Proc. Natl. Acad. Sci. USA
Vol. 76, No. 12, pp. 6542–6546, December 1979
Genetics

Operon-specific regulation of ribosomal protein synthesis in *Escherichia coli*

(recombinant DNA/gene expression/*lac* promoter)

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Communicated by Masayasu Nomura, September 24, 1979

ABSTRACT **We have cloned a DNA fragment harboring the genes for ribosomal proteins L2, L4, and L23 on a plasmid vector that contains a *lac* operator and promoter. The cloned ribosomal protein genes are now under the control of *lacOP*. Addition of a *lac* inducer to these cells results in a specific 5- to 10-fold increase in the synthesis of the proteins corresponding to the cloned genes. Within 10 min of this induction, the synthesis of ribosomal proteins S3, S19, L3, L16, L22, and L29 stops almost completely. The genes for all these proteins reside in the same chromosomal operon as L2, L4, and L23. We have seen no dramatic effect on the synthesis of any other ribosomal proteins. Thus, the induction of L2, L4, and L23 results in a specific and rapid decrease in the expression of all (or almost all) genes in their own transcription unit.**

The synthesis of ribosomes in *Escherichia coli* is regulated as a function of the growth condition; that is, fast growing cells devote a higher fraction of their energy and mass to ribosome synthesis than do more slowly growing cells (1). An additional feature of this control is that the individual ribosomal proteins (r-proteins) normally are regulated in parallel. The various r-proteins are synthesized in equimolar amounts (except L7/L12) even though the r-protein genes are organized into at least 11 transcription units (here called operons) (2, 3). The regulation of ribosome synthesis has been described phenomenologically in great detail (3, 4), but our understanding of the molecular mechanisms behind the regulatory phenomena is only beginning to emerge. We do know, however, that the cells can regulate at least three processes during ribosome formation: transcription (5, 6), translation or mRNA turnover (7, 8), and degradation of complete rRNA and r-proteins (2, 6, 9, 10).

One approach to elucidate the molecular mechanisms regulating r-protein synthesis is to develop a system in which the coordinate synthesis of r-proteins can be perturbed in a conditional and well-defined manner. To achieve this, we have constructed strains in which the synthesis of one or several r-proteins can be specifically increased. We have found that the over-synthesis of L2, L4, and L23 (over-synthesized as a group) leads immediately to an almost complete cessation of synthesis of proteins from all (or almost all) genes in the operon that harbors the L2, L4, and L23 genes. The synthesis of no other r-proteins appears to be affected in such a dramatic way. Thus, the increased expression of a few r-protein genes results in a specific and rapid decrease in the expression of an entire operon.

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EXPERIMENTAL PROCEDURES

The two media used were LB-broth (11) and AB-minimal medium (12). Where indicated, the media were supplemented with ampicillin at 35 μg/ml or oxytetracycline at 20 μg/ml. Two strains of *E. coli* K12 were used (relevant genotypes are indicated in brackets): LL308 [F'*pro*,*lacI*^{q}*z*$^{\Delta M15}$/Δ(*lac-pro*), *recA*] and LL309 [F'*pro*,*lacI*^{q}*z*$^{\Delta M15}$/Δ(*lac-pro*), *recA*$^{+}$]. Supercoiled plasmid DNA was purified from chloramphenicol-treated cultures by gentle lysis, precipitation of high molecular weight DNA, and banding in CsCl/ethidium bromide gradients (13). Strains constructed for this work and all additional procedures are described in the text and in the legends to figures and tables. All experiments were performed in compliance with the NIH Recombinant DNA Research Guidelines, Part II (P1 and EK1 containment levels).

RESULTS

We wished to investigate the regulatory response provoked in *E. coli* when the coordinate synthesis of the individual r-proteins is disrupted by a specific increase in the rate of synthesis of one or a few r-proteins under conditions in which the stimulus has no *direct* effect on the synthesis of any other r-proteins. To achieve this we inserted DNA fragments containing r-protein genes into a plasmid vector that carries the promoter–operator region from the lactose operon. The cloning vectors used were pBGP120 (14) and pOP203 (F. Fuller, personal communication). Both of these vectors have a single site for *Eco*RI cleavage situated close to a *lac* promoter (*lacP*). Transcription initiated at *lacP* is directed towards the *Eco*RI site. Thus, a fragment inserted at this site can be transcribed by RNA polymerase molecules initiating at *lacP*.

