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Ribosomal Protein S4 Acts in *trans* as a Translational Repressor to Regulate Expression of the α Operon in *Escherichia coli*†

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Ribosomal protein (r-protein) S4 is the translational repressor which regulates the synthesis rates of r-proteins whose genes are in the α operon: r-proteins S13, S11, S4, and L17. In a strain having a mutation in the gene for r-protein S4 (*rpsD*), the mutant S4 fails to regulate expression of the α operon, resulting in specific and significant overproduction of r-proteins S13, S11, and S4. This confirms and extends similar observations made with *rpsD* mutants (M. O. Olsson and L. A. Isaksson, Mol. Gen. Genet. **169**:271–278, 1979) before post-transcriptional regulation of r-protein synthesis was proposed and is consistent with the established regulatory role of r-protein S4. The *rpsD* mutant has been used to study the question of whether regulatory r-proteins function in *trans* or strictly in *cis* as translational repressors. The mutant strain was lysogenized with one or two specialized transducing phages carrying a wild-type S4 gene to obtain strains which were diploid or triploid with respect to the α operon. The wild-type and mutant forms of S4 were separated by two-dimensional polyacrylamide gel electrophoresis, which allowed accurate measurement of the relative contributions of r-proteins from different α operons within a single cell. We found that expression of r-proteins from the chromosomal α operon containing the *rpsD* allele was reduced when the wild-type S4 was present, with the effect being greater in the triploid strain than in the diploid strain. We conclude that the wild-type S4 acts in *trans* as a translational repressor to regulate expression from the chromosomal α operon.

The synthesis rates of most ribosomal proteins (r-proteins) in exponentially growing *Escherichia coli* are identical and respond coordinately to changes in environmental conditions (for reviews, see references 15 and 28). Based on gene dosage experiments in which we compared the synthesis rates of r-proteins with the synthesis rate of r-protein mRNA, we proposed a model of post-transcriptional feedback regulation to explain the coordinate and stoichiometric synthesis of r-proteins (12). We hypothesized that r-protein synthesis and ribosome assembly are coupled so that when r-protein synthesis rates exceed those needed for ribosome assembly, "free" r-proteins interact with their mRNA and block further translation. This model has been confirmed and refined by both in vitro and in vivo experiments. These experiments have identified specific r-proteins (S4, S7, S8, L1, L4, and L10) as "translational repressors" which selectively inhibit the synthesis of some or all of the r-proteins whose genes are in the same transcription unit (operon) as the repressor r-protein (3, 8–10, 13, 37–39, 41).

The α operon, for example, contains the genes for r-proteins S13, S11, S4, and L17 and RNA polymerase subunit α. It has been shown that S4 specifically inhibits synthesis of itself, S13, and S11 in in vitro experiments utilizing a DNA-dependent protein synthesis system (37). In in vivo experiments in which the S4 gene (together with the S11 gene) has been fused to the *lac* promoter on a multicopy plasmid, induction of S4 synthesis causes a specific decrease in the synthesis rates of S13, L17, and presumably a decrease in the synthesis rates of S4 and S11 from the chromosomal α operon (8; D. Bedwell, D. Dean and M. Nomura, unpublished experiments).

In our original gene dosage paper (12), we considered available information on the dominance of wild-type Str^s alleles over mutant Str^r alleles (5). Based on this information, we suggested that the translational repressor r-proteins would act mainly on mRNA at the site of their

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synthesis (*cis* regulation) and not on homologous mRNA coming from a different genome in a merodiploid strain (*trans* regulation). In this paper, we describe the results of experiments designed to study the question of whether this *cis* model of regulation is correct. We isolated an *E. coli* regulatory mutant which had an alteration in the gene for r-protein S4 (*rpsD*) and showed an increase in the relative synthesis rates of r-proteins S13, S11, and S4. This mutant is similar to those described previously by Olsson and Isaksson (32, 33). The *rpsD* mutant strain was lysogenized with one or two specialized transducing phages to obtain strains that were diploid or triploid with respect to the α operon. We present evidence that in such diploid or triploid strains, the wild-type S4 functions as a translational repressor to regulate the synthesis rate of the chromosomal mutant S4. We concluded that r-protein S4 (and presumably all other repressor r-proteins) acts in *trans* to regulate gene expression and not strictly in *cis* as we originally suggested (12). In addition, we discuss the altered pattern of r-protein synthesis in our *rpsD* mutant and in those studied by Olsson and Isaksson (33) in relation to the regulatory role of S4 established previously.

MATERIALS AND METHODS

Bacterial strains. A list of the *E. coli* K-12 strains used in this study is given in Table 1.

Construction of λ α-*lac*1. λ α-*lac*1 is a hybrid transducing phage constructed in vitro by fusion of the *lacZ* gene with a 2,000-base-pair *Eco*RI-*Hin*dIII fragment derived from λ *spc*1 (H. de Boer, this laboratory). The 2,000-base-pair fragment contains the promoter for the α operon (Pα) and the genes for r-proteins S13, S11, and S4; it does not contain the genes for RNA polymerase subunit α or r-protein L17 (see λ *spc*2 in Fig. 1 for a map of this region). The left arm of λ α-*lac*1 was obtained by digesting λRS205-7 with *Hin*dIII. λRS205-7 carries a fused *trp-lac* operon originally constructed by the W205 fusion (24) and was obtained from K. Bertrand and W. S. Reznikoff. The right arm of λ α-*lac*1 was obtained by digesting λ459 with *Eco*RI. λ459 was obtained from N. E. Murray and is a Δ*srl*1-2 phage having the normal *att* site and *c*I857 on the right arm (27). Ligation of the right and left vector arms with the 2,000-base-pair λ *spc*1 fragment produced a hybrid phage with the *lacZ* gene fused to Pα in the correct orientation. λ α-*lac*1 was isolated as a plaque-forming phage which produced Lac$^+$ lysogens after infection of strain B2550. The structure of the phage was confirmed by analysis of the DNA with various restriction enzymes (Fig. 1).

