribo-

somes to the mRNA, or preventing the progression from a preinitiation complex to an initiation-competent complex. In this connection, it should be noted that the current secondary structure model for this region of the *spc* message predicts that the Shine–Dalgarno sequence for the L5 gene is not base-paired (Fig. 8) and hence may be available even if S8 is bound.

The seven r-protein genes downstream from L5 (i.e., S14 through L15) are not regulated directly by S8 binding to the *spc* mRNA, but rather by an indirect effect of the inhibition by the r-protein of L5 translation (94, 99). This effect is not due to transcription regulation, because measurements of mRNA synthesis during the autogenous response to S8 oversynthesis show that excess S8 has no effect on the transcription rate (99; J. M. Zengel and L. Lindahl, unpublished). Rather, translation of the downstream genes appears to be coupled to (i.e., dependent on) translation of the upstream L5 gene. When the initiation codon of the L5 gene is mutated to a termination codon, translation of the downstream genes is decreased (99).

Northern analysis of accumulated mRNA showed that oversynthesis of S8 also results in site-specific processing and increased turnover of the *spc* mRNA, presumably due to an indirect effect of the reduced translation (99). The increased rate of mRNA degradation may help to propagate the autogenous regulation through the long *spc* operon (99), because translational coupling may not be absolute. Thus, the increased mRNA turnover rate could serve the same function achieved by the transcription regulation (attenuation) in the S10 operon (Section III,A).

The L14 and L24 genes are upstream from the target for S8 and their translation is not directly inhibited by S8 *in vitro* (27). Nevertheless, the synthesis of L14 and L24 is temporarily repressed *in vivo* when S8 is oversynthesized (54). Because excess S8 leads to fragmentation of the *spc* mRNA, and because one of these fragments carries the L14 and L24 gene sequences, it was proposed that L14 and L24 synthesis is regulated by retroregulation (54). That is, S8-mediated inhibition of translation triggers nucleolytic cleavage of the *spc* mRNA near the S8 binding site, and degradation of the resulting L14–L24 mRNA fragment. Consistent with this proposal, the repression of L14 and L24 synthesis is diminished in a mutant defective for polynucleotide phosphorylase and RNase II (54), two 3'-to-5' exonucleases that have been implicated in mRNA degradation.

E. *rif* Region

1. GENETIC AND REGULATORY ORGANIZATION

The rifampicin region (*rif*) contains the genes for the four r-proteins, L11, L1, L10, and L12, as well as for the RNA polymerase subunits β and β' (Fig. 1). Two major promoters drive transcription of these genes: P_{L11} at the

beginning of the gene cluster and P_{L10} between the second and third gene (15, 16, 100, 101). There is little or no termination of transcription after the second gene, so that most or all polymerases continue to the end of the cluster (15–17). Because the genes for L10 and L12 are transcribed from both promoters, they are transcribed at a higher rate than the upstream L11 and L1 genes (16). Transcription is partially terminated by an attenuator between the L12 and β genes, such that the β and β' genes are transcribed only about one-fifth as frequently as the r-protein genes (15, 16, 102, 103). Finally, all transcripts are terminated at the end of the cluster (102). Further complexity is added to the expression of this region by a site for RNase III cleavage immediately upstream from the attenuator between the L12 and β genes (Fig. 1), although processing at this site has no clear effect on gene expression (103, 104).

Even though the four r-protein genes in the *rif* region are partially cotranscribed, they are regulated by two separate autoregulatory translation repression circuits. The two proximal genes are regulated by r-protein L1 (27, 29), whereas the two distal r-protein genes are regulated by L10, probably in a complex with L12 (105–108). Expression of the β and β' genes is not affected by the two regulatory r-proteins. Rather, these two genes are auto-genously regulated by both transcriptional and translational processes in response to RNA polymerase activity. (For recent discussions of the regulation of β and β' , see 109–112.)

