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In vitro expression of *Escherichia coli* ribosomal protein genes: Autogenous inhibition of translation

(protein synthesis/λ transducing phages/immunoprecipitation)

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ABSTRACT *Escherichia coli* ribosomal protein L1 (0.5 μM) was found to inhibit the synthesis of both proteins of the L11 operon, L11 and L1, but not the synthesis of other proteins directed by λ*rif*^{d}*18* DNA. Similarly, S4 (1 μM) selectively inhibited the synthesis of three proteins of the α operon, S13, S11, and S4, directed by λ*spc1* DNA or a restriction enzyme fragment obtained from this DNA. S8 (3.6 μM) also showed preferential inhibitory effects on the synthesis of some proteins encoded in the *spc* operon, L24 and L5 (and probably S14 and S8), directed by λ*spc1* DNA or a restriction enzyme fragment carrying the genes for these proteins. The inhibitory effect of L1 was observed only with L1 and not with other proteins examined, including S4 and S8. Similarly, the effect of S4 was not observed with L1 or S8, and that of S8 was not seen with L1 or S4. Inhibition was shown to take place at the level of translation rather than transcription. Thus, at least some ribosomal proteins (L1 S4, and S8) have the ability to cause selective translational inhibition of the synthesis of certain ribosomal proteins whose genes are in the same operon as their own. These results support the hypothesis that certain free ribosomal proteins not assembled into ribosomes act as "autogenous" feedback inhibitors to regulate the synthesis of ribosomal proteins.

In exponentially growing *Escherichia coli* cells, synthesis rates of all ribosomal proteins (r-proteins) (except L7/L12) appear to be identical and coordinately regulated in response to environmental conditions (for reviews, see refs. 1–3). Despite extensive studies on this subject, molecular mechanisms involved in the regulation have remained unknown. We previously examined gene dosage effects on the synthesis of r-proteins encoded by the genes in the *spc* region (72 min) of the *E. coli* chromosome (4). It was found that the rates of transcription of the r-protein genes increase in proportion to the increase in gene dosage, but that the rates of r-protein synthesis do not increase relative to the synthesis rates of other r-proteins whose genes exist outside the *spc* region in a single copy per genome. From these experimental results we suggested that free r-proteins inhibit the translation of their own mRNA and that, as long as the assembly of ribosomes removes r-proteins, the corresponding mRNA escapes this feedback inhibition. We have tested this hypothesis by using an *in vitro* protein-synthesizing system with various template DNA molecules carrying r-protein genes. We have found that some r-proteins (S4, S8, and L1) selectively inhibit the synthesis of certain r-proteins whose genes are in the same operon as their own and that this "autogenous" inhibition is, in fact, at the level of the translation of mRNA rather than at the level of transcription.

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MATERIALS AND METHODS

DNA. Transducing phages used as sources of DNA, DNA extraction, and the preparation of restriction enzyme fragments have been described (5, 6). Fig. 1 shows the structures of the transducing phages. The 10% and 5% *Eco*RI fragments of λ*fus3* were obtained from pNO1001 (14) and hybrid phage Ch3S45i$^{\lambda}$ (15), respectively.

Ribosomal Proteins. The proteins were purified and stored in the presence of 6 M urea (6, 16). Before use for *in vitro* studies, r-proteins, except for L1, were dialyzed against 4 M urea/1 M KCl/2 mM potassium phosphate, pH 6.5/1 mM dithiothreitol and then against KUP buffer (1 M urea/1 M KCl/2 mM potassium phosphate, pH 6.5/1 mM dithiothreitol). L1 was found to be unstable in KUP buffer and was kept in 6 M urea at a sufficiently high protein concentration to allow dilution of the urea to less than 50 mM when added to protein-synthesis reactions.

