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Translational regulation is responsible for growth-rate-dependent and stringent control of the synthesis of ribosomal proteins L11 and L1 in *Escherichia coli*

(translational repression/site-directed mutagenesis)

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ABSTRACT **The physiological importance of translational regulation in controlling the synthesis of ribosomal proteins from the L11 ribosomal protein operon was determined for the classical regulatory phenomena of growth rate dependence and stringent control. Translational regulation of the L11 operon by ribosomal protein L1, the L11 operon-specific translational repressor protein, was abolished by introducing a chromosomal mutation that causes an alteration of the site where L1 interacts with L11 operon mRNA. It was found that abolishing translational regulation of the L11 operon also abolished growth-rate-dependent regulation and stringent control of the L11 operon ribosomal proteins without affecting the normal regulation of ribosomal proteins from other operons that are not regulated by L1. These results show that both growth-rate-dependent control and stringent control of ribosomal protein synthesis in the L11 operon are a direct result of translational regulation.**

The *Escherichia coli* ribosome is a complex subcellular organelle consisting of three RNA molecules and 52 different ribosomal proteins (r proteins). It is known that the rate of synthesis of ribosomes is closely tied to the overall rate of translation so that the amount of ribosomes produced per unit amount of cell mass increases with increasing growth rate and usually matches the amount necessary to sustain a given rate of translation. In addition, the synthesis rates of most or all r proteins are coordinately regulated to match the level of synthesis of complete ribosomes, with little excess r protein production. Consequently, the rate of synthesis of most or all r proteins relative to total cellular protein also increases linearly with growth rate, and this observed regulation is referred to as growth-rate-dependent control of r protein synthesis (for reviews, see refs. 1 and 2).

The translational feedback regulation model was originally proposed to explain the observed coordinated and balanced synthesis of r proteins (3). According to this model, the synthesis of r proteins is coupled with the assembly of ribosomes so that when r protein synthesis rates exceed those needed for ribosome assembly, further synthesis of r proteins is inhibited by certain free r proteins acting as translational repressors. The model postulates that most or all r protein mRNAs are synthesized in excess over the amount needed for the proper level of r protein translation and that, since rRNA synthesis is presumably the rate-limiting step in ribosome synthesis under most growth conditions, translational feedback regulation brings the r protein synthesis rate down to the proper level to match rRNA synthesis. The synthesis rate of r proteins would then be determined by the rate of rRNA synthesis, not by the rate of r protein mRNA synthesis, and this linking of r protein synthesis to rRNA synthesis would explain the observed coordinate regulation of the synthesis of the various ribosomal components observed under most growth conditions. Although the presence of translational feedback regulation had been convincingly demonstrated for many r proteins (for a review, see ref. 2), the possibility has still existed that the major regulation of r protein synthesis takes place at the level of transcription and that translational regulation plays only a minor role, perhaps a role in fine-tuning synthesis rates or a regulatory role under certain transient nonequilibrium conditions (see, e.g., refs. 4 and 5). For example, as originally proposed (6), growth-rate-dependent control of r protein synthesis might be largely achieved at the level of the initiation of transcription. Stringent control of r protein synthesis (7) might also take place at the level of transcription, as originally suggested on the basis of the analysis of pulse-labeled radioactive RNA (8), even though a different interpretation, based on translational regulation and its effects on mRNA stability, is now possible (see also *Discussion*).

In this paper, we describe the results of experiments designed to examine directly the physiological significance of translational regulation for the two classical regulatory phenomena of r protein synthesis mentioned above: growth-rate-dependent control and stringent control. The L11 operon coding for r protein L11 and L1 and regulated by L1 was used as an experimental system. By constructing strains carrying a chromosomal target site mutation that disrupts translational regulation by L1, we have been able to conclude that translational regulation is fully responsible for the correct growth-rate-dependent control and stringent control of the synthesis of these r proteins.

MATERIALS AND METHODS

The bacterial strains used are all *E. coli* K-12 derivatives and are described in Table 1 together with plasmids. Oligodeoxynucleotide d(TATATCAC*CC*GGAGCC) was described in our previous studies (ref. 9; italicized sequence *CC* represents the mutational alteration from the wild-type sequence GG). Radioactive oligodeoxynucleotide was prepared essentially as described by Zoller and Smith (12) and used as a probe to detect the presence of the MN2 mutation. Preparation of chromosomal DNA (13), Southern blotting, and hybridizations (14) were performed essentially according to the published procedures. Other procedures are described in the legends to figures and tables.

