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Genetics

Effects of ribosomal mutations on the read-through of a chain termination signal: Studies on the synthesis of bacteriophage λ *O* gene protein *in vitro**

(λ *cII* gene protein/amber mutations/*in vitro* suppression/streptomycin-resistance mutation/bacteriophage Qβ coat protein)

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ABSTRACT **In a DNA-dependent protein-synthesizing system that contains streptomycin-sensitive ribosomes, λ DNA directs the synthesis of two proteins that are products of the *O* gene. The larger is produced as a result of read-through of a UGA termination codon. In the system containing streptomycin-resistant ribosomes this read-through protein is not synthesized, indicating that the mutational alteration in the ribosomal protein S12 restricts the read-through. The mutant ribosomes also fail to synthesize the read-through coat protein of RNA phage Qβ. In addition, the mutant ribosomes restrict suppression of amber mutations *in vitro*, similar to their effect *in vivo*.**

One of the most extensively studied *Escherichia coli* ribosomal protein genes is *strA*. This gene codes for ribosomal protein S12 (1). It is known that many of the streptomycin-resistance (Str-R) mutations in this gene cause additional phenotypic alterations in the mutants (pleiotropic effects). For example, extensive *in vivo* studies by Gorini and his coworkers have demonstrated that many mutations in the *strA* gene decrease the *in vivo* efficiency of suppression of nonsense and missense mutations (2, 3). This is called "restriction" of suppression.

We have recently analyzed several Str-R mutants that exhibit various pleiotropic effects and have shown that some Str-R mutations cause decreases in the rate of protein chain elongation (analyzed *in vivo*) (23) as well as alterations in the efficiency and specificity of protein chain initiation (analyzed *in vitro*) (4). We have now found that these same mutations affect an aspect of protein chain termination; wild-type ribosomes allow read-through of certain chain termination signal(s), while ribosomes from Str-R mutants do not.

Our *in vitro* system is a bacteriophage λ DNA-directed protein-synthesizing system. We have found that two proteins synthesized in this system (using Str-S ribosomes) are coded for by the λ *O* gene. One of them, which is a read-through protein from gene *O*, is not synthesized when the ribosomes from the most restrictive Str-R mutant are used in the system.

MATERIALS AND METHODS

Phages λ*c*I857*S*7 (called "λ" in this paper), λ*c*I857*c*II-*am*60*am*41 ("λ*c*II*am*") (5), λ*c*I857*Oam*125 ("λ*Oam*125"), and λ*cIam*6*Oam*29 ("λ*Oam*29") (6) were used in this work. DNA-dependent protein synthesis *in vitro* as well as analysis of products was performed as described previously (7, 8), expect for the source of ribosomes. Ribosomes were prepared from *E. coli* K-12 strain PR-C600 (*str*^{s}) (4, 23), strain PR-SM3 (isogenic to strain PR-C600, but carrying a Str-R mutation (4, 23), *E. coli* B strain L44 (*str*^{s}, *arg*$^-$, *leu*$^-$), strain L44-401 (isogenic to strain L44, but carrying the *strA1* allele) and strain L44-2 (isogenic to strain L44, but carrying the *strA2* allele). Strain L44 was a gift from the late L. Gorini, and the other two derivatives were constructed by phage P1 transduction in this laboratory (4). Unfractioned "amber suppressor tRNA" and "UGA suppressor tRNA" were prepared from *E. coli* K-12 strain Ymel [SuIII$^+$ (*supF*); ref 9] and *E. coli* K-12 strain CAJ64 (10), respectively, by phenol extraction of whole cells (11). A commercial unfractionated tRNA from *E. coli* B (Calbiochem) was used as "Su° tRNA." The amount of these tRNAs used was 30 μg/40 μl total reaction volume. Phage Qβ RNA was prepared according to the method of Gesteland and Boedtker (12). Approximate molecular weights of proteins were estimated from comparison of their mobility in polyacrylamide gel electrophoresis with those of several reference proteins with known molecular weights (13). Relative amounts of radioactive proteins in gels were estimated by tracing suitable autoradiograms with a Joyce-Loebl densitometer.