Protein Synthesis *in Vitro*. DNA-dependent protein synthesis was carried out as described (6, 17) except that NH_4^+, K^+, and $CaCl_2$ concentrations were changed to 45, 68, and 1.5 mM, respectively. r-Proteins were diluted to the appropriate concentrations in 0.5× KUP buffer immediately before addition. Four microliters of this diluted solution (or 0.5× KUP without r-proteins) was then added to reaction mixtures, which contained all other components. The resulting urea concentration (50 mM) had no apparent effect on the reactions. Concentrations of r-proteins L1, S4, and S8 used in most of the experiments were 0.5, 1, and 2 μg in a final volume of 40 μl, corresponding to 0.5, 1, and 3.6 μM, respectively. Concentrations of ribosomes and RNA polymerase in the system were 0.75 and 0.06 μM, respectively. Template concentrations were 0.0017 μM for λ*spc1* and λ*rif*^{d}*18* DNA, 0.0035 μM for 10% *Eco*RI fragment, and 0.016 μM for 5% *Eco*RI fragment. Proteins were labeled with [^{35}S]methionine (Amersham, 70 mCi/μmol, 15 μM; 1 Ci = 3.7 × 10^{10} becquerels). Incubation was performed at 37°C for 60 min.

Immunoprecipitation. Samples were heated for 2 min at 90°C in 2 vol of $NaDodSO_4$ sample buffer (18) and then diluted 1:4 in the same buffer without $NaDodSO_4$ to give a $NaDodSO_4$ concentration of 0.5%. Excess amounts of rabbit antisera raised against purified r-proteins (6, 19) were added and incubated on ice for 1–2 hr. About 0.2 ml (10 times the volume of the antisera used) of a 10% suspension of *Staphylococcus aureus* (Cowan I strain) ghosts (IgGSorb, The Enzyme Center, Boston, MA) was used to precipitate IgG–antigen complexes (20).

Abbreviations: r-protein, ribosomal protein; KUP buffer, 1 M urea/1 M KCl/2 mM potassium phosphate, pH 6.5/1 mM dithiothreitol.