2. REGULATION OF THE L11 OPERON

The two-gene L11 operon (Fig. 1) is regulated at the translational level by the product of the downstream gene, L1 (27, 29). Genetic studies showed that the target for L1 control is upstream from the L11 gene (113–115). Enzymatic structure probing (116) and phylogenetic studies (117–119) identified a structure in the L11 leader RNA homologous to the site of L1 binding in 23-S rRNA. This region contains two short helices separated by an internal loop (Fig. 9). Although binding of L1 to this region of the leader has not been directly demonstrated, the correspondence between the 23-S binding site and the presumed binding site on the mRNA was confirmed by mutational studies. Base changes in the presumed target region of the leader that reduced or eliminated the sensitivity to L1 repression (115) were compared to analogous changes introduced into the L1 binding site on a fragment of 23-S rRNA. Specific base changes had very similar effects on the affinity of L1 for 23-S rRNA and L1-mediated repression of translation (120). Thus, similar features on both RNAs are recognized by the regulatory protein.

Two observations demonstrate that translation regulation of the downstream L1 gene is accomplished by translational coupling to the upstream

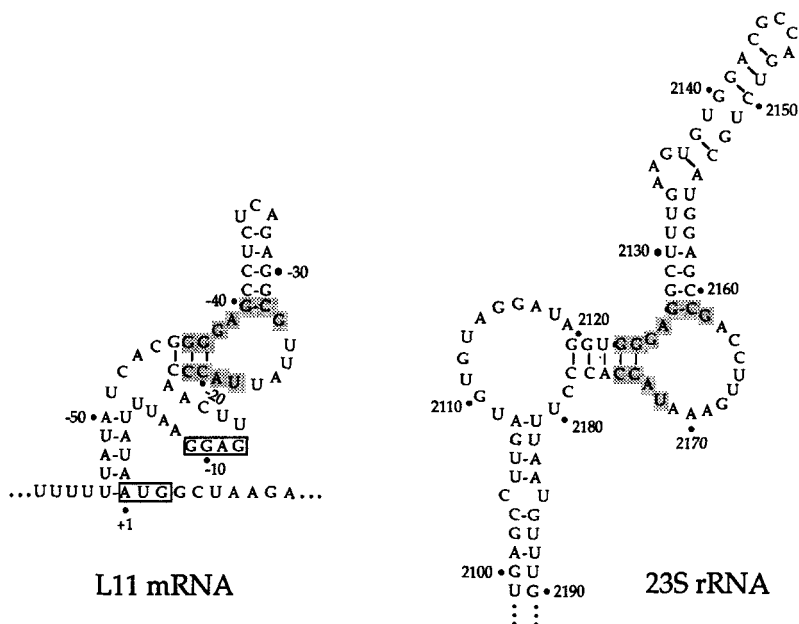


FIG. 9. Targets for L1 on L11 mRNA and 23-S rRNA. The secondary structure of the L11 mRNA is based on enzymatic structure probing (116) and phylogenetic studies (121). The structure of the L1 target on 23-S rRNA is based on the structure of Branlant *et al.* (119). The Shine-Dalgarno sequence and AUG initiation codon for the L11 gene are indicated by open boxes. The regions of similarity between mRNA and 23-S rRNA (115, 120) are indicated by shading.

L11 gene. First, deletion of the translation initiation region for the L11 gene blocks translation of both L11 and L1 (113). Second, mutations in the leader that eliminate L1-mediated control of L11 also relieve translational repression of L1 (114). Efficient translation of the L1 gene requires ribosomes to traverse almost to the end of the upstream L11 gene; in the absence of L11 translation, translation of the L1 gene is inhibited by the RNA sequence in the distal part of the L11 gene (121). The mechanism of this inhibition is not clear. One possibility is that the inhibitory sequence contributes to a secondary structure that sequesters the L1 ribosome binding site. However, not all point mutations resulting in partial relief of L1 repression are consistent with this model (121).