λ α-*lac*1 was crossed with λpapa to replace the *c*I857 allele from λ459 with the *c*I$^+$ allele of λpapa so that lysogens could be grown at 37°C (A. Miura, this laboratory). λ α-*lac*1 *c*I$^+$, the resulting recombinant phage, is used in the present study.

Construction of λ *trkA*, λ *spc*2, and λ α-*lac*1 *c*I$^+$ lysogens. Lysogens of NO2514 and NO2515 were constructed by standard phage techniques. λ *trkA* lysogens contained λpapa as helper, and λ *spc*2 lysogens contained either λpapa or λ α-*lac*1 *c*I$^+$ as helper. The structures of λ *trkA* and λ *spc*2 are shown in Fig. 1. λ *trkA* and λ *spc*2 lysogens were isolated by selecting for expression of the *aroE*$^+$ allele carried on

TABLE 1. Bacterial strains

Designation	Description	Source, method of construction, or reference
B2550 (= NO2183)	Δ(*lacOZ*) *lacY*^{c} Δ(*trpEA*)2 *tna-2* Nal^r	K. Bertrand, W. S. Reznikoff
NO816	F$^-$ *aroE malA thi*	Laboratory collection
NO2143	F$^-$ *gal thi trkA401 kdpABC5 aroE lacY lacZ rpsL* (Str^r)	Laboratory collection
NO2206	Same as B2550, but *rpsL*	Spontaneous streptomycin-dependent mutant of B2550
NO2207	Same as B2550, but *rpsD rpsL*	Spontaneous streptomycin-independent "revertant" of NO2206
NO2246	Same as NO816, but *aroE*$^+$ *rpsD*	Transduction of NO816 by P1 (NO2207), selecting *aroE*$^+$; screen for hypersensitivity to streptomycin
NO2514	Same as NO2143, but *trkA*$^+$ *rpsL*$^+$	Transduction of NO2143 by P1 (NO2246), selecting for *trkA*$^+$; screen for *aroE* Str^s
NO2515	Same as NO2143, but *trkA*$^+$ *rpsD rpsL*$^+$	Transduction of NO2143 by P1 (NO2246), selecting for *trkA*$^+$; screen for *aroE* Str^s, temperature sensitivity
NO2543	λpapa, λ *trkA* double lysogen of NO2514	This work
NO2544	λpapa, λ *spc*2 double lysogen of NO2514	This work
NO2545	λ α-*lac*1 *c*I$^+$, λ *spc*2 double lysogen of NO2514	This work
NO2546	λpapa, λ *trkA* double lysogen of NO2515	This work
NO2547	λpapa, λ *spc*2 double lysogen of NO2515	This work
NO2548	λ α-*lac*1 *c*I$^+$, λ *spc*2 double lysogen of NO2515	This work