As a source of r-protein genes we used DNA from λ*fus*3, a specialized transducing phage carrying genes for 27 r-proteins, EF-Tu, and EF-G (Fig. 1; refs. 15 and 16). Hybrid plasmids, in which *Eco*RI restriction fragments of λ*fus*3 DNA are inserted at the *Eco*RI sites of the two vectors, were constructed as described in the legend to Fig. 1. To minimize the probability of cloning "mutant" versions of the DNA fragment, we avoided genetic selection in the cloning procedure. The orientation of the inserted fragment was determined by mapping of cleavage sites for restriction enzymes other than *Eco*RI in the insert and in the vector DNA. Only plasmids in which the sense strand of the inserted r-protein genes is connected to the sense strand of the *lac* operon were used for further studies. We succeeded in cloning three different *Eco*RI fragments of λ*fus*3 in the appropriate orientation: the 4.6% harboring intact structural genes

Abbreviations: r-protein, ribosomal protein; IPTG, isopropylthiogalactoside.

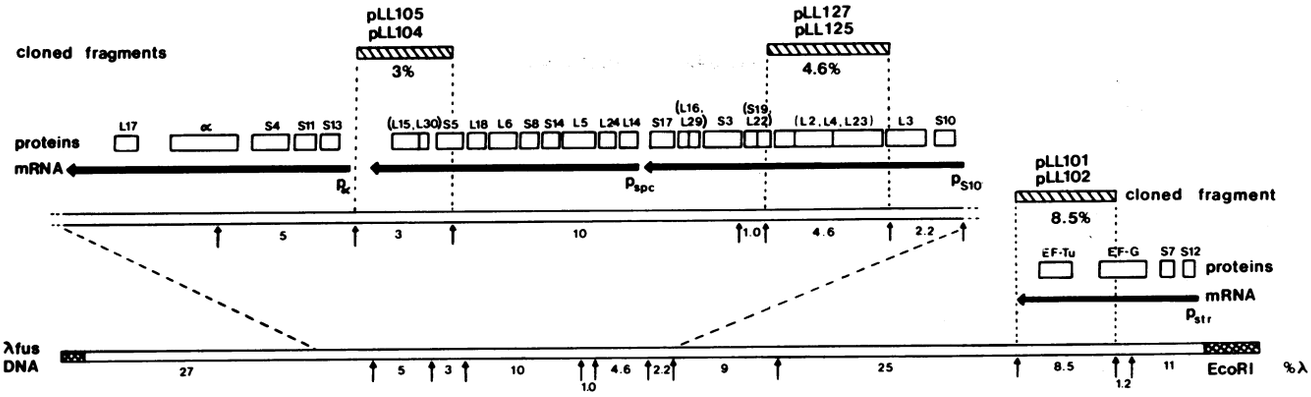

FIG. 1. Map of λ*fus*3 genome. The transducing phage λ*fus*3 carries the "streptomycin region" of the *E. coli* genome, including genes for 27 r-proteins, EF-Tu, and EF-G. The cross-hatched areas indicate λ DNA and open areas indicate *E. coli* DNA. Cleavage sites for *Eco*RI are indicated by vertical arrows. The four transcription units (operons) in this region are indicated by solid horizontal arrows originating at each promoter (P_x). For details of the map, see refs. 15 and 16. To construct hybrid plasmids carrying λ*fus*3 *Eco*RI fragments, 3 μg of vector DNA and 1.5 μg of λ*fus*3 DNA were digested separately with *Eco*RI restriction endonuclease, mixed, and treated with DNA ligase essentially as described (17). The ligation mixtures were used to transform *E. coli* K12 (18), and transformants were selected on plates containing ampicillin at 35 μg/ml (pBGP120) or oxytetracycline at 20 μg/ml (pOP203). To identify clones containing hybrid plasmids, the electrophoretic mobility in agarose gels of plasmids from different colonies were compared. Plasmids migrating more slowly than the vector were digested with *Eco*RI to identify the cloned fragment. The orientation of the inserted fragment was determined by mapping of recognition sites for other restriction endonucleases. Fragments that were cloned in the proper orientation (i.e., in which the sense strand of the cloned fragment can be transcribed from *lacP*) are indicated above the map along with the designations for the corresponding hybrid plasmids (see also Table 1). (The size of each fragment is indicated in %-λ units, where 1%-λ is approximately 500 base pairs.)

for r-proteins L2, L4, and L23, the 3.0% carrying genes for L15 and L30, and the 8.6% carrying the gene for EF-Tu (see Fig. 1). One plasmid, pLL126, appears to consist of two pOP203 structures and two 4.6% *Eco*RI fragments. Only one of these 4.6% fragments is connected with its sense strand to a *lac* sense strand (D. Mueckl and L. Lindahl, unpublished experiments).