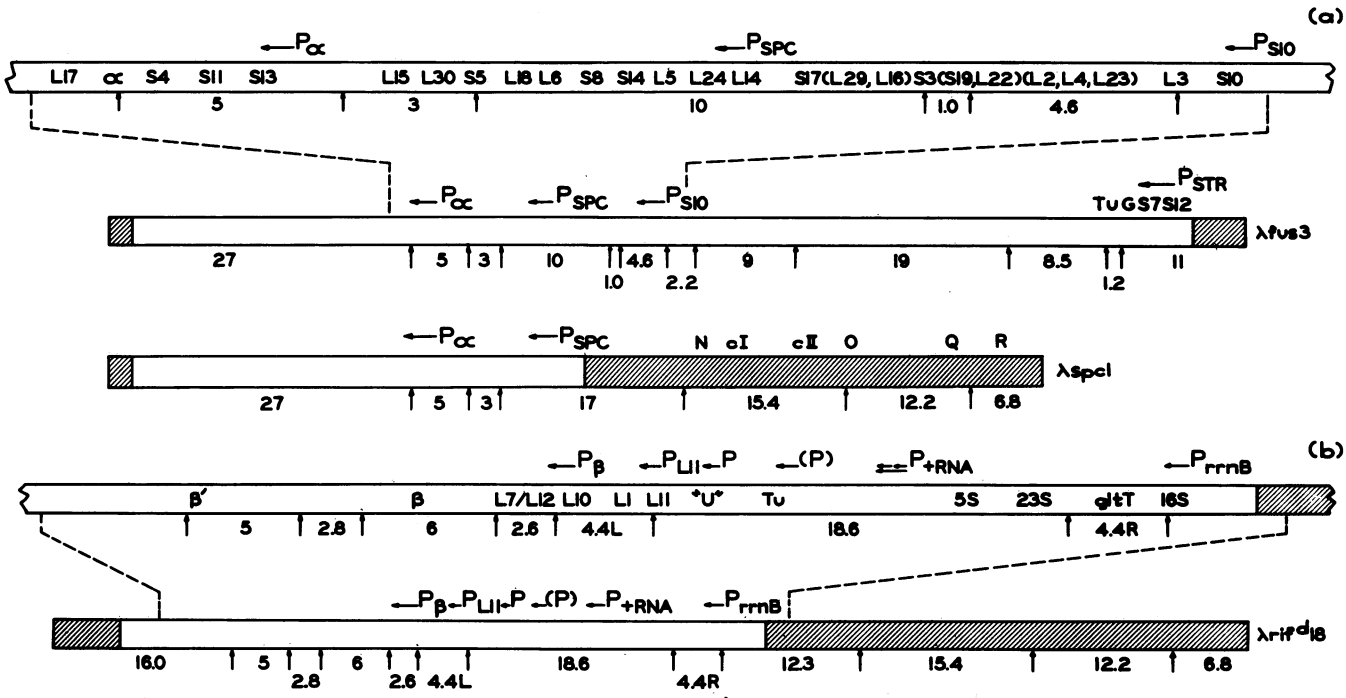

FIG. 1. Ribosomal protein genes carried by λ*fus3* and λ*spc1* (*a*) and λ*rif*^{d}*18* (*b*). Locations of bacterial genes and their promoters and *Eco*RI cleavage sites are shown (see refs. 5–13). The genes are indicated by the name of the gene product. Bacterial DNA is represented by the open areas and λ DNA by the hatched areas. The sizes of *Eco*RI fragments are given as a percentage of the λ genome (1% λ = 480 base pairs).

Precipitates were washed twice with 10 mM sodium phosphate, pH 7.2/0.15 M NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% $NaDodSO_4$ and then heated in $NaDodSO_4$ sample buffer at 90°C for 2 min. Samples were centrifuged, and radioactive proteins in the supernatant were analyzed by $NaDodSO_4$/13.5% polyacrylamide gel electrophoresis.

RESULTS

Inhibition of *in Vitro* r-Protein Synthesis by Purified r-Proteins. Among several r-proteins examined, we found that purified L1, S4, and S8 had preferential inhibitory effects on the DNA-dependent *in vitro* synthesis of r-proteins and that their inhibitory effects were different from each other. Fig. 2 shows that L1 (0.5 μg in 40-μl reaction mixtures) inhibited the synthesis of L1 and L11, S4 (1 μg) inhibited the synthesis of S4, S11, and S13, and S8 (2 μg) inhibited the synthesis of L5 and L24 and that these inhibitions were specific. The specificity was demonstrated in two respects. For example, L1 (0.5 μg) selectively inhibited the synthesis of L1 and L11 directed by λ*rif*^{d}*18* DNA, but not (or only very weakly) the synthesis of other proteins, such as L10, L7/L12, Tu, and several λ proteins including O and cII proteins encoded by the same template (Fig. 2*a*). This inhibitory effect of L1 was not observed when S4 or S8 was added instead of L1 (Fig. 2*a*). Similarly, the inhibitory effect of S4 (or S8) was not observed when the other two r-proteins were added (Fig. 2 *b–d*; see below).

The inhibitory effect of S4 on the synthesis of S4, S11, and S13 was studied with either λ*spc1* DNA or the 5% *Eco*RI fragment as the template (see Fig. 1). In the former case, the inhibition of S4 synthesis was shown as a reduction of the intensity of a radioactive band corresponding to both S4 and λ proteins when the protein products were directly analyzed by $NaDodSO_4$ gel electrophoresis (Fig. 2*b*, lane 3). Inhibition of S13 synthesis, but not S11 synthesis, was also shown by such direct analysis (see the legend to Fig. 2). Inhibition of the synthesis of S4, S11, and S13 was clearly demonstrated when the products were analyzed by $NaDodSO_4$ gel electrophoresis after immunoprecipitation with specific antisera (Fig. 3) or when the 5% *Eco*RI fragment was used as template (Fig. 2*c*). The 5% *Eco*RI fragment carries the genes for S13, S11, and S4, but not other genes identified on λ*spc1* (ref. 6; Fig. 1). Thus, the pattern of radioactive protein products was much simpler and the inhibition by S4 could be easily recognized.