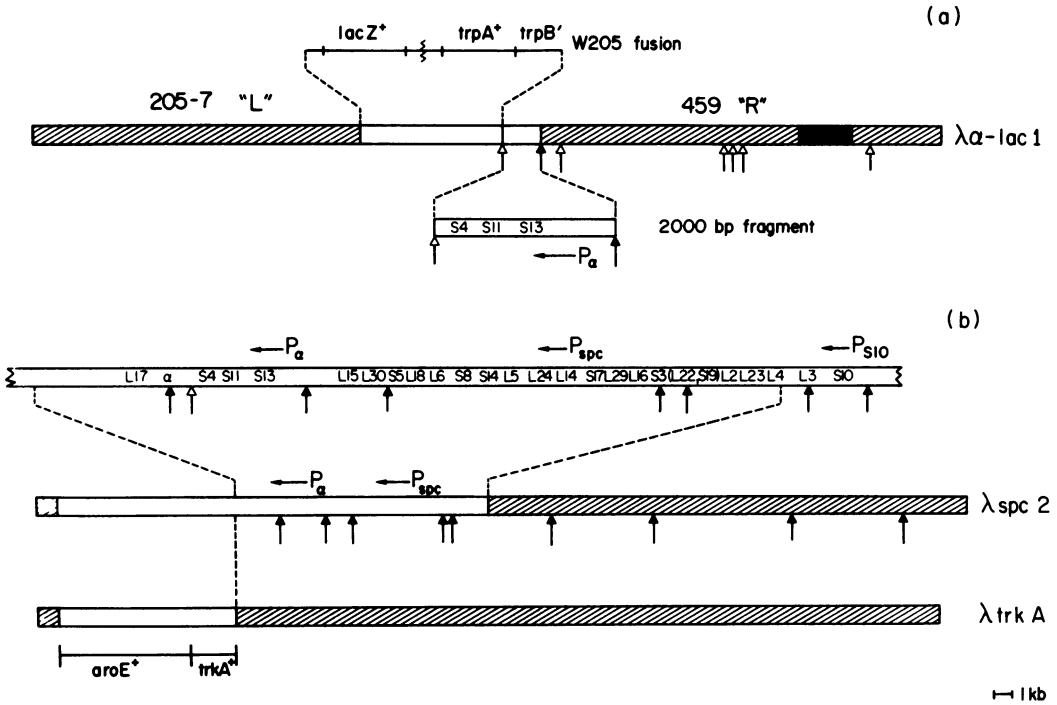

FIG. 1. Structures of specialized transducing phages. (a) Structure of λ α-*lac*1. 205-7 "L" is the left arm of λ derived from digestion of λRS205-7 with *Hin*dIII (ϕ). 459 "R" is the right arm of λ derived from digestion of λ459 with *Eco*RI (ϕ). Symbols: ▨, phage DNA; □, bacterial DNA; ■, deletion of phage DNA in λ459. The 2,000-base-pair *Eco*RI-*Hin*dIII fragment is from λ *spc*1 and is identical to that shown for λ *spc*2 in (b). (b) Structures of λ *spc*2 and λ *trkA*. Symbols: ▨, phage DNA; □, bacterial DNA. *Eco*RI (ϕ) and *Hin*dIII (ϕ) sites for λ *spc*2 bacterial DNA are shown (22). The bacterial DNA of λ *spc*2 contains a single *Hin*dIII site (ϕ) between the S4 and α genes.

these phages. λ α-*lac*1 cI^+ lysogens were isolated by screening λ-resistant cells for those producing β-galactosidase. Since the recipient strains are *lacY* in addition to *lacZ*, $LacZ^+$ lysogens could not be directly selected on standard indicator plates. Production of β-galactosidase was shown by spraying colonies with toluene followed by the addition of 1 ml of 10 mM *o*-nitrophenyl-β-D-galactopyranoside (23). $LacZ^+$ cells turn bright yellow within several minutes, whereas $LacZ^-$ cells remain white.

The presence of λ *trkA* or λ *spc*2 and λ α-*lac*1 cI^+ prophage was confirmed by the production of $aroE^+$ and $lacZ^+$ transducing phage, respectively, after induction of lysogens with 1 μg of mitomycin C per ml.

Growth conditions. LB medium contained 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1 ml of 1 N NaOH per liter. For growth and selection of streptomycin-dependent strains, LB was supplemented with 100 μg of streptomycin per ml. Streptomycin sensitivity was used to distinguish *rpsD rpsL* double mutants from *rpsD* $rpsL^+$ single mutants in the transduction experiments; the former are more sensitive, whereas the latter are less sensitive to streptomycin than an $rpsD^+$ $rpsL^+$ strain (32). For determining the degree of streptomycin sensitivity, LB plates were supplemented with 1, 3, 5, and 10 μg of streptomycin per ml. For growth of *trkA* mutants, the medium was supplemented with 0.1 M KCl.

ABG and ABG-K2.5 minimal plates were used for selecting $aroE^+$ and $trkA^+$ transductants, respectively. ABG plates contained AB medium (6) supplemented with 0.2% glucose and 1 μg of thiamine per ml. ABG-K2.5 plates contained less potassium than ABG plates, thus allowing growth of *kdpABC5* strains, but not *kdpABC5 trkA* double mutants (11). ABG-K2.5 medium was made the same as ABG, except that sodium salts were substituted for potassium salts in AB medium and KCl was added to a final concentration of 2.5 mM. A 10-ml amount of aro mix (5 mg of tyrosine, 5 mg of phenylalanine, 2 mg of shikimic acid, 0.5 mg of *p*-aminobenzoic acid, and 0.5 mg of *p*-hydroxybenzoic acid per ml) was added per liter of minimal ABG medium for growth of *aroE* strains. Details of the labeling conditions are given in footnote *a* of Table 2.

Preparation and analysis of r-proteins. To analyze unlabeled r-proteins from various strains, 1 liter of cells was grown in LB medium at 37°C to approximately 5×10^8 cells per ml. Cells were harvested by centrifugation and washed with TMAI (10 mM Tris-hydrochloride [pH 7.5], 30 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol). Washed cells were resuspended in 7.5 ml of TMAI and lysed by sonication. Cellular debris was removed by centrifugation at 10,000 rpm for 1 h. 70S ribosomes were pelleted by centrifuging the supernatant for 4 h at 30,000 rpm in a

TABLE 2. Relative synthesis rates of r-proteins in haploid, diploid, and triploid strains[a]

r-Protein[b]	Relative synthesis rate of r-proteins					
	NO2514 control strains			NO2515 mutant strains		
	Haploid	Diploid	Triploid	Haploid	Diploid	Triploid
L1	1.00	1.00	1.00	1.00	1.00	1.00
L2	0.82	0.97	0.87	0.92	1.00	1.01
L14	0.85	1.06	1.00	1.01	1.15	1.10
L17	0.95	0.95	0.93	**1.19**	1.37	1.11
S3	0.91	0.98	0.96	0.84	1.04	1.02
S4w	0.82	0.94	0.89		**0.90**	**0.91**
S4m				**1.98**	**0.80**	**0.43**
S7	0.95	0.97	1.00	0.98	0.99	1.01
S11	0.93	1.09	1.07	**1.89**	**1.74**	**1.32**
S12	1.12	1.13	1.13	1.09	1.07	1.16
S13	0.93	1.14	1.10	**1.87**	**1.62**	**1.32**