The L1 gene can be activated by deleting most of the L11 gene, showing that, in the absence of L11 translation, the ribosome binding site is intrinsically capable of accepting ribosomes from the pool of free ribosomes. Nevertheless, *in vivo* translation of the L1 gene in the deletion mutants is less

efficient than the translation of the gene from the wild-type L11/L1 message. This suggests that the L1 translation initiation site works better if ribosomes already associated with the mRNA are "delivered" to the L1 binding site from the upstream L11 gene (121). Translation of the L1 gene in the absence of L11 translation can also be improved by substituting adenines for guanines in the region immediately upstream of the Shine–Dalgarno sequence of the L1 gene (121). A similar improvement in the translation efficiency has been observed for the ϕ 1 phage gene VII, also translationally coupled to its upstream gene (122). In the latter system, it was suggested that ribosomes interact with the mRNA upstream from the Shine–Dalgarno region during the formation of the translation initiation complex, and that guanines in this upstream region reduce the ability of the mRNA to associate with ribosomes (122).

3. REGULATION OF THE L10 OPERON

L10 (or the "L8" complex, containing one copy of L10 and four copies of L12) represses translation of the L10 and L12 genes both *in vivo* and *in vitro* (105–108). The L10–(L12)₄ complex binds specifically to the L10 leader (31). The secondary structure of the L10 leader has been determined by chemical structure probing both *in vitro* and *in vivo* (123) and the target for the L10 repressor complex was mapped by genetic and mRNA protection experiments (123–126). The L10–(L12)₄ target on 23-S rRNA has been mapped by chemical as well as phylogenetic studies (127) to a region adjacent to the L11 binding site (Fig. 10). No experiments have directly addressed the question of similarities between the mRNA and 23-S rRNA targets, but one possible region of similarity is shown in Fig. 10.

The target for the translational repressor on the L10 leader is unusual among translational repressor sites in that it is located 120–160 bases upstream from the initiation codon of the L10 gene. To explain this long-distance interaction it was proposed (126) that the binding of L10–(L12)₄ to the leader induces a change in the secondary structure leading to sequestering of the Shine–Dalgarno sequence of the L10 gene and hence to inhibition of translation. However, subsequent studies of the mRNA structure failed to confirm the predicted structural switch (123). Thus the mechanism for the translation repression of the L10 gene is still unknown. It is noteworthy that the chemical modification of certain regions of the L10 leader is particularly sensitive to the presence of Mg^{2+} , suggesting that these bases are involved in tertiary structure formation (123).

Translation of the L12 gene is coupled to translation of the L10 gene (106, 128), even though the two genes are separated by a 66-base intercistronic region. Together with the translationally coupled S10 and L3 genes, which are separated by 32 bases (67), this demonstrates that genes do not have to be