The inhibitory effect of S8 was studied with λ*spc1* DNA (Fig. 2*b*) or the 10% *Eco*RI fragment (Fig. 2*d*) as template. The selective inhibition of the synthesis of L5 was clearly demonstrated with λ*spc1* DNA. The 10% *Eco*RI fragment carries the *spc* operon genes, L14, L24, L5, S14, S8, L6, and L18 (Fig. 1), but the strong synthesis of only the first three gene products (L14, L24, and L5) was observed. S8 (2 μg) inhibited the synthesis of L24 partially and that of L5 strongly. The synthesis of L14 was not significantly inhibited (Fig. 2*d*). The effects of S8 on the synthesis of other proteins could not be studied.

The genes for L1 and L11 constitute a single transcription unit (the "L11 operon") (7–9). Thus, L1 (0.5 μg) inhibits the expression of all the genes in this operon. In addition to S4 and S8, we found that L7 (8 μg), L12 (8 μg), and L11 (3 μg) did not cause any inhibition of the synthesis of L1 and L11. L11, unlike L1, does not appear to be involved in the regulation of the expression of the L11 operon. However, because all of our purified proteins were prepared in the presence of urea, the negative results (without positive control results) could simply be due to denaturation of the proteins, and we could not rigorously exclude the possibility that L11 or "nascent" L11 just synthesized inhibits its own synthesis. Similar reservations also apply to the negative results obtained with other proteins.

The genes for S4, S11, and S13 are in the same operon ("α operon"; refs. 5 and 10, Fig. 1). This operon also contains the genes for L17 and RNA polymerase subunit α (5, 10). The synthesis of both α and L17 was only very weakly inhibited by S4 (1–2 μg) (Figs. 2*b* and 3). These weak inhibitory effects are probably not significant; higher concentrations of S4 cause inhibition of the synthesis of other proteins, including λ proteins, which we consider as nonspecific inhibition. Similar nonspecific