[a] Haploid strains were λpapa, λ *trkA* double lysogens; diploid strains were λpapa, λ *spc*2 double lysogens; and triploid strains were λ *spc*2, λ α-*lac*1 *cI*$^+$ double lysogens. Cells were grown at 37°C in AB medium supplemented with 0.4% glucose and 1 μg of thiamine per ml (ABG medium). At a density of approximately 2×10^8 cells per ml, cultures were pulse-labeled for 1 min with [^{3}H]lysine (0.441 nmol/ml; 68 μCi/nmol) followed by a 1-min chase with excess nonradioactive lysine (500 μg/ml). At the end of the labeling period, cells were chilled rapidly on ice and washed twice with 50 mM Tris-hydrochloride (pH 8.0). The growth rates of all strains under these conditions were 1.3 doublings per h, except for that of the *rpsD* haploid strain NO2515 (λpapa, λ *trkA*). The growth rate of this strain was 0.5 doublings per h. Each ^{3}H-labeled sample was divided; half was mixed with ^{14}C-labeled *rpsD* cells, and half was mixed with ^{14}C-labeled wild-type cells. ^{14}C-labeled cultures were prepared by growing cells in ABG medium containing [^{14}C]lysine (5.85 nmol/ml; 342 mCi/mmol) for at least three generations, and these were used as an internal standard for calculating the synthesis rates of individual r-proteins. Proteins were extracted from the mixed cells and separated by two-dimensional gel electrophoresis. After staining and destaining of the gels, individual r-protein spots were cut, dried, and oxidized in a Packard sample oxidizer to separate the ^{3}H and ^{14}C. The ^{3}H/^{14}C ratio was calculated for each r-protein, and these ratios were normalized to that for r-protein L1. The normalized values are given and are the average value from four samples: ^{14}C-labeled mutant cells were used in two samples, and ^{14}C-labeled wild-type cells were used in two samples. The same ^{3}H/^{14}C values were obtained regardless of the type of ^{14}C-labeled cells used, except in the case of L17 and S7. The values for these two r-proteins are those obtained by using wild-type ^{14}C-labeled cells as an internal standard. r-Protein S7 appears to be turning over faster in the mutant strain than in the wild-type strain, so that the amount of ^{14}C-labeled S7 is less in the uniformly labeled mutant cells than in the wild type cells. As a result, all of the ^{3}H/^{14}C values obtained for S7 by using the ^{14}C-labeled mutant cells as a normalization standard are about 30% greater than the values shown. L17 was overproduced slightly in the mutant strain relative to the wild-type strain, and the overproduced protein was stable (similar to previous observations with L11 [21]). ^{3}H/^{14}C values for L17 with mutant cells as a reference are thus slightly lower than those with wild-type cells. The values for r-proteins in the α operon which are pertinent to the discussion in the text are in boldface type.

[b] S4w, Wild-type S4; S4m, mutant S4.

Beckman 40 rotor. The 70S ribosome pellet was suspended in 0.25 ml of TMAI and stored at −60°C.

For two-dimensional gel analysis, r-proteins were extracted from crude 70S ribosomes with 67% acetic acid (18) and precipitated with 5 volumes of acetone (1). Samples for two-dimensional gels contained 5 to 10 absorbancy at 260 nm equivalents of crude ribosomes and were suspended in a final volume of 30 μl of gel sample buffer (60 mM Tris, 75 mM boric acid, 6 mM dithiothreitol, 8 M urea). Two-dimensional polyacrylamide gel electrophoresis was done as described by Howard and Traut (20).

To measure the synthesis rates of r-proteins, total cellular protein was extracted from labeled cells as follows. ^{3}H-labeled and ^{14}C-labeled cells were mixed, washed with 50 mM Tris-hydrochloride (pH 8.0), and suspended in 50 μl of lysis solution (1.2 ml 50 mM Tris-hydrochloride [pH 8.0], 150 μl of 0.1 M EDTA [pH 7.1], 60 μl of lysozyme [2 mg/ml]). Cells were lysed by freeze-thawing six times, and proteins were extracted with acetic acid and precipitated with acetone as described above. Labeled protein pellets were suspended in 30 μl of sample buffer containing unlabeled r-proteins (5 absorbancy at 260 nm equivalents from NO2514 and 5 absorbancy at 260 nm equivalents from NO2515) and were separated by two-dimensional gel electrophoresis.

RESULTS

Isolation of an *rpsD* mutant. The *rpsD* mutant used in this study was isolated by the method of Olsson and Isaksson (32), which is based on the following observations. An *E. coli* mutant having a mutation in the gene for r-protein S12 (*rpsL*) which renders it streptomycin dependent (2) will become phenotypically streptomycin independent through acquisition of an additional mutation. Many of the secondary mutations are in *rpsD* (*ramA*), the gene for r-protein S4 (19, 32). Strains having such an *rpsD* mutation are

usually temperature sensitive, and this temperature sensitivity has been correlated with a failure to assemble 30S ribosomal subunits at the restrictive temperature (30). In most cases, the mutant protein has been found to be shorter than the wild-type protein and to have considerable alteration of amino acid sequence at the carboxy-terminal end (14).

A spontaneous streptomycin-dependent mutant (NO2206) was isolated by plating cells of strain B2550 on LB medium containing 100 μg of streptomycin per ml. Spontaneous streptomycin-independent revertants of NO2206 were isolated by plating cells in the absence of streptomycin at 30°C, and single colonies were screened for a temperature-sensitive phenotype at 42°C. Analysis of the r-proteins of one such temperature-sensitive revertant (NO2207) revealed the presence of an S4 spot with altered electrophoretic mobility on a two-dimensional polyacrylamide gel (Fig. 2). The mutant S4 protein was approximately 15% shorter than the wild-type protein as judged from the mobility on a sodium dodecyl sulfate-polyacrylamide gel, and peptide analysis has shown that the missing 15% corresponds to the carboxy-terminal end of the wild-type S4 (G. Craven, personal communication). Although the exact nature of the *rpsD* mutation has not been characterized, these observations suggest that the mutation causes premature termination of translation of the S4 mRNA.