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Abbreviations: r protein, ribosomal protein; kb, kilobase(s); Mops, 4-morpholinepropanesulfonic acid.

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Table 1. Bacterial strains and plasmids

Designation	Comments
Bacterial strains	
N02580	GM-1 derivative with *lacZ*::Tn*9*G8 on F′ [GM-1 is *ara*, Δ(*lac–pro*), *thi*/F′ *lacI*^{q}, *lacPL8*]; from Jeffrey Miller
N02383	(= CH931) Hfr (the same origin of chromosome transfer as HfrH), *lysA*, *polA1*; from Charles Hill
N03280	Spontaneous *rif*^{r} (*rpoB*) mutant of N02383
N03282	Made by insertion of plasmid pN02698 into the chromosome of N03280 by homologous recombination in the L11 operon; places the L1 gene under *lac* promoter (P_{lac}) control (see text); requires isopropyl thiogalactoside for growth
N03291	N02580 carrying pN02699
N03292	N02580 carrying pN02700
N03293	*ara*, Δ(*lac–pro*), *thi*, *rif*^{r}/F′ *lacI*^{q}, *lacPL8*, *lacZ*::Tn*9*G8/pN02699; constructed from N03291 by P1 transduction from N03280
N03294	*ara*, Δ(*lac–pro*), *thi*, *rif*^{r}, *rplK*MN2/F′ *lacI*^{q}, *lacPL8*, *lacZ*::Tn*9*G8/pN02699; constructed from N03291 by P1 transduction from pool of isopropyl thiogalactoside-independent revertants of N03282; carries the MN2 mutation in the L11 operon leader (see text)
N03401	*rif*^{r} derivative of CP78 (F$^+$, *thi*, *leu*, *thr*, *his*, *arg*, *mal*, *xyl*, *ara*, *gal*, *relA*$^+$); made by P1 transduction from N03280
N03402	*rif*^{r}, *rplK*MN2 derivative of CP78 that carries the MN2 mutation in the L11 operon leader; made by P1 transduction from N03294
N03403	*rif*^{r} derivative of CP79 (F$^+$, *thi*, *leu*, *thr*, *his*, *arg*, *mal*, *xyl*, *ara*, *gal*, *relA*); made by P1 transduction from N03280
N03404	*rif*^{r}, *rplK*MN2 derivative of CP79 that carries the MN2 mutation in the L11 operon leader; made by P1 transduction from N03294
Plasmids	
pN02698	Transcriptional fusion of the *lac* promoter (P_{lac}) to mutant L11 operon; includes entire untranslated L11 operon leader sequence containing the MN2 point mutation, the entire L11 gene, and the first 25 bases of L1 gene; created from pN02597 (9) by deleting between the two *Sph* I sites
pN02767	Same as pN02698 except that it contains the wild-type L1 target site; created from pN01587 (10) by deleting between the two *Sph* I sites
pN02699	*rplK–lacZ* translational fusion under P_{lac} control; contains the entire wild-type L11 operon leader sequence and the first 12 codons of the L11 gene fused in-phase to the *lacZ* gene; created by replacing the *Pst* I fragment in pSKS105 (11) with the *Pst* I fragment from pN01587 (10) containing P_{lac} and the beginning of the L11 structural gene; places the L11–β-galactosidase fusion under transcriptional control of P_{lac} and translational control of L1
pN02700	Same as pN02699 except the L11 operon fragment is from plasmid pN02597 (10) containing the MN2 leader mutation; places the L11–β-galactosidase fusion under the transcriptional control of P_{lac} but *not* under the translational control of L1

RESULTS

Transfer of an L1 Target Site Mutation to the Chromosome. In our previous studies, the target site for L1 repressor on L11 operon mRNA was defined both *in vitro* and *in vivo* by deletion analysis and site-directed mutagenesis, and several mutations that make L11 operon mRNA insensitive to translational regulation by L1 were created on multicopy plasmids (9, 10). We have transferred one such mutation called MN2 (a two-base substitution, see the legend to Fig. 1) to the chromosomal copy of the L11 operon, as schematically shown in Fig. 1.