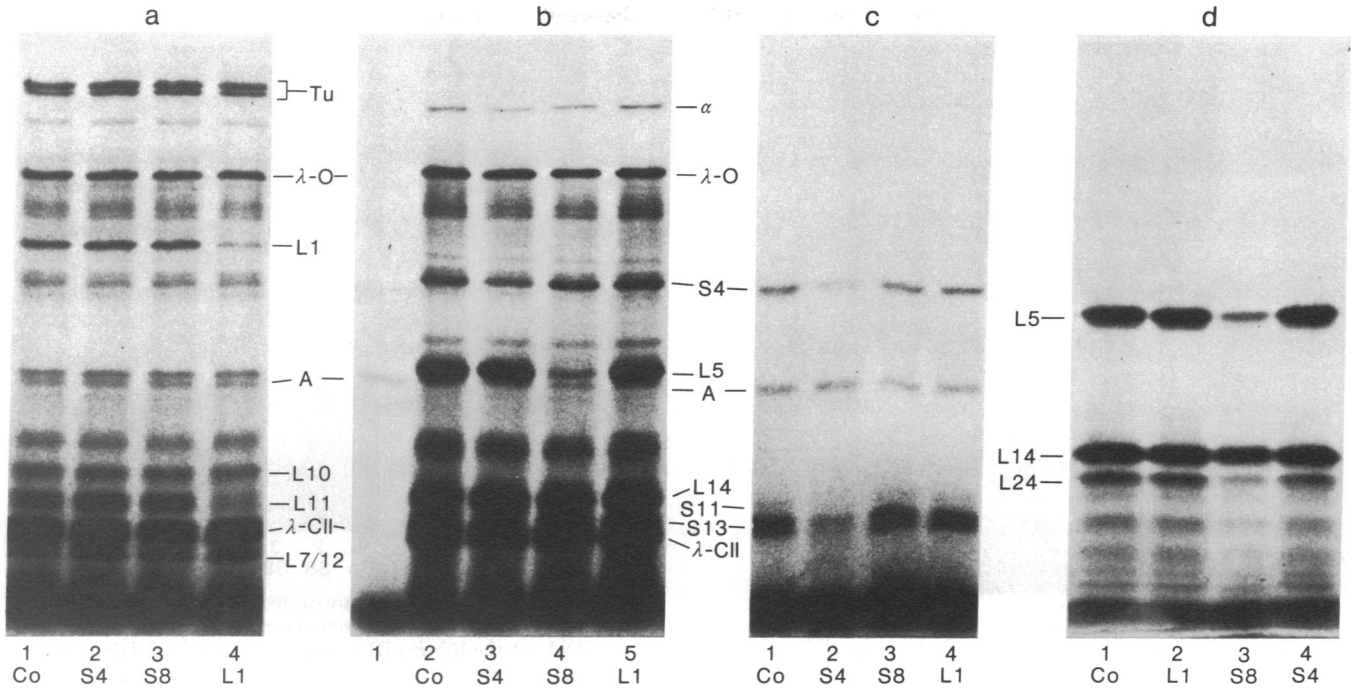

FIG. 2. Effects of S4, S8, and L1 on the synthesis of r-proteins directed by λ*rif*^{d}*18* (*a*), λ*spc1* (*b*), 5% *Eco*RI (*c*), and 10% *Eco*RI (*d*) DNA. Proteins S4 (1 μg), S8 (2 μg), and L1 (0.5 μg) were added to the reaction mixtures as indicated. Three microliters of reaction mixtures were analyzed by $NaDodSO_4$/12% polyacrylamide gel electrophoresis (18) followed by autoradiography. The reaction mixture in the absence of template DNA (*b*, lane 1) produced an unidentified radioactive protein indicated as A. Other radioactive proteins identified are indicated. Identification was done by comigration with reference proteins or by specific immunoprecipitation or both (see also refs. 6, 7, 13, and 21). Autoradiograms *a*, *b*, and *c* were from the same experiment; *d* was from another experiment. The radioactive S11 bands were clearly seen in the original film of *c*, and the inhibition of S11 synthesis by S4 was observed. This was clearly shown by immunoprecipitation (see Fig. 3). Similarly, inhibition of S13 synthesis by S4, clearly shown in *c* and in Fig. 4, could be easily seen in the original film of *b*.

inhibition was observed with higher concentrations of L1 and S8. Preliminary experiments showed that L17 does not cause selective inhibition of the synthesis of L17 and that neither S13 nor S11 inhibits the synthesis of S13, S11, or S4.

The genes for L5 and L24 are in the same operon (the *spc* operon) as the gene for S8 (refs. 5 and 11, Fig. 1). However, S8 did not inhibit the synthesis of L14, whose gene is the first gene of the *spc* operon. In preliminary experiments, radioactive proteins synthesized with λ*spc1* DNA as template were analyzed by immunoprecipitation. It was found that S8 inhibited the synthesis of S14 and S8 (in addition to L5 and L24), but not that of L6, S5, and L30 (and L14). It appears likely that S8 inhibits the expression of a cluster of genes (from L24 through S8 in Fig. 1) in the *spc* operon, but not that of all the genes in the operon. The question remains as to whether expression of the genes in the *spc* operon, which is not inhibited by S8, is inhibited by any other r-proteins.

Reaction Step Affected by r-Proteins. The inhibitory effects of L1, S4, and S8 described above took place at the level of mRNA translation. The *in vitro* protein synthesis was carried out in two steps. In the first incubation step, ribosomes were omitted and synthesis of mRNA was allowed but not its translation. After the first incubation step, transcription reactions were terminated by DNase treatment or by the addition of rifampin. Proteins (S4, S8, and L1) were added either during the first step (transcription) or after the termination of this step. The second step (translation) was then initiated by the addition of ribosomes and methionine. Appropriate controls showed that there was no protein synthesis directed by DNA in the first step and that no transcription took place in the second step (see the legend of Fig. 4). If the observed inhibition is at the level of transcription rather than translation, we would expect to observe the inhibition only when the proteins were added in the first step, but not when they were added in the second step only. As seen in Fig. 4, full inhibitory effects were observed when the proteins were added in the second step only. We conclude that proteins L1, S4, and S8 exert inhibitory effects at the level of mRNA translation. Effects at the level of transcription, if any, appear to be small compared to the effects at the level of translation. S8 inhibited the expression of the L24 and L5 genes, but not the L14 gene, the first structural gene of the operon. Similarly, S4 greatly inhibited the synthesis of only S13, S11, and S4; the synthesis of distal proteins of the operon, α and L17, was not selectively inhibited by S4. These results are consistent with the above conclusion that the inhibition is at the level of translation rather than transcription. We have not examined the question of whether the observed translational inhibitory effects are reversible or whether they involve mRNA inactivation followed by degradation in this *in vitro* system. Regardless of the mechanisms involved, we call the observed post-transcriptional inhibitory effects "translational" inhibition.