The *rpsD* mutation of NO2207 was separated from the *rpsL* mutation by P1 transduction and was subsequently introduced into a genetic background appropriate for the selection and maintenance of diploid and triploid strains. NO2514 and NO2515 are the resulting isogenic *aroE rpsD*$^+$ and *aroE rpsD* strains, respectively, which were used in the experiments described below. It should be noted that our *rpsD* mutant strains grew much better at 37 than at 30°C, and that temperature sensitivity was more striking when growth was compared at 37 and 42°C rather than at 30 and 42°C. All of the experiments described in this paper were done at 37°C.

Comparison of the synthesis rates of r-proteins in *rpsD* and *rpsD*$^+$ haploid strains. The relative synthesis rates of r-proteins in *rpsD* mutants has previously been examined by Olsson and Isaksson (33). They found that the relative synthesis rates of r-proteins S4, S12, and S13 were elevated in *rpsD* mutant strains, and they suggested that S4 might be involved in regulating the synthesis rates of the promoter-proximal r-proteins in both the *str* and α operons. Their work, however, was done before the model of post-transcriptional regulation of r-protein synthesis was proposed, so it was important to confirm and possibly extend their findings by using our *rpsD* mutant. It appeared to us that *rpsD* mutants might be regulatory mutants which had lost the translational repressor activity of the wild-type S4. We reasoned that if this were indeed the

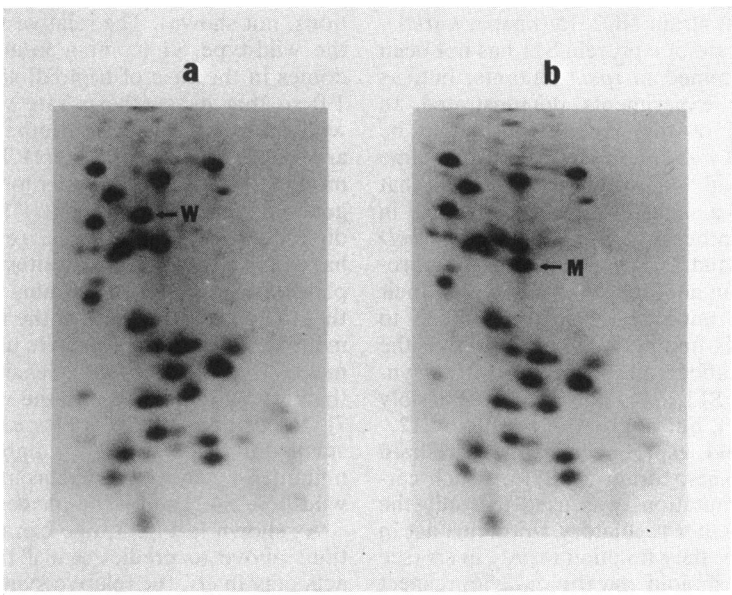

FIG. 2. Two-dimensional gel electrophoresis of 70S r-proteins. r-Proteins were extracted from crude 70S ribosomes containing 10 absorbancy at 260 nm equivalents of (a) wild-type ribosomes from NO2514 and (b) mutant ribosomes from NO2515. W, wild-type form of r-protein S4; M, mutant form of r-protein S4. Only the basic sides of the gels are shown.

case, then we should see overproduction only of those r-proteins encoded by the α operon (S13, S11, S4, and L17).

The synthesis rates of r-proteins in NO2514 (λpapa, λ *trkA*) and NO2515 (λpapa, λ *trkA*) were measured by pulse-labeling exponentially growing cultures for 1 min with [^{3}H]lysine and chasing for 1 min with an excess of nonradioactive lysine. ^{3}H-labeled cells were mixed with cells uniformly labeled with [^{14}C]lysine, and the ratio of ^{3}H to ^{14}C in individual r-proteins was determined (see footnote *a* of Table 2 for details). The results are presented in Table 2 as ^{3}H/^{14}C values which have been normalized to the value for r-protein L1. The values in Table 2 thus represent the synthesis rates of individual r-proteins relative to the synthesis rate of r-protein L1 in each strain. L1 was chosen as the normalization standard because it separates well from other r-proteins on two dimensional gels, and because its gene is located in the *rif* region at 89 min on the *E. coli* chromosome rather than in the *str-spc* region at 72 min (28). The addition of identical ^{14}C-labeled cells to different ^{3}H-labeled samples allowed direct comparison of the synthesis rates of r-proteins in different strains as well as within a given strain. In the experiments done here, all ^{3}H-labeled samples were divided so that r-proteins could be analyzed by using both mutant and wild-type ^{14}C-labeled cells (see below and footnote *a* of Table 2).