Plasmid pN02698 contains a transcriptional fusion of the *lac* operon promoter to the L11 gene and the first 25 bases of the L1 gene and includes the MN2 mutation in the L1 target site on the L11 operon leader. pN02698 was inserted into the chromosome of a *polA* strain (N03280) by homologous recombination to give strain N03282. Strain N03282 thus contains two copies of the L11 gene but only one complete copy of the L1 gene, and this single L1 gene is under the *lac* promoter control. When the *lac* promoter is not induced the growth rate of the N03282 is extremely depressed, presumably from lack of L1 (however, see ref. 15). Integration of pN02698 in the chromosomal L11 operon was confirmed by cotransduction of *rif*^{r} (*rpoB*) and *amp*^{r} (data not shown) and by Southern hybridization (Fig. 2).

A culture of N03282 was enriched for those cells that had lost the integrated *amp*^{r} gene and *lac* promoter by homologous recombination by growing for approximately 10 generations in the absence of both the *lac* operon inducer (isopropyl thiogalactoside) and ampicillin. Phage P1 grown on this revertant pool was used to transduce strain N03291 to *rif*^{r}. This strain carries, on a multicopy plasmid (pN02699), a gene coding for a L11–β-galactosidase fusion protein that is under the *lac* promoter control but retains the wild-type target site for L1 repressor. Thirty *rif*^{r} transductants were screened for cotransduction of the MN2 mutation, which would lead to overproduction of L1 (and L11) and lowered β-galactosidase activity upon induction of the synthesis of the L11–β-galactosidase fusion protein under conditions of submaximal induction (induction with 0.5 mM phenylethyl thiogalactoside; see ref. 17). Three transductants showed a decrease in β-galactosidase activity to approximately 10%, indicating cotransduction of the MN2 mutation. One of the transductants (N03294) was chosen for further study, and the presence of the MN2 mutation was confirmed by the following experiments. First, phage P1 was grown on this strain and transduction of N03291 to *rif*^{r} was repeated and compared with transduction of another strain (N03292) to *rif*^{r}. This strain, N03292, is identical to N03291 except that the gene coding for the L11–β-galactosidase fusion protein carries the MN2 mutation in the target site for L1 repressor. Indeed, it was observed that most of *rif*^{r} transductants derived from N03291 showed the lowered β-galactosidase phenotype, whereas none of *rif*^{r} transductants from N03292 showed this reduction. Second, the presence of the MN2 mutation on the chromosome was directly demonstrated by Southern analysis using a synthetic oligodeoxynucleotide with the mutant sequence as a radioactive probe (Fig. 2).

Growth-Rate-Dependent Control of r Protein Synthesis. We have tested directly for the involvement of translational regulation in the growth-rate-dependent control of the synthesis of r proteins L11 and L1. The differential synthesis rates of L11 and L1 (relative to total protein synthesis rate) were measured at three different growth rates in a strain carrying the MN2 chromosomal mutation (N03294) and in an isogenic control strain without the mutation (N03293). In the control strain the synthesis rates of both L1 and L11 and two