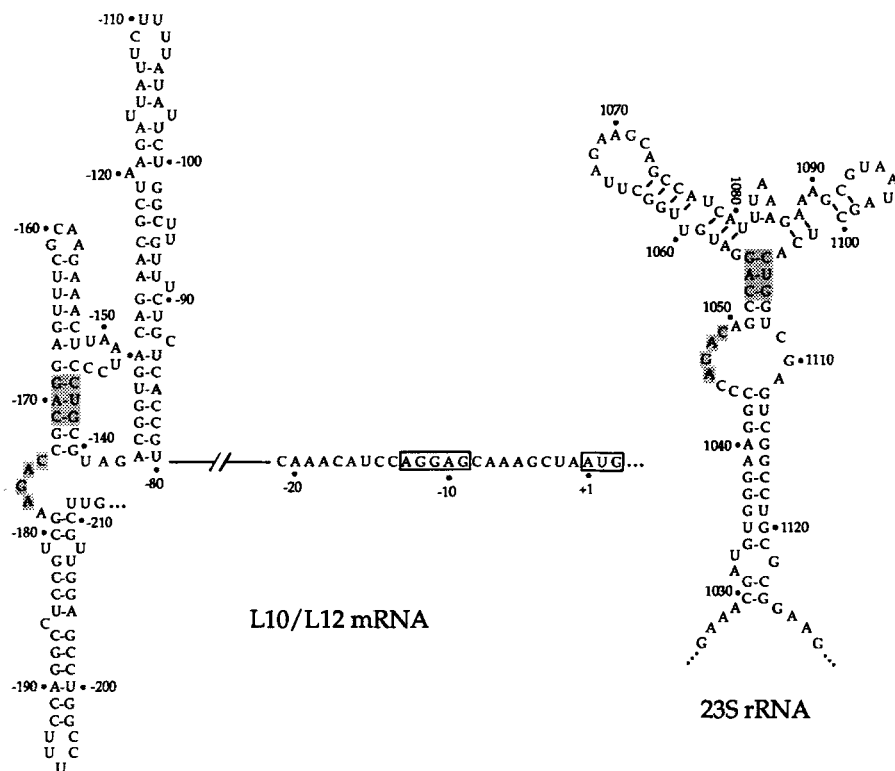


FIG. 10. Targets for L10–(L12)₄ on mRNA and 23-S rRNA. The secondary structure of L10 mRNA (123) in the region of the regulatory target for the L10–(L12)₄ protein complex (31, 124, 126) is shown on the left. The Shine–Dalgarno sequence and AUG initiation codon for the L10 gene are indicated by open boxes. The secondary structure of the L10–(L12)₄ binding site in the 23-S rRNA (127) is shown on the right. The shaded regions indicate possible regions of homology shared by the two targets.

located within a few bases of each other in order to be translationally coupled. Recent studies show that the coupling of L12 and L10 depends on sequences not only in the intercistronic region, but also in the proximal part of the L10 gene, suggesting that long-distance base-pairing is involved (128). Perhaps the entire mRNA is condensed when the translation of the L10 gene is blocked, thereby permanently inactivating the mRNA.

F. S20 Operon

The S20 protein is encoded by a monocistronic operon transcribed from two tandem promoters (129). This r-protein regulates its own translation as

shown both by *in vitro* translation experiments (130) and *in vivo* gene dosage (131), and by S20 oversynthesis studies (132). It was originally thought that the S20 operon conforms to the paradigm of homologous target sites for the regulatory protein on mRNA and rRNA because of primary sequence similarities between the translation initiation region of the S20 leader and the 5' region of 16-S rRNA harboring the S20 binding site (131, 133). However, this similarity appears to be fortuitous, because mutations in this region of the leader reduce the translation efficiency but have no specific effect on S20-mediated translation repression (132). Furthermore, subsequent analysis of the S20 binding site in 16-S rRNA revealed no S20 footprints in the regions homologous to the leader (134), and mutations in these regions also had no effect on S20 binding (135). Finally, it has not been possible to demonstrate binding of S20 to purified leader RNA (35).

The S20 gene initiates with a UUG codon. A change to the traditional AUG initiation codon eliminates translational repression of the S20 gene. This observation, together with the inability to demonstrate S20 binding to its mRNA, prompted the suggestion that the target for S20 action in repressing its own synthesis may be the 30-S::S20-mRNA complex, rather than the "naked" mRNA (132). An alternative model emerges from recent experiments that suggest that 30-S ribosomal subunits lacking S20 initiate protein synthesis relatively inefficiently on normal initiation codons (136). It is tempting to speculate that at limiting S20 synthesis, S20-less 30-S subunits accumulate. These deficient subunits may initiate translation at the UUG initiation codon in the S20 gene more efficiently than complete subunits, thereby favoring the synthesis of S20. Because S20-less 30-S particles can be converted into normal 30-S subunits (136), repression of the S20 gene could be accomplished by addition of S20 to S20-less particles.