The hybrid plasmids were transformed into strain LL308 or LL309, both of which contain a normal complement of r-protein genes on the chromosome, as well as F′ carrying the *laci*^{q} gene to provide sufficient *lac* repressor to ensure complete repression of all copies of the hybrid plasmid. In the absence of a lactose inducer, the plasmid-borne copies of the r-protein genes remain unexpressed and essentially all r-protein synthesized is derived from the chromosomal genes. The regulation of r-protein synthesis should therefore be unaffected by the presence of the hybrid plasmid. Addition of a *lac* inducer activates the plasmid-borne r-protein genes, resulting in the excessive synthesis of a specific subset of the r-proteins, but having no *direct* effect on the synthesis of other r-proteins. Thus, after induction we can study any immediate regulatory response made by the cells in an effort to re-establish a balanced r-protein synthesis.

Table 1. Effect of unbalanced r-protein synthesis on ability of cells to form colonies

Plasmid	Fragment cloned	Genes on cloned fragment	Host	+IPTG*/−IPTG
pBGP120 derivatives				
pLL101	8.6%	EF-Tu	LL308	1
			LL309	1
pLL104	3.0%	L15, L30	LL308	1
			LL309	1
pLL125	4.6%	L2, L4, L23	LL308	4×10^{-4}
			LL309	15×10^{-4}
pOP203 derivatives				
pLL102	8.6%	EF-Tu	LL308	1×10^{-4}
pLL105	3.0%	L15, L30	LL309	1
pLL127	4.6%	L2, L4, L23	LL308	$<10^{-4}$

The hybrid plasmids were contained in the indicated host. Cultures were grown overnight in minimal medium supplemented with glucose and ampicillin or oxytetracycline. Fresh overnight cultures were diluted and spread on AB minimal glucose plates with or without 1 mM isopropylthiogalactoside (IPTG) as the inducing agent and incubated at 37°C. After 36–48 hr, colonies were counted.

* +IPTG/−IPTG indicates the number of colonies formed on plates with IPTG relative to the number of colonies on plates without IPTG.

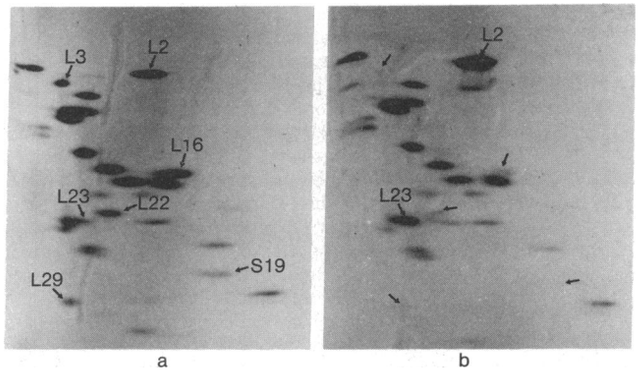

FIG. 2. Gel electrophoretic analysis of r-proteins synthesized before and after over-production of L2, L4, and L23 has been induced. Aliquots of strain LL313 were pulse-labeled with [^{35}S]methionine before (*a*) and 20 min after (*b*) addition of 1 mM IPTG. The cells were harvested, lysed, and extracted with acetic acid. (Details of these procedures are given in the legend to Table 2.) The total protein extract was electrophoresed in two dimensions by using the procedure of Kaltschmidt and Wittmann (19) with the following modifications: (*i*) The sample was layered on top of the first-dimension gel and electrophoresed with the anode on the top. Thus, "acidic r-proteins" are not displayed. (*ii*) The thickness of the gel was decreased to 0.8 mm for the first dimension and to 1.5 mm for the second dimension. (*iii*) The first-dimension gel included 4% Nonidet P-40. (*iv*) The second-dimension gel was formed with 20% acrylamide and 1.1% *N,N′*-diallyltartardiamide. After electrophoresis the gels were stained, destained, dried, and exposed to a Kodak No-Screen x-ray film for 5 days. The pictures show these autoradiograms. Pertinent protein spots are identified on *a*. Proteins whose synthesis was arrested after the induction of the plasmid-borne r-protein genes are indicated by unlabeled arrows on *b*.