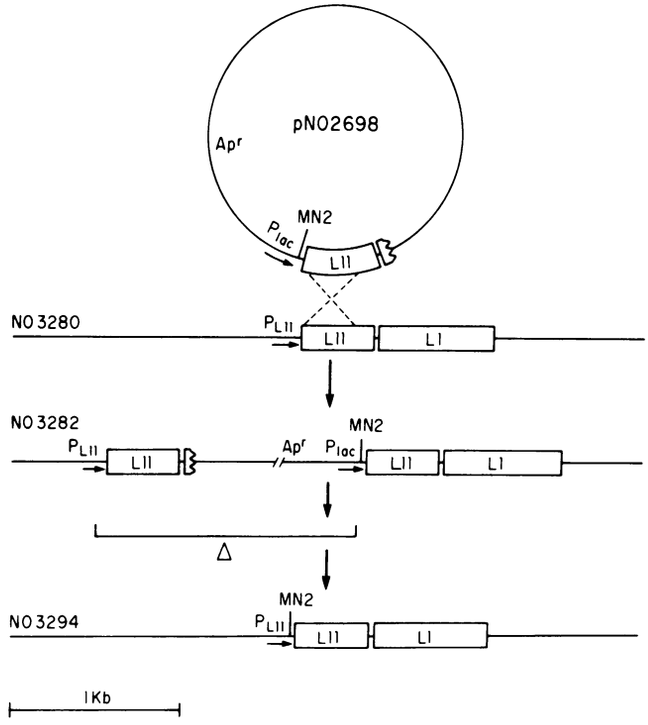

FIG. 1. Construction of a strain (N03294) carrying the L1 target mutation, MN2, in the chromosomal L11 operon. For explanation, see text and Table 1. The MN2 mutation is the mutation carried by pN02597 previously (9). It is a two-base substitution (GG to CC) at a site 45 nucleotides preceding the AUG codon of the L11 gene, and it disrupts a proposed double-stranded stem structure at the L1 target site of the L11 mRNA (9). Ap^r, ampicillin resistance; kb, kilobase.

other r proteins not in the L11 operon and not regulated by L1 (L3 and L6) showed the classical growth rate dependence (Fig. 3*A*). In strain N03294, carrying the MN2 mutation, the differential synthesis rates of L1 and L11 were elevated at all three growth rates and clearly did not show the normal dependence on growth rate, while two control r proteins analyzed (L3 and L6) showed the normal growth rate dependency (Fig. 3*B*). The results demonstrate that the translational regulation is indeed responsible for the growth-rate-dependent control of the synthesis of L11 and L1 and that in the absence of translational regulation there is no other alternative mechanism able to properly regulate the synthesis rates of L11 and L1 during exponential growth.

Stringent Control of r Protein Synthesis. Stringent control was initially defined for rRNA and tRNA synthesis; during amino acid starvation the synthesis rate of stable RNA is depressed in *relA*$^+$ strains but not in *relA*$^-$ strains. Subsequently, it was found that during amino acid starvation the differential synthesis rate of r proteins (relative to total protein) decreases in *relA*$^+$ strains and increases in *relA*$^-$ strains, and hence, r protein synthesis is also under stringent control (7). We have used the MN2 chromosomal mutation to examine whether the stringent control of the synthesis of r proteins L11 and L1 takes place at the level of transcription, or alternatively, if it is a consequence of translational regulation that links their synthesis to rRNA synthesis.

Four isogenic strains were constructed from the classical *relA*$^+$/*relA*$^-$ pair, CP78 and CP79. One *relA*$^+$/*relA*$^-$ pair (N03401 and N03403) carries the wild-type L1 target site and was used as controls. The other *relA*$^+$/*relA*$^-$ pair (N03402 and N03404) carries the MN2 target site mutation in the L11 operon. Starvation for isoleucine was induced in the four strains by valine addition (19) and the differential synthesis

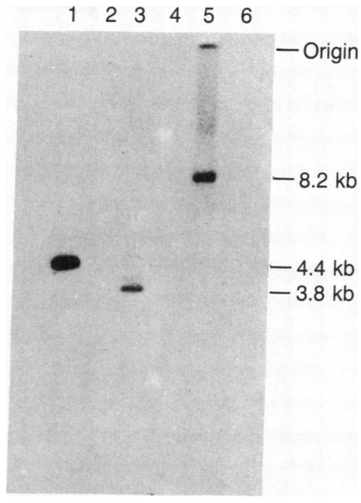

FIG. 2. Detection of the MN2 mutation by Southern hybridization technique. A ^{32}P-end-labeled 16-base oligodeoxynucleotide [d(TATATCACCCGGAGCC); *CC* represents the mutational alteration from the wild-type GG] homologous to the L1 target site with the MN2 mutation was hybridized to chromosomal DNAs that had been digested with restriction enzymes and separated by electrophoresis on a 0.7% agarose gel. Hybridization conditions were chosen so that the probe would hybridize to DNA fragments containing the L1 target site with the MN2 mutation but not with DNA fragments containing the wild-type L1 target site. Lane 1 is 0.01 μg of *Sph* I-digested plasmid pN02698 containing the MN2 mutation. Lane 2 is 0.01 μg of *Sph* I-digested plasmid pN02767, which is identical to pN02698 except that it does not contain the MN2 target site mutation. Lane 3 is 10 μg of *Sma* I-digested total chromosomal DNA from strain N03294 containing the MN2 mutation in the chromosomal L11 operon. Lane 4 is 10 μg of *Sma* I-digested total chromosomal DNA from strain N03293, the parent strain of N03294 without the MN2 mutation. Lane 5 is 10 μg of *Sma* I-digested total chromosomal DNA from strain N03282, which contains plasmid pN02698 integrated into the chromosomal L11 operon. Lane 6 is 10 μg of *Sma* I-digested total chromosomal DNA from strain N03280, the parent of N03282 without plasmid pN02698 integrated into the chromosome. The sizes of the DNA fragments containing the MN2 mutation expected from Fig. 1 and the DNA sequences (10) are 4.4 kb for the fragment in lane 1, 3.8 kb for that in lane 3, and 8.2 kb for that in lane 5, as indicated in the figure. In parallel to the samples shown, λ*rif*^{d}18 DNA digested with *Sma* I was run as size markers (not shown; cf. ref. 16). The positions of the three radioactive DNA fragments were consistent with the expected sizes as judged from the positions of the size markers.