In agreement with the data of Olsson and Isaksson, we found that the relative synthesis rates of S4 and S13 were elevated about 90% in the *rpsD* mutant strain NO2515 (λpapa, λ *trkA*). The synthesis rate of r-protein S11 has not been previously examined in *rpsD* mutants, but, as predicted, our experiments demonstrated an overproduction of this protein. It should be noted that S11 was overproduced to the same extent as S13 and S4. In contrast, we found that there was only a slight increase (about 20%) in the relative synthesis rate of L17 in the *rpsD* strain. Olsson and Isaksson observed overproduction of S12 in addition to S4 and S13 in their *rpsD* mutants, but our experiments failed to substantiate this finding. We conclude that the *rpsD* mutation affects the regulation of the synthesis rates of S13, S11, and S4 (and possibly L17; see below), but not that of r-protein S12.

Diploid-triploid experiments with specialized transducing phages. Strain NO2515, which carries the *rpsD* mutation, was used to study the question of whether regulatory r-proteins act in *trans* or whether they function mainly in *cis* (see above). Strains diploid and triploid with respect to the α operon were constructed by lysogenizing this strain with specialized transducing phages carrying the wild-type S4 gene (λ *spc*2, or λ *spc*2 and λ α-*lac*1 *c*I$^+$; see below and Fig. 1). The haploid strain contains one mutant (*rpsD*) α operon, the diploid strain contains one mutant and one wild-type (*rpsD*$^+$) α operon, and the triploid strain contains one mutant and two wild-type α operons. The copy number of the α operon refers to the portion from the promoter through the S4 gene (see below). Since the mutant S4 can be clearly separated from the wild-type S4 by two-dimensional gel electrophoresis (Fig. 2), it is possible to examine the effect of the wild-type S4 synthesized from the phage genomes on the "unregulated" synthesis of the mutant S4 from the chromosome. Using the model of translational regulation, we made quantitative predictions as to what the synthesis rates of α operon r-proteins should be if *trans* or *cis* regulation by the wild-type S4 were occurring in the diploid and triploid strains. These predictions are presented schematically in Fig. 3 and are based on the following assumptions. (i) The S13, S11, and S4 cistrons are sequentially translated (34, 40), and their relative synthesis rates are identical. S4 acts at or near the beginning of the S13 cistron mRNA and regulates S11 and S4 translation as a consequence of this primary action (29, 40). (ii) The mutant S4 does not have any significant activity as a translational repressor, and as a consequence the relative synthesis rates of S13, S11, and S4 in the mutant haploid strain increase by a factor of about two under our experimental conditions (see above). (iii) In diploid and triploid strains containing both forms of S4, only the wild-type S4 gets incorporated into ribosomes (33; our observations, not shown). The relative synthesis rate of the wild-type S4 (coming from two phage genomes in the case of triploid) should always be 1.0 so that its synthesis rate will be balanced with the synthesis rates of other r-proteins that are not regulated by S4. (iv) The ratio of the mutant S4 gene copy number to the wild-type S4 gene copy number is about 1:1 and 1:2 in the diploid and triploid strains, respectively. (We have not determined the sites of transducing phage integration in the strains used. Assuming that the phages integrate at the normal λ attachment site, their copy numbers under our experimental conditions would be about 20% lower than if they integrated into the *str-spc* region [4, 7]. In our predictions, we have disregarded this small difference in copy number between the mutant S4 gene on the chromosome and the wild-type S4 gene on the phages.)

As shown in Fig. 3, one can use the assumptions above to predict that if the wild-type S4 acts only in *cis*, the relative synthesis rate of the mutant form of S4 should not be affected by the presence of the wild-type protein and should remain at about 2 in both the diploid (*rpsD*/*rpsD*$^+$) and triploid (*rpsD*/*rpsD*$^+$/*rpsD*$^+$) strains.

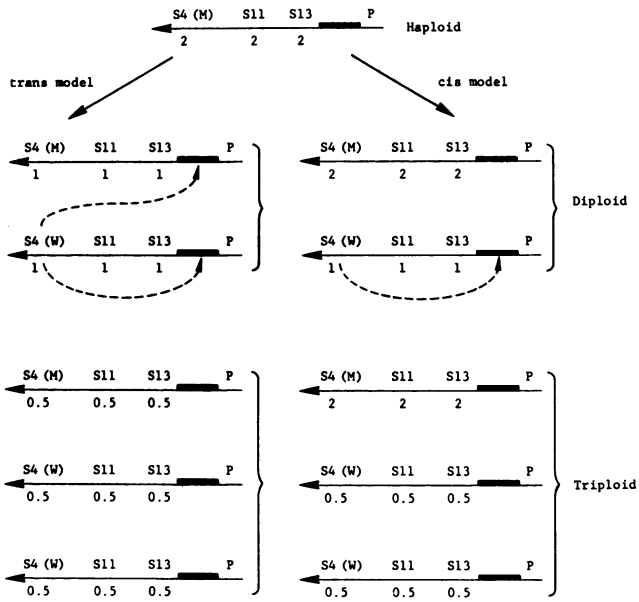

FIG. 3. r-Protein synthesis rates predicted by both *trans* and *cis* regulation by r-protein S4. The first three genes in the α operon are shown. M, mutant form of r-protein S4; W, wild-type S4; P, α promoter; ←, direction of mRNA transcription and protein translation; ■, segment corresponding to the presumed mRNA target site for the binding of the wild-type S4; --►, interaction of the wild-type S4 with the target site(s) in a diploid strain if it acts in *trans* (*trans* model) or strictly in *cis* (*cis* model). A more detailed explanation is given in the text.