DISCUSSION

Our initial inference of the presence of post-transcriptional feedback regulation in r-protein synthesis came from the experiments on the effects of gene dosage on the synthesis of r-proteins and r-protein mRNA (ref. 4; see also refs. 14 and 22). Experimental results reported in this paper confirm this inference and demonstrate that some r-proteins (S4, S8, and L1) have specific inhibitory effects on the synthesis of certain r-proteins whose genes are in the same operon as their own. The inhibitory effects may be called "autogenous" (see ref. 23). We believe that the observed *in vitro* effects are relevant to the mechanism involved in the regulation of r-protein synthesis *in vivo*. In addition to the above gene-dosage effect experiments,

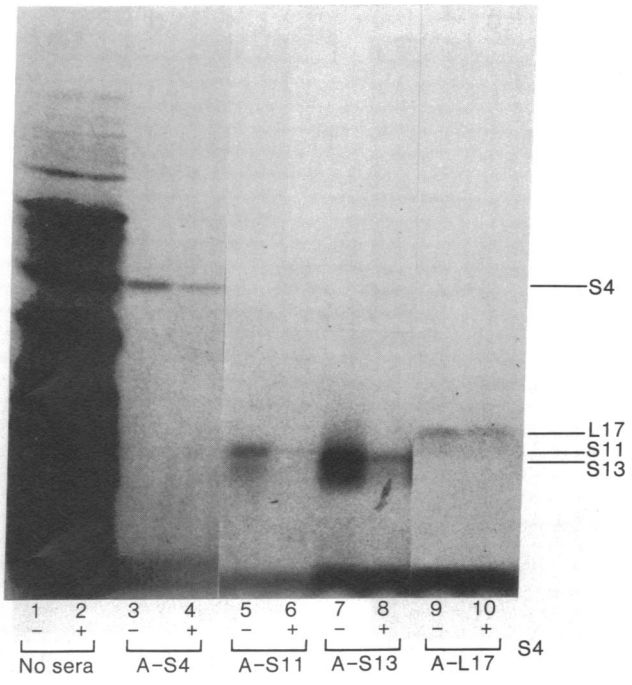

FIG. 3. Immunoprecipitation analysis of radioactive S4, S11, S13, and L17 synthesized in the presence of λ*spc1* DNA as template. The reaction was carried out with or without the addition of S4 (2 μg) as indicated. After the reaction, S4 was added to the reaction mixture without S4. Portions (2 μl for detection of S4, 10 μl for the others) were treated with excess anti-S4 (A-S4), anti-S11 (A-S11), anti-S13 (A-S13), and anti-L17 (A-L17) as indicated, and then *Staphylococcus* ghosts were added. The precipitated radioactive proteins were then analyzed by $NaDodSO_4$ gel electrophoresis followed by autoradiography.