RESULTS

λ-DNA-directed protein synthesis *in vitro*

DNA from phage λ was used as a template in our standard DNA-directed *in vitro* protein-synthesizing system, and the [^{35}S]methionine-labeled proteins were analyzed using sodium dodecyl sulfate/polyacrylamide gel electrophoresis followed by autoradiography. As can be seen in Fig. 1, slots 1 and 2, there is one clear difference between the protein pattern obtained with ribosomes from the Str-S strain PR-C600 (slot 1) and that with ribosomes from the Str-R mutant strain PR-SM3 (slot 2). The PR-C600 ribosomes produced a protein indicated as O′, while the PR-SM3 ribosomes did not (see also Fig. 2, slots 1 and 2).

The following experimental results demonstrate that both this protein (called O′, molecular weight about 36,500) and the protein (indicated as O, molecular weight about 34,500) revealed just below the O′ protein band are encoded by the gene *O*, and that the O′ protein is synthesized by "read-through" of a chain termination codon(s) by the Str-S (but *not* by the Str-R mutant) ribosomes.

(*a*) When DNA from λ*Oam*125 was used as template, neither O′ nor O protein was synthesized by either the parent (Fig. 1, slot 11) or the mutant ribosomes (slot 12). Instead, a new single protein species (indicated as A in Fig. 1) was produced by both kinds of ribosomes. This new protein species is probably an amber fragment coded for by the *O* gene but prematurely

Abbreviations: Str-R and Str-S, streptomycin resistant and sensitive phenotypes, respectively; Su° and Su$^+$, wild-type and suppressor-positive phenotypes, respectively.

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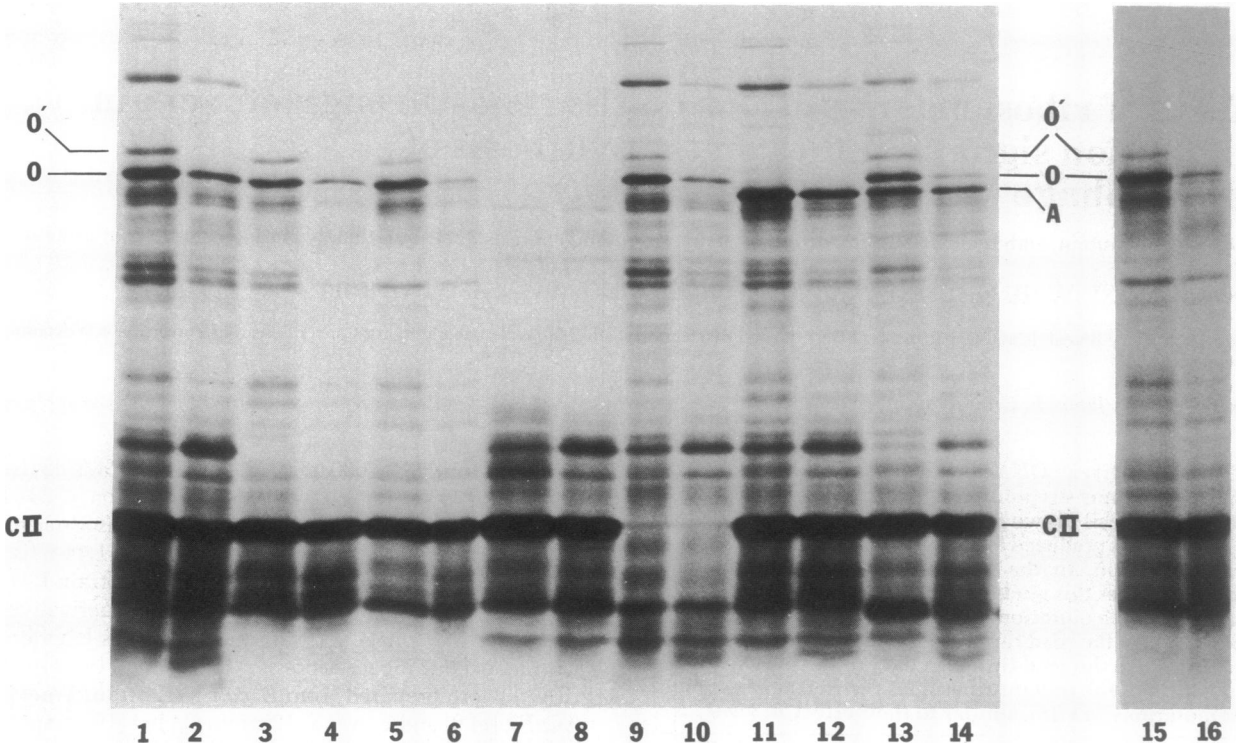