rates of r proteins were compared with the synthesis rates before valine addition (Table 2).

It was found that the differential synthesis rates of the control proteins (L3, L5, L6, and S14) were decreased in the *relA*$^+$ strains and increased in the *relA*$^-$ strains, indicating that the control proteins are under stringent control both in the strains with the wild-type L1 target site and in the strains with the MN2 target site mutation. L1 and L11, on the other hand, showed the typical stringent response as seen for the control proteins in the strains with the wild-type L1 target site, while in the strains with the MN2 target site mutation the differential synthesis rates of L1 and L11, which are higher than the corresponding values in the control strains in the absence of valine, stayed the same or decreased only slightly in both the *relA*$^+$ and *relA*$^-$ strains after valine addition. This indicates that in the presence of the MN2 target site mutation the synthesis of L1 and L11 is no longer stringently controlled. Thus, we conclude that stringent control does not act directly on transcription of the genes for r proteins L1 and L11; rather, it is a consequence of translational regulation that links r protein synthesis to rRNA synthesis.

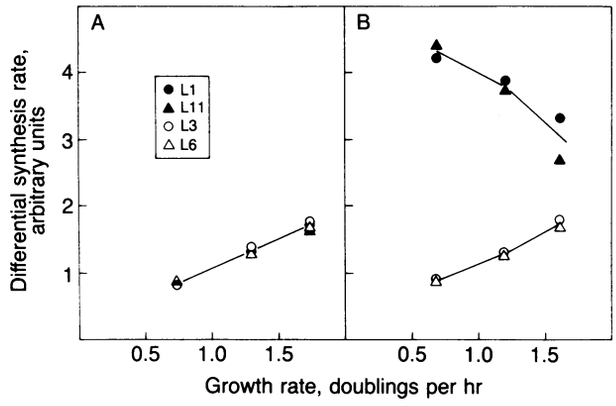

FIG. 3. Growth rate dependence of the differential synthesis rates of ribosomal proteins. Two isogenic strains, one with the MN2 mutation in the L1 target site (N03294; *B*) and another without this mutation (N03293; *A*) were grown in three different media at 37°C and the differential synthesis rates of r proteins (relative to the synthesis rate of total protein) were measured. The three media used were 4-morpholinepropanesulfonic acid (Mops) minimal media (18) supplemented with thiamin at 0.1 μg/ml, ampicillin at 50 μg/ml, and (*i*) 0.4% glucose with 18 amino acids (omitting lysine and proline) each at 40 μg/ml, (*ii*) 0.4% glucose with no amino acids, or (*iii*) 0.4% glycerol. At cell density of about 2×10^8/ml, 2.5-ml aliquots of each culture were pulse-labeled for 1 min with 100 μCi of [^{3}H]lysine (88.7 Ci/mmol; 1 Ci = 37 GBq) and chased for 1 min with nonradioactive lysine (final concentration 100 μg/ml). ^{3}H-labeled cultures were mixed with an equal volume of a long-term ^{14}C-labeled N03293 reference culture that was grown in Mops medium supplemented with thiamin at 0.1 μg/ml, 0.4% glycerol, and ampicillin at 50 μg/ml and labeled for several generations with [^{14}C]lysine (319 mCi/mmol) at 4 μCi/ml. Cells were lysed by heating in sodium dodecyl sulfate, and selected r proteins were immunoprecipitated, separated by polyacrylamide gel electrophoresis, and oxidized, and ^{14}C and ^{3}H were determined as described previously (9). The relative differential synthesis rate of the individual proteins in the different growth media was calculated by dividing the ^{3}H/^{14}C ratio for a specific r protein by the ^{3}H/^{14}C ratio in total protein; thus, the values obtained in this way represent (rate of synthesis of r protein *i*/rate of synthesis of total protein) in a given medium normalized to the value (amount of r protein *i*/the amount of total protein) in glycerol medium.