G. S1 Operon

S1 is the only r-protein encoded by this operon, but the gene may be partially cotranscribed with the downstream *hip* gene coding for host-integration protein (38) (Fig. 1). Upstream from the S1 gene, there is an open reading frame (ORF-25) coding for a 25-kDa protein of unknown function (137). Several promoters located upstream and within ORF-25 contribute to the transcription of the S1 gene (137). Gene dosage experiments (37) showed that the S1 gene is regulated at the posttranscriptional level. Subsequent experiments with S1 gene fusions and oversynthesis of S1 *in trans*, as well as with *in vitro* translation, demonstrate that S1 is the effector for this regulation (38).

The mechanism for regulating S1 synthesis is of considerable interest, because S1 is not a primary rRNA binding protein like the other r-proteins that have been identified as autogenous repressors. S1 also differs from other

r-proteins by rapidly cycling on and off the ribosome. The protein has a general affinity for RNA, especially oligo(U)-containing sequences (138). S1 also interacts with 16-S rRNA at the 3' region (139). However, proteolytic fragments of the protein, which have only weak affinity for RNA, still bind efficiently to 30-S ribosomes, suggesting that S1 incorporation into the 30-S subunit is due mainly to protein-protein interactions (140). Furthermore, fragments of r-protein S1 lacking the domain(s) for strong RNA binding still repress S1 synthesis (38). These results indicate that S1 may repress its own translation without direct binding to mRNA.

H. L20 Operon

The genes for r-proteins L35 and L20 form a complex transcription unit with the genes for translation initiation factor-3 (IF3) and threonyl-tRNA synthetase (Fig. 1). Four promoters have been identified, but three are internal, mapping within the threonyl-tRNA synthetase (*thrS*) and IF3 (*infC*) genes (21, 22). Thus, the *thrS* gene, which occupies the 5'-most position in the transcription unit, is transcribed from just one promoter, whereas the two r-protein genes at the 3' end are transcribed from all four promoters (Fig. 1). There is a terminator between *infC* and the r-protein genes that stops about half the RNA polymerases (21, 22). The genes for the subunits of phenylalanyl-tRNA synthetase are immediately downstream from the *thrS-infC-L35-L20* cluster, but these genes are transcribed from a separate promoter and are regulated independently by an attenuator (141-143).

Even though the *thrS*, *infC*, L35, and L20 genes are partially cotranscribed, they are subject to three distinct circuits of autogenous regulation, all at the translational level. Each *thrS* and *infC* product controls its own synthesis (144-146), whereas L20 is the repressor of its own gene and the gene for L35 (22, 147).

The autorepression of *thrS* translation depends on the secondary structure of the *thrS* leader RNA, which mimics the structure of threonyl-tRNA (148). Binding of threonyl-tRNA synthetase to the leader RNA prevents the ribosome from forming a translation initiation complex, presumably because ribosomes and threonyl-tRNA synthetase cannot bind simultaneously (149). The phenotypes of mutations in the leader RNA and in the threonyl-tRNA synthetase suggest that the same region of the protein interacts with both tRNA and mRNA leader (148, 150). Thus the regulation of the *thrS* gene follows the paradigm proposed for the translational repression of r-protein genes. The translational repression of the *thrS* gene has been the subject of a recent review (151).

The translational autorepression of the *infC* gene requires its unusual initiation codon, AUU: a mutation changing it to the standard initiation codon AUG results in loss of regulation (152). The translation initiation site of

the *infC* gene also lacks a recognizable Shine–Dalgarno region. It was proposed that the regulation of translation of this gene may depend on an interaction between the region upstream of the AUU initiation codon and an internal portion of the 16-S rRNA (153), but this hypothesis has not yet been tested experimentally. IF3 functions in translation initiation by helping to ensure that stable ternary complexes containing 30-S, mRNA, and tRNA are formed only at true initiation codons (154, 155). Given that IF3 regulation depends on the unique initiation codon, it is tempting to speculate that the role of IF3 in preventing incorrect initiation complexes from forming may also be involved in the control of its own synthesis.