FIG. 1. λ-DNA-directed synthesis of proteins *in vitro*. Proteins were synthesized in the *in vitro* system with DNA from λ (slots 1 and 2), 13.8% *Hin*dIII fragment from λ (slots 3 and 4), 4.5% *Hin*dIII-*Sma* fragment (slots 5 and 6; and 15 and 16), 15.4% *Eco*RI fragment (slots 7 and 8), and DNA from λ*c*II*am* (slots 9 and 10) and λ*Oam*125 (slots 11–14). Controls that did not receive DNA were also analyzed; no radioactive band was detected (not shown). Samples 13 and 14 had amber suppressor tRNA. Ribosomes were either Str-S PR-C600 (slots 1, 3, 5, 7, 9, 11, 13, and 15) or Str-R PR-SM3 ribosomes (slots 2, 4, 6, 8, 10, 12, 14, and 16). The samples were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (ref. 8), followed by autoradiography. The concentration of polyacrylamide was 15%. For O′, O, and A, see the *text*. The band "cII" was identified as the product of the *c*II gene; this protein was not synthesized when DNA from the *c*II amber phage was used as a template (see slots 9 and 10). Samples 15 and 16 are identical to 5 and 6, respectively, but were photographed with a longer exposure. Electrophoresis was from the top to the bottom.

chain-terminated because of the amber mutation. Addition of an amber suppressor tRNA preparation to the *in vitro* system containing wild-type ribosomes (slot 13) restored the production of both O and O′ proteins, whereas in the system containing PR-SM3 ribosomes (slot 14) it only restored the production of the O protein, and even then only to a limited extent (see below).

Results of similar experiments using DNA from a phage with a different amber mutation in the *O* gene (*Oam*29) are shown in Fig. 2 (slots 7 and 8, compared to slot 1). Presence of the *Oam*29 mutation abolished synthesis of both O′ and O (slot 8), and addition of the amber suppressor tRNA preparation to the *in vitro* system restored synthesis of both proteins (slot 7). In this case, no new radioactive protein band was observed; presumably the amber fragments were too small and moved too fast to be detected as a separate band on the gel, or possibly the fragments might have been degraded. This explanation is consistent with the relative mapping positions of *Oam*29 and *Oam*125 (14).

(*b*) Several DNA fragments were prepared from λDNA using various restriction enzymes (see Fig. 3). It was found that the *Hin*dIII 13.8% fragment and the *Hin*dIII-*Sma* 4.5% fragment were able to direct the synthesis of both O′ and O proteins in the presence of PR-C600 ribosomes [Fig. 1, slots 3 and 5 (and 15)], while only O protein, but not O′ protein, was produced in the presence of Str-R mutant PR-SM3 ribosomes [slots 4 and 6 (and 16)]. On the other hand, the *Eco*RI 15.4% fragment failed to direct the synthesis of either O′ or O proteins (Fig. 1, slots 7 and 8). It is known that the *Eco*RI-sensitive site between the