DISCUSSION

The present work shows that translational regulation of r proteins L11 and L1 (and presumably most or all other r proteins) is responsible for their growth-rate-dependent control and stringent control, and hence is physiologically very important. Concerning the growth-rate-dependent control, the results of previous analyses of the expression of the *galK* gene (or *lacZ* gene) fused to one of several r protein promoters have already indicated that the characteristic growth-rate-dependent control is not caused by the promoter activity. This conclusion was obtained with respect to the promoters of the *spc* operon, the α operon, and the S10 operon (20, 21). The present work gives a more direct demonstration of the involvement of the translational regulation in the growth-rate-dependent control of synthesis of r proteins in the L11 operon.

Stringent control of r protein synthesis was previously thought to act at the level of transcription. This conclusion was based on the analysis of radioactive RNA labeled for 1 min at 36°C under conditions of partial inhibition of aminoacyl-tRNA synthesis (8). Since it is now known that an increase in translational repression causes a decrease in the half-life of r protein mRNA (3, 22–24), the apparent change in r protein mRNA synthesis rate observed in the earlier experiments may have been due to a large decrease in mRNA half-life caused by translational repression, with rRNA synthesis directly under stringent control and r protein synthesis indirectly linked to rRNA synthesis through translational regulation.

Table 2. Relative differential synthesis rates of r proteins during amino acid starvation

	Relative differential synthesis rate (valine-treated/untreated)			
	Strains with normal regulation		Strains with MN2 mutation	
r protein	*relA*$^+$ (N03401)	*relA*$^-$ (N03403)	*relA*$^+$ (N03402)	*relA*$^-$ (N03404)
L1	0.45	2.44	*0.80*	*0.75*
L11	0.53	3.29	*1.04*	*0.93*
L3	0.38	1.70	0.30	2.07
L5	0.55	2.34	0.49	2.27
L6	0.43	1.21	0.42	1.55
S14	0.62	2.25	0.56	2.39

The values in the table are the differential synthesis rates for the valine-treated cultures divided by the values for the corresponding culture without valine, and they represent the average of three separate experiments, except for L5 and S14, which are the values obtained from a single experiment. The italicized values indicate the breakdown of stringent control of L1 and L11 synthesis in the presence of the MN2 mutation. The variation from protein to protein in relative differential synthesis rates during the stringent response seen in *relA*$^-$ strains has been observed previously (7). Strains N03401, N03402, N03403, and N03404 were grown at 37°C in Mops minimal media supplemented with thiamin at 0.1 μg/ml, 0.4% glucose, and 15 amino acids (omitting lysine, valine, isoleucine, serine, and alanine) at 40 μg/ml each. At an OD_{600} of 0.2, 2.5-ml aliquots of each culture were pulse labeled for 2 min with 100 μCi of [^{3}H]lysine (88.7 Ci/mmol) and chased for 2 min with nonradioactive lysine at a final concentration of 100 μg/ml. Valine was added to another 5-ml aliquot of each culture to a final concentration of 100 μg/ml to induce isoleucine starvation. After 15 min these aliquots were pulse labeled for 2 min with 200 μCi of [^{3}H]lysine and chased for 2 min as above. Each ^{3}H-labeled culture was mixed with 2.5 ml of a ^{14}C-labeled reference culture of N03401 grown in the same medium in the presence of [^{14}C]lysine (319 mCi/mmol) at 4 μCi/ml for several generations. The samples were processed as described in the legend to Fig. 3. The ^{3}H/^{14}C ratio for each r protein was calculated and the values were normalized to the ^{3}H/^{14}C ratio of total protein in the sample to obtain differential synthesis rates. The differential synthesis rates obtained for the valine-treated cultures were then divided by the corresponding values for the untreated cultures.

After completion of the present work, a paper by Friedman *et al.* (25) appeared, suggesting that the promoter for the S10 r protein operon is responsible for the stringent control of the synthesis of r proteins in this operon. It remains to be seen whether the apparent discrepancy between our conclusion and that obtained for the S10 operon indicates a basic difference in regulation between the S10 operon and other r protein operons as noted previously by these authors (26, 27).

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