In contrast, if the wild-type S4 functions as a translational repressor in *trans* as efficiently as in *cis*, the relative synthesis rate of the mutant S4 should be about 1 in the diploid strain and about 0.5 in the triploid strain. Both models predict that the relative synthesis rates of S13 and S11 should be equal to the sum of the synthesis rates of both forms of S4. They should therefore be about 3 in both the diploid and triploid strains if the *cis* model is correct, and they should be about 2 and 1.5 in the diploid and triploid strains, respectively, if the *trans* model is correct.

For actual experiments, haploid, diploid, and triploid double lysogens of NO2514 and NO2515 were constructed by using λpapa, λ *trkA*; λpapa, λ *spc*2; and λ *spc*2, λ α-*lac*1 *cI*$^+$, respectively. Figure 1 shows the structures of the specialized transducing phages λ *trkA*, λ *spc*2, and λ α-*lac*1 *cI*$^+$. It can be seen that λ *spc*2 carries the entire *spc* and α operons with their bacterial promoters as well as the *trkA*$^+$ and *aroE*$^+$ genes. λ *trkA* does not carry any r-protein genes, but does have the bacterial *trkA*$^+$ and *aroE*$^+$ genes and was used as a control for λ *spc*2. λ *trkA* and λ *spc*2 lysogens were constructed and maintained by selecting AroE$^+$ transductants. λ α-*lac*1 *cI*$^+$ carries the α promoter (Pα) and the genes for r-proteins S13, S11, and S4; it does not have the genes for the α subunit of RNA polymerase or r-protein L17. Since λ α-*lac*1 *cI*$^+$ does not carry the distal part of the α operon, only the synthesis rates of the first three r-proteins in the α operon were considered in the predictions discussed above. The presence of λ α-*lac*1 *cI*$^+$ prophage in NO2514 or NO2515 was confirmed by the production of β-galactosidase.

The relative synthesis rates of r-proteins in the control NO2514 and mutant NO2515 haploids, diploids, and triploids were measured by pulse-labeling cells with [^{3}H]lysine. Both mutant and wild-type haploid cells uniformly labeled with [^{14}C]lysine were used as an internal standard for comparison of the relative synthesis rates of r-proteins (see footnote *a* of Table 2 for details). The results are given in Table 2 as ^{3}H/^{14}C values which have been normalized to the value for r-protein L1. In the control haploid, diploid, and triploid strains, the relative synthesis rates of all of the r-proteins measured were identical. The absence of gene dosage effects in diploid and triploid strains confirms the results of previous gene dosage experiments (12, 16, 31).

In the mutant strains, the results obtained are very close to those predicted by assuming that the wild-type S4 acts in *trans* as a translational repressor. In the diploid strain NO2515 (λpapa, λ *spc*2), the relative synthesis rates of the wild type and mutant forms of S4 were identical and were both close to 1.0. The data for the relative synthesis rates of r-proteins S11 and S13 were also consistent with *trans* regulation by S4 since

the predicted values of 2 were close to the observed values of 1.7. In the mutant triploid NO2515 (λ α-*lac*1 *cI*$^+$, λ *spc*2), the synthesis rates of the wild-type and mutant forms of S4 were also those predicted by the *trans* model. The relative synthesis rate of the mutant S4 was about 0.4, which is close to the predicted value of 0.5; the relative synthesis rates of S11 and S13 (about 1.3) were also close to the values of 1.5 predicted by the *trans* model. Table 3 summarizes these relevant data and compares them to both the *cis* and *trans* model predictions. Based on the results presented here, we conclude that the wild-type S4 acts in *trans* to regulate the translation of mRNA synthesized from the chromosomal α operon containing the *rpsD* allele.

DISCUSSION

Role of S4 in regulating the synthesis of the α operon r-proteins. Previous in vitro and in vivo work has established that S4 is the translational repressor which regulates the synthesis of r-proteins in the α operon: S13, S11, S4, and probably L17 (8, 37). In agreement with the regulatory role of S4, we have found that a mutational alteration of this protein results in a significant and specific increase in the synthesis rates of r-proteins S13, S11, and S4. This finding confirms the earlier observation of Olsson and Isaksson that S4 and S13 are overproduced in *rpsD* mutants (33) and, in addition, demonstrates a comparable overproduction of r-protein S11.

In vivo induction experiments have indicated that L17 synthesis is regulated by r-protein S4 (8), but in vitro experiments have failed to substantiate this (37). Assuming that the in vivo data accurately reflect the steady-state regulation of α operon r-proteins, we expected to see an overproduction of L17 comparable to that of S13, S11, and S4 in the *rpsD* haploid strain. Even though only a slight increase (about 20%) in the synthesis rate of L17 was observed in the *rpsD* mutant, we think it is significant because of the following observation. We have found that the synthesis rates of all α operon r-proteins increase with increasing gene dosage when only the mutant form of S4 is present, so that the marginal overproduction of L17 seen in the *rpsD* mutant becomes significant (about 70%) in an *rpsD*/*rpsD* merodiploid (our unpublished experiments). This demonstrates that L17 synthesis is affected by the S4 mutation, and we conclude that L17 is translationally regulated by r-protein S4. An explanation as to why the increase in L17 synthesis is slight compared with that of S13, S11, and S4 in the *rpsD* mutant is as follows. The exact nature of the *rpsD* mutation is not known, but the fact that the mutant S4 protein is missing 15% of the wild-type protein at the carboxy-terminal end suggests that premature termination of translation occurs in the mutant. We believe that this causes a polar effect on the expression of the downstream L17 gene, leading to less efficient synthesis of L17 relative to S13, S11, and S4.