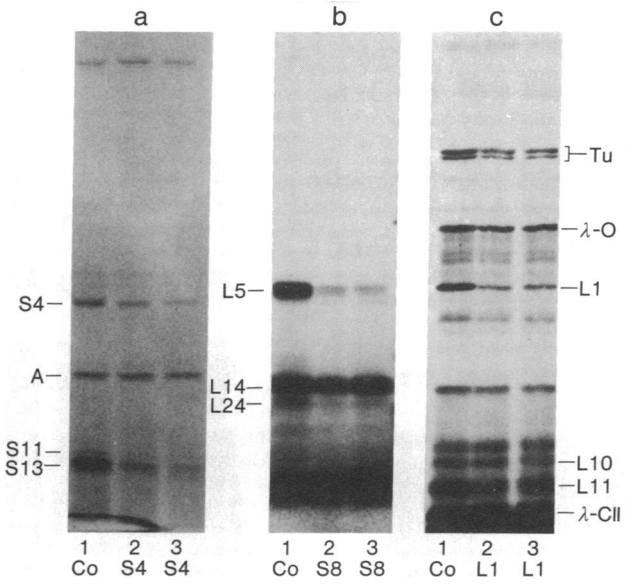

FIG. 4. Translational inhibitory effects of S4, S8, and L1 on the synthesis of r-proteins. *In vitro* protein synthesis was carried out with λ*spc1* DNA (*a*), the 10% *Eco*RI fragment (*b*), or λ*rif*^{d} *18* DNA (*c*). The first incubation was carried out for 30 min at 37°C in a 20-μl reaction mixture identical to those used for coupled transcription–translation except that ribosomes and methionine were omitted. The transcription reactions were terminated by DNase treatment (10 μg/ml for 2 min at 37°C) in *b* or by rifampin addition (25 μg/ml in the final reaction mixture) in *a* and *c*. Indicated r-proteins were added either during the first incubation (lane 2 in *a* and *b*, lane 3 in *c*) or after the termination of this step (lane 3 in *a* and *b*, lane 2 in *c*). Control tubes did not receive the r-proteins (lane 1). The second step was then initiated by addition of 20 μl of complete reaction mixture containing ribosomes and [^{35}S]methionine (but lacking DNA), and incubation was done for 40 min at 37°C. Radioactive proteins synthesized were analyzed by $NaDodSO_4$/12% polyacrylamide gel electrophoresis followed by autoradiography. In one control reaction, the first incubation was carried out at 0°C and, in another, ribosomes were omitted during the second incubation as well as the first. The only radioactive band observed in both controls was A.

there are some other considerations that support this belief. First, the observed *in vitro* inhibitory effects are sufficiently strong to explain the virtual absence of a free r-protein pool in exponentially growing *E. coli* cells. For example, in the present *in vitro* system, 0.5 μg of L1 in 40 μl of the reaction mixture can cause a near maximal inhibition of the synthesis of L1. This concentration corresponds to 0.5 μM. If the volume of an *E. coli* cell growing with the doubling time of 30 min is 10^{-15} liters, the presence of only 300 free L1 molecules uniformly distributed in the cell would inhibit L1 synthesis almost completely. The number of ribosomes in such a cell is about 7×10^4 (e.g., ref. 1), so this corresponds to about 0.4% of the L1 present in the complete ribosomes. This is consistent with the observations that the size of the free r-protein pool in exponentially growing *E. coli* is very small and represents at most a few percent of the r-proteins in the finished ribosomes (24, 25).

Second, Olson and Isaksson (26) reported that in a temperature-sensitive mutant of *E. coli* with a mutation in the S4 gene, small but significant overproduction of S4, S12, and S13 (and possibly S7) takes place at a permissive temperature and that the overproduced proteins are rapidly degraded. Using a similar temperature-sensitive S4 mutant, we have also observed small overproduction of S4, S11, S13, S12, and S7 (C. S. Jinks and M. Nomura, unpublished experiments). Although the apparent overproduction of S12 and S7 needs further investigation, the overproduction of S4, S11, and S13 could be due to a defect in the presumed inhibitory function of the mutant S4. Further experimental evidence of the presence of autogenous regulation in r-protein gene expression was recently obtained by Lindahl and Zengel (27). They fused a cluster of three r-protein genes (the L2, L4, and L23 genes carried by the 4.6% *Eco*RI fragment of λ*fus*3; see Fig. 1) to the promoter for the *lac* operon carried by a hybrid plasmid. They showed that addition of an inducer of the *lac* operon resulted in an overproduction of L2, L4, and L23, and this overproduction led to the inhibition of the synthesis of all or most of the proteins encoded by the genes of the S10 operon on the chromosome. Although we have not studied the effects of L2, L4, or L23 on gene expression *in vitro*, the observed phenomenon may involve a mechanism similar to the autogenous inhibition described in this paper. However, Lindahl and Zengel have suggested the possibility that the observed inhibition takes place at the level of transcription rather than at a post-transcriptional step. Clearly, this system deserves further investigation.