Table 2. Synthesis of individual r-proteins after enhancement of synthesis of L2/L4/L23

	Time after IPTG addition, min									
	Strain LL310					Strain LL313				
r-proteins	0	5	10	20	30	0	5	10	20	30
S4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
S5 + L11	1.0	1.2	1.1	0.97	1.0	1.0	1.3	1.8	2.0	2.0
S7	1.0	0.83	0.95	0.94	0.92	1.0	0.87	1.0	1.3	1.0
S8	1.0	1.3	1.1	1.2	0.91	1.0	0.84	0.92	0.99	0.92
S9 + S11	1.0	1.0	0.85	0.90	0.75	1.0	0.89	1.1	0.93	0.89
S13	1.0	1.2	1.0	1.2	1.1	1.0	1.1	0.74	1.2	1.1
S14	1.0	0.93	0.93	1.1	1.1	1.0	0.89	0.75	1.0	0.70
S15,S16,S17	1.0	0.97	0.85	1.0	1.1	1.0	0.85	0.83	1.3	1.3
S19	1.0	1.1	1.3	0.98	1.2	1.0	0.25	0.00	0.08	0.11
S20	1.0	1.1	1.6	0.98	0.99	1.0	0.93	0.85	0.73	1.6
L1	1.0	1.2	0.80	0.90	1.08	1.0	1.0	1.2	1.2	1.4
L2	1.0	1.1	0.94	0.99	0.83	1.0	7.4	9.9	10.5	7.8
L3	1.0	1.1	0.93	0.86	0.80	1.0	0.10	0.06	0.14	0.06
L6	1.0	0.61	0.60	0.72	0.71	1.0	1.5	0.75	1.0	0.77
L13	1.0	0.95	0.97	1.0	0.98	1.0	1.2	1.1	1.5	1.4
L14 + L15	1.0	0.84	0.87	0.89	1.00	1.0	1.1	0.77	1.1	1.0
L16	1.0	0.83	0.83	0.79	0.74	1.0	0.41	0.00	0.16	0.12
L17	1.0	0.96	0.96	0.92	0.91	1.0	1.0	0.76	0.90	0.99
L18	1.0	1.4	1.4	1.4	1.4	1.0	1.1	1.4	1.2	1.3
L19	1.0	0.98	0.92	0.90	0.87	1.0	0.83	0.90	2.0	1.7
L22	1.0	0.94	0.95	0.91	0.93	1.0	0.28	0.15	0.48	0.30
L23	1.0	0.94	0.93	0.93	0.83	1.0	4.8	6.6	5.7	6.0
L25	1.0	0.72	0.67	0.68	0.62	1.0	1.1	0.97	0.99	0.77
L29	1.0	—	—	0.77	0.90	1.0	0.57	—	0.34	0.24

(*i*) *Growth and labeling of cells.* Strains LL310 (pBGP120/LL308) and LL313 (pLL125/LL308) were grown overnight in glycerol minimal media supplemented with ampicillin. The overnight cultures were diluted 1:100 into glycerol minimal medium without ampicillin and grown at 37°C to a density of 0.4 A_{450} units (about 10^8 cells per ml). A 2-ml aliquot of the culture was removed and mixed with 30 μCi of [^{35}S]methionine (800 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) at 37°C; 1 min later nonradioactive methionine was added to a concentration of 25 μg/ml and incubation was continued for an additional 90 sec. The aliquot was then poured over ice and the cells were harvested. The remainder of the culture was then induced by addition of IPTG to 1 mM; at the indicated times, new 2-ml aliquots were removed and labeled and harvested as described above. (*ii*) *Extraction of protein.* The cells were washed with 1 ml of 10 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/30 mM NH_4Cl, resuspended in 20 μl of the same buffer, and kept at −70°C until used. The cell suspension was thawed and mixed with 20 A_{260} units of salt-washed 70 S ribosomes and 20 μl of "reference cells" (to allow adjustment for recovery differences among the various samples) that had been labeled with approximately 100 μCi of [^{3}H]leucine until all radioactivity was incorporated. The mixture was frozen and thawed five times. The resulting lysate was brought to 0.2 M $MgCl_2$ and extracted with 2 vol of acetic acid at 0°C. The precipitate was removed by centrifugation, and protein in the supernatant was precipitated by addition of 2 vol of acetone. The precipitate was dried, redissolved in 25 μl of sample buffer (9 M urea/4% Nonidet P-40/0.15 M 2-mercaptoethanol/2.5 mM Na_2 EDTA/50 mM boric acid) and incubated at 37°C for approximately 1 hr. This incubation was necessary to bring the r-proteins into monodisperse solution after the precipitation. (*iii*) *Analysis of r-proteins.* Approximately 10 μl of the sample was subjected to two-dimensional gel electrophoresis as described in the legend of Fig. 2. After electrophoresis the gel was stained with Coomassie blue, destained, and dried. The stained spots were cut out and extracted with H_2O_2 as described by Pedersen *et al.* (20). The extract was mixed with Liquiscint (National Diagnostics) and the radioactivity was measured by scintillation spectrometry. (*iv*) *Data treatment.* The ^{35}S-to-^{3}H ratio (= R_i) was calculated for each individual spot. Because the isotope ratio for S4 (R_{S4}) was affected very little by the addition of IPTG to the culture, R_{S4} was used to standardize all R_i values—i.e., the R_i values for all spots in a given gel were divided by the R_{S4} value for the S4 spot in that gel. Finally, all R_i/R_{S4} values were normalized to the value obtained in the preinduced cells. The numbers in the table, therefore, give the ratio R_i/R_{S4} (induced) to R_i/R_{S4} (preinduced). Because R_{S4} is virtually unaffected by the induction, the numbers are approximately equal to the relative differential synthesis rates for each of the analyzed proteins. Underlined values indicate significant differences between strains LL313 and LL310. The solid underlines indicate values for proteins whose genes map in the S10 operon.