The details of the molecular mechanism for L20-mediated translation repression of the L35 and L20 genes are still lacking. However, a region internal to the *infC* gene is known to be essential (22), suggesting that translation repression depends on binding of L20 to a translated region of the mRNA that lies well upstream from the regulated cistrons. Recent experiments confirmed that L20 directly inhibits translation of the promoter-proximal L35 gene, and indirectly, by translational coupling, blocks translation of the downstream L20 gene (147). Genetic and RNA structure studies suggest that translation of the L35 gene prevents formation of a “coupling structure” that sequesters the ribosome initiation site for the L20 gene (147).

I. *str* Operon

The *str* operon contains genes for r-proteins S12 and S7 and the elongation factors G and Tu [Fig. 1; an additional gene for EF-Tu is located elsewhere on the chromosome (9)]. *In vitro* experiments show that S7 inhibits expression of its own and the EF-G genes but has little or no effect on synthesis of S12 and EF-Tu (52). However, S7 *in vivo* also represses synthesis of S12 (and possibly EF-Tu), although not to the same extent as the inhibition of S7 and EF-G synthesis (M. Nomura, personal communication). In analogy with the *spc* operon, the target for S7 is not upstream from the most proximal gene, but rather maps between the genes for S12 and S7. Translation of the S7 gene is directly inhibited whereas the repression of S12 synthesis appears to be mediated by retroregulation, i.e., enhanced degradation of mRNA upstream from the S7 target site (M. Nomura, personal communication).

Experiments with a construct consisting of the S12 gene followed by an S7'/*lacZ'* fusion showed that mutations in the S12 gene that abolish its translation reduce synthesis of the fusion protein by about 90% (M. Nomura, personal communication). These results indicate that translation of the S7 gene is coupled to translation of the upstream S12 gene, but the coupling is incomplete, similar to what was found for the S10 and L3 genes (see Section III,A,5). When S7 is oversynthesized in *trans*, expression of the S7'/*lacZ'*

fusion gene is inhibited if there is an intact S12 gene upstream. However, if translation of the S12 gene has been genetically blocked, the 10% residual fusion protein synthesis is not further reduced by excess S7. The interpretation of these results is that S7 specifically inhibits translation of the S7 gene that is due to coupling to the S12 gene, but not translation due to ribosomes entering at the intercistronic junction (M. Nomura, personal communication).

IV. Epilogue

A. Physiological Implications

Can the autogenous control mechanisms discussed above account for the regulation of ribosomal protein synthesis under various growth conditions? Perhaps more importantly, can we prove experimentally that the autogenous control is necessary *and* sufficient to account for the phenomenological description of ribosome synthesis? The model in Fig. 2 invites the speculation that regulation of r-protein synthesis is simply piggybacked on the regulation of rRNA synthesis, because r-protein synthesis can only proceed when sufficient rRNA sites are available to sequester the inhibitory regulatory r-proteins from the pool of free proteins.

Several experiments demonstrate that the coupling of r-protein and rRNA synthesis mediated by autogenous control does indeed help to balance r-protein synthesis with the total biosynthesis of the bacterial cell. For example, induced oversynthesis of 16-S or 23-S rRNA leads to enhanced expression of the r-protein genes that are in operons autogenously regulated by 30-S or 50-S r-proteins, respectively (156). Also, the synthesis of r-protein L11 is increased in mutant cells that do not synthesize L1, the autogenous control protein for the bicistronic L11/L1 operon (157, 158). In addition, steady-state growth rate regulation as well as stringent control (preferential inhibition of the synthesis of ribosomal components during amino-acid deprivation) of the L11 operon is disrupted in cells with a mutated mRNA target for L1 binding (159). These experiments all suggest that autogenous control is necessary for normal physiological regulation of r-protein synthesis, but do not exclude the contribution of other mechanisms.