Our experiments strongly suggest that not all the r-proteins encoded by an operon participate in the regulation of expression of genes of that operon. For example, the L11 operon appears to be autogenously regulated by L1, but apparently not by L11, and this regulation is at the level of translation. What could then be the mechanism to ensure balanced synthesis of these two proteins *in vivo*? One plausible model is to assume that translational efficiencies of L11 and L1 *in vivo* are identical, presumably because every ribosome initiated at the beginning of the L11 message continues to translate the L1 message and no independent translational initiation takes place at the beginning of the L1 message. The fact that only three nucleotides exist between the L11 and L1 genes (12) supports such an assumption. Thus, the use of the L1 protein only (without using L11) as a feedback inhibitor can explain that the synthesis of both

L1 and L11 is balanced with the synthesis of ribosomes. Our *in vitro* experiments give some support for this specific model. We have examined the effects of L1 on the synthesis of L1 with the *Eco*RI 4.4% L fragment from $\lambda rif^{d}18$ as the template. This fragment has the intact L1 gene but not its promoter nor the 5′ end of the L11 gene (ref. 12; see Fig. 1) and has the ability to direct the synthesis of L1 *in vitro* (13). Only a very weak inhibition of L1 synthesis was observed (our unpublished experiments). It appears that the presence of the missing 5′ end is required for the maximum interaction between L1 and L11–L1 mRNA.

As discussed above, the unit of autogenous translational regulation is probably identical to the unit of transcription for the L11 operon. However, this does not appear to be the case in other operons. S4 inhibits the *in vitro* expression of the S13, S11, and S4 genes, but probably not the α and L17 genes; similarly, S8 inhibits the expression of the L24 and L5 genes, but not the L14 gene, the first structural gene of the operon. Although further experiments are needed to find out whether and how the synthesis of L17 or L14 is regulated, it appears likely that some transcription units are subdivided into units of translational regulation as suspected previously (4).

Finally, we note similarities between the present system and the phage T4 gene 32 system, which involves autogenous translational regulation (28–30). The gene 32 protein has a strong affinity for single-stranded DNA and, to a lesser extent, for single-stranded RNA. Gold and his coworkers (29, 30) 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 gene 32 protein onto available intracellular single-stranded DNA. Ribosomal proteins S4, S8, and L1 studied in this work are among the "initial binding proteins" in the *in vitro* ribosome assembly reaction and display strong and specific binding to rRNA (e.g., see refs. 31–33). This affinity is almost certainly stronger than their affinity to their own mRNA, and the autogenous inhibition *in vivo* presumably takes place only after the titration of these r-proteins onto newly synthesized rRNA. Thus, the autogenous regulation model discussed in this and previous papers (4, 34) is formally analogous to the gene 32 system. It appears that such regulation is of wide occurrence, as discussed previously (4, 23, 30, 34).

Note Added in Proof. We have now shown, in a strain bearing a multicopy plasmid with the S4 gene fused to the *lac* promoter, that the overproduction of S4 *in vivo* specifically inhibits the synthesis of S13 and, to a lesser extent, L17. Inhibition of L17 synthesis may result from a polar effect, not observed *in vitro*, caused by inhibition of translation of promoter proximal mRNA of the S13, S11, and S4 genes. Similarly, by fusing the L1 gene to the *lac* promoter, we have shown that overproduction of L1 leads to specific inhibition of L11 synthesis (D. Dean and M. Nomura, unpublished data).

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