We first determined whether the unbalanced r-protein synthesis caused by induction of the plasmid-borne r-protein genes has any effect on cell viability. As seen from Table 1, the ability of the cells to form colonies was strongly reduced when the expression of the L2, L4, L23 gene group was enhanced, whereas excessive expression of the L15, L30 genes had no effect on the growth of the cells. We conclude that specific enhancement of the expression of some but not all r-protein genes is harmful to the cells. In the case of EF-Tu, induction of the gene on pBGP120 did not affect the colony formation, but induction of the gene on pOP203 did. We have no data to explain this discrepancy, but we suspect that more EF-Tu is synthesized from pLL102 than from pLL101 because pOP203 carries an "up promoter" mutation in *lacP* (F. Fuller, personal communication).

The dramatic decrease in viability after induction of the L2, L4, L23 group prompted us to determine the rates of synthesis of individual r-proteins in induced cells. Strains LL313 (pLL125/LL308) and LL310 (pBGP120/LL308) were used for this experiment. Aliquots of the cultures were pulse labeled with [^{35}S]methionine immediately before and at various times after addition of the *lac* inducer. Total protein was extracted and subjected to two-dimensional electrophoresis to display the r-proteins (see legends of Table 2 and Fig. 2 for details). As seen from the autoradiograms in Fig. 2, the synthesis of a number of r-proteins is strongly decreased after the enhancement of the synthesis of the L2, L4, L23 group. To get quantitative data, we determined the amount of radioactivity in each spot of the two-dimensional gels. From this data we calculated the rate of synthesis of each r-protein relative to the rate of synthesis of S4

(this protein was chosen as a standard because its rate of synthesis appeared to be almost unaffected by the induction). All data for a given protein were then normalized to the value obtained for uninduced cells (see legend of Table 2 for details). As seen from Table 2 the synthesis of L2 and L23 was enhanced 5- to 10-fold after the induction of the plasmid-borne genes. Because L4 is an acidic protein, it is not displayed in the gels used here, but we suspect that the synthesis of L4 is affected in a way similar to L2 and L23.

As predicted by the autoradiograms (Fig. 2), the synthesis of S19, L3, L16, L22, and L29 rapidly decreased after induction. The synthesis of these proteins was decreased by 70–90% within 10 min (Table 2). The genes for the proteins whose synthesis was decreased all map in the S10 operon, the operon harboring the chromosomal copies of the L2, L4, and L23 genes (see Fig. 1). The rates of synthesis of the remaining genes in the S10 operon, S3, S10, and S17, have not been measured. The extraction procedure we used in the experiment reported in Table 2 appears to cause preferential loss of S3. However, in other experiments in which proteins were concentrated by lyophilization rather than by acetone precipitation we obtained better recoveries of S3 and found that the synthesis of S3 is also decreased strongly by the induction of the plasmid-borne L2, L4, L23 genes (data not shown). Protein S17 cannot be analyzed because it overlaps on the gel with S16, and S10 runs too close to the edge of the gel to allow us to identify this spot conclusively. Thus, all of the proteins from the S10 operon for which we have data show a drastically decreased synthesis after the induction. We have obtained essentially the same results with a strain harboring the plasmid pLL127 (see Table 1).