Experiments with the S10 operon suggest that even though autogenous control is necessary, it is not sufficient for normal *in vivo* regulation. Read-through at the S10 attenuator (see Section III,A) is transiently increased when cells are rapidly shifted from one growth medium to another supporting higher growth rates (57), consistent with the involvement of the attenuation mechanism in modulating S10 operon expression immediately after a

“nutritional shift-up.” However, read-through at the S10 attenuator does not remain at the increased level after the shift-up, nor does it change significantly at various steady-state growth rates (57).

Thus, the attenuation control mechanism provides a “quick fix” during a nutritional transition state, but apparently does not play a major role in regulating S10 operon expression during steady-state growth. Indeed, other experiments show that the 5' end of the S10 operon, including the promoter, leader, and proximal one-and-one-half structural genes, contains the information for autogenous control induced by oversynthesis of the regulatory r-protein L4, but is insufficient for normal steady-state growth rate regulation (160). These results indicate that mechanisms in addition to those described in this review contribute to the physiological control of r-protein synthesis. Classic experiments suggested that the lifetime of r-protein mRNA changes as a function of the growth rate (161). Perhaps the “missing” mechanisms involve control of mRNA stability.

B. Future Directions

We expect that the study of r-protein synthesis will take several directions in the future. Certainly there is still much to be learned about the details of the molecular mechanisms. It is already clear that such work will be relevant not only to the specific genes studied, but also to broader questions about transcription termination, translation initiation, and mRNA processing.

Analysis of the regulation of r-protein synthesis in different bacterial species will undoubtedly be a valuable additional source of information. There is already much known about the organization of r-protein genes in non-*E. coli* systems, but relatively little understanding of their regulation. Interestingly, the most prominent r-protein gene clusters, the *str-spc* and *rif* regions, are preserved in systems as diverse as gram-positive bacteria, archaeobacteria, and chloroplasts, even though insertions, deletions, and transpositions have occurred (reviewed in 2, 162–164). However, although the gene sequences and order are often highly conserved, the transcription organization is often different. For example, the *spc*-alpha gene cluster is largely preserved in *B. subtilis* (165–168), but the alpha promoter appears to be absent (168). In addition, the *rif* region in several species contains the L1, L10, and L12 genes in one operon and the L11 gene in a monocistronic transcription unit; in some species the latter gene is even genetically unlinked from the three other genes (162, 163).

Variations in the transcription organization of r-protein genes among species imply that the regulatory schemes must differ from those of *E. coli*. Experimental evidence for this is already appearing. The S4 gene controls its own translation in *B. subtilis* (169, 170), but this gene is not part of the S10–

spc-alpha cluster in this organism (171). Rather, it is in an unlinked monocistronic transcription unit. In addition, the structures forming the S8 binding site in the *E. coli spc* mRNA are not preserved in *B. subtilis spc* mRNA, at least not in the same genetic location (165). Furthermore, the existence in several organisms of a transcription unit with both L1 and L10 genes, both of which code for regulatory proteins in *E. coli*, also suggests that the *E. coli* "rules" cannot be universal.

The emerging field of phylogenetic analysis of r-protein synthesis promises not only to expand the diversity of the known repertoire of regulatory mechanisms, but it also might yield clues about how these mechanisms evolved. For example, how and why were certain r-proteins recruited (and perhaps in some cases decommissioned) as autogenous regulators? A particularly interesting question is whether the autogenous regulatory systems all evolved from one ancestor system, or emerged independently of each other.

In summary, the genetic organization and expression of r-protein genes in an organism as "simple" as *E. coli* represent a complex microcosm of regulatory mechanisms. Even though excellent progress has been made in understanding some of the molecular details of their regulation, there are still many outstanding questions to be answered before we fully understand the regulation of this global multigene system.

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