The results obtained with our *rpsD* mutant demonstrate that regulation by r-protein S4 is essential for balancing the synthesis rates of r-proteins in the α operon with the synthesis rates of all other r-proteins under steady-state growth conditions. A similar conclusion was reached in the analysis of a strain which is apparently deficient in the regulatory r-protein L1 (21, 36). We have suggested that the capacity of r-protein synthesis from mRNA is in excess of that needed for ribosome assembly under the growth conditions used, and that this becomes evident when the post-transcriptional regulatory mechanisms are disrupted as in the L1-deficient and *rpsD* mutant strains. Consistent with this idea is the recent demonstration that the characteristic growth rate-dependent regulation of r-proteins synthesized from the α and *spc* operons is not determined by changes in transcription rates from these promoters and thus must be determined by post-transcriptional mechanisms (25).

TABLE 3. *cis-trans* test of S4 repressor activity: comparison of the predicted and observed r-protein synthesis rates in NO2515 mutant strains

r-Protein	Haploid	Synthesis rate of r-proteins					
		Diploid			Triploid		
		Predicted		Observed	Predicted		Observed
		cis	*trans*		*cis*	*trans*	
S13	2.0	3.0	2.0	1.6	3.0	1.5	1.3
S11	2.0	3.0	2.0	1.7	3.0	1.5	1.3
S4w		1.0	1.0	0.9	1.0	1.0	0.9
S4m	2.0	2.0	1.0	0.8	2.0	0.5	0.4
All other r-proteins	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Interaction of S4 with rRNA and mRNA. The *rpsD* mutant strains studied by Olsson are temperature sensitive due to a defect in ribosome assembly at the restrictive temperature. S4 is one of the first r-proteins to bind to 16S rRNA in in vitro reconstitution of 30S subunits (26). It has been reported that a mutant form of S4, which is similar to those studied by Olsson and by us, has a lower affinity for its binding site on 16S rRNA than does the wild-type r-protein in vitro (17). Consistent with the in vitro data is the finding that only the wild-type form of S4 is incorporated into ribosomes in the *rpsD*/*rpsD*$^+$ merodiploid strains (our data) (33), even though both forms of S4 are synthesized at the same rate. The presumed target site for S4 action on α-operon mRNA has structural homology with the S4 binding site on 16S rRNA, and it has been suggested that the same structural feature of S4 is involved in the interaction with both rRNA and mRNA (29). Although this has not been proven, our data support this idea since mutational alterations in r-protein S4 appear to affect the affinity of S4 for both 16S rRNA and the mRNA regulatory target site.

Regulation in *trans*. The exact mechanism of translational repression and its relief by competing rRNA synthesis is not known, but there are two possibilities that should be considered. One is that, under steady-state growth conditions, "free" repressor r-proteins are released from mRNA and then interact with either rRNA or mRNA target sites, depending on the availability of rRNA. According to this model, repressor r-proteins would be expected to function not only in *cis* but also in *trans* on any mRNA with the proper target site. Another possibility is that, upon completion of their synthesis, repressor r-proteins interact immediately with the target site on their own mRNA, and complete release of such r-proteins from their message requires coupling with ribosome assembly. This second possibility implies that repressor r-proteins act only in *cis* and not in *trans*.

From experiments in which r-protein genes have been fused to the *lac* or *ara* operon promoters (8–10, 38, 41) on a multicopy plasmid, it is clear that regulatory r-proteins can act in *trans* to stop synthesis of r-proteins whose genes are on the chromosome. In most of these cases, however, the target sites for the regulatory r-proteins are removed from the fused operon, resulting in an unregulated two- to fourfold overproduction of the induced r-protein. Translational repression under steady-state growth conditions occurs without there being any detectable overproduction of regulatory r-proteins, so the question still remained as to whether regulatory r-proteins act in *trans* or mainly in *cis* under normal steady-state growth conditions. The present study was designed to answer this question of *cis* versus *trans* regulation. By using an *rpsD* mutant strain as a background for constructing strains diploid and triploid with respect to the α operon, it was possible to measure the relative contributions of r-proteins from different α operons in a single cell. From the experimental data presented in this paper, we conclude that S4 acts in *trans* as a translational repressor, and we assume that this is true of all other regulatory r-proteins. Presumably, under steady-state growth conditions, small amounts of free regulatory r-proteins exist in the cellular pool which can interact with either rRNA for ribosome assembly or with mRNA for translational repression.

We originally suggested that translational repression would occur mainly in *cis* (12). A *cis*-type of autogenous regulation was considered to explain data concerning the phenotypic dominance of antibiotic-sensitive alleles over antibiotic-resistant alleles in Str^r/Str^s or Spc^r/Spc^s merodiploid strains (5, 35; see reference 12 for discussion). We now know, however, that repressor r-proteins function as efficiently in *trans* as in *cis*, and we would predict that antibiotic sensitive and resistant alleles should be expressed equally in merodiploid strains. In view of our expanded knowledge of the regulation of r-protein synthesis, we think that the problem of dominance deserves re-examination.

The presence of a post-transcriptional mechanism for regulating the synthesis of most of the r-proteins in the *str-spc* and *rif* regions of the *E. coli* chromosome has been firmly established. Some essential features of our regulatory model have been discussed in previous papers (29, 40) and were used as assumptions in the present study to predict the relative synthesis rates of α-operon r-proteins in diploid and triploid strains (Fig. 3 and above). The excellent agreement found between the observed values and those predicted by a *trans* model of repressor action gives further support to the validity of the essential features of the translational regulation model.

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