For most other r-proteins, we observed no significant differences between LL313 and LL310 (see Table 2). However, we did observe several reproducible changes after induction of LL313. For example, the synthesis of L13 and L19 appeared to be somewhat increased after induction, although this effect was not observed until 20 min after induction and therefore could be a secondary effect. Also, the radioactive spot corresponding to L6 appeared to shift to a position closer to the S5/L11 spot. This shift may explain the increased values observed for S5 + L11 (Table 2). Finally, we observed minor differences in several other unidentified radioactive spots. The significance of the changes observed for these proteins whose genes map outside the S10 operon is not clear.

We also analyzed the synthesis of rRNA by hybridization of pulse-labeled RNA to λ*ilv*5 DNA (21). Little or no effect on the relative differential rates of rRNA synthesis was observed after induction of the plasmid-borne L2, L4, and L23 genes (data not shown). However, the induction affected rRNA maturation and ribosome assembly. Ribosomal RNA synthesized after induction coelectrophoresed with precursor 23S rRNA and precursor 16S (17S) rRNA. These RNA molecules were incorporated into ribosomal particles that sedimented more slowly than the mature 50S and 30S subunits (data not shown).

DISCUSSION

We have shown that a specific enhancement of the expression of a small group of r-protein genes, coding for L2, L4, and L23, results in a large decrease in the synthesis of all, or almost all, the r-proteins whose genes map in the same operon. The increased expression of these three genes has little effect on the synthesis of any other r-proteins that we analyzed. We believe that our results disclose an alternative mechanism for regulating r-protein synthesis: the accumulation of one or several r-proteins (or perhaps their respective mRNA) can specifically control the synthesis of all other proteins whose genes map in the same operon.

Many questions regarding this operon-specific control of r-protein synthesis must be answered before we understand its significance in relation to the overall regulation of ribosome synthesis. For example, we do not know if *all* proteins encoded by a given operon can serve as regulators. However, it appears that two proteins from another transcription unit, the "*spc* operon" (see Fig. 1) are not involved in regulating the expression of this operon. Enhanced synthesis of these two proteins, L15 and L30, does not affect cell viability (Table 1). Furthermore, we have not observed an effect on the synthesis of any r-proteins (other than increased synthesis of L15 and L30) after induction of these two genes (data not shown). These results suggest that perhaps only one or several r-proteins from a given operon are responsible for regulating the operon. Obviously, additional experiments are necessary to settle this question. For example, we would like to determine if all three r-protein genes from the 4.6% fragment, coding for L2, L4, and L23, are necessary for the regulatory response and whether enhanced expression of any other genes in this operon can elicit the same response. We would also like to know whether all r-protein operons are subject to the type of control described here for the S10 operon.

Nomura and coworkers have proposed a model for regulation of r-protein synthesis involving a feedback mechanism in which r-proteins can recognize and specifically inactivate (and degrade) their own mRNA (8). Their model, based on a study of gene dosage effects, therefore postulates a posttranscriptional regulation of r-protein synthesis. We have no compelling reason for expecting a similar posttranscriptional control mechanism in our system, because our strains still contain only the normal dosage of promoters for r-protein operons. In fact, our preliminary hybridization experiments suggest that the regulation may be predominantly at the level of transcription.

Other unanswered questions concern the actual effectors involved in the operon-specific control. For example, does the r-protein(s) actually participate in the reaction with the (still undefined) regulatory target? Are any nonribosomal molecules involved in the regulation? If the r-proteins themselves are involved in mediating the regulatory response, the ribosome assembly process may be the mechanism that coordinates the synthesis of protein from the different r-protein operons. Because the assembly of ribosomes consumes equimolar amounts of all r-proteins, the pool sizes of free r-protein (proteins not incorporated into ribosomal particles) could immediately provide signals for rectifying any imbalance in the synthesis of proteins from the various r-protein operons.

The skillful technical assistance of Dawn Mueckl and Mark Denson is greatly appreciated. We are grateful to D. Gelfand, M. Nomura, F. Fuller, W. Gilbert, and B. Müller-Hill for bacterial strains. This work was supported by grants from the National Institute for Allergy and Infectious Diseases and the New York State Health Research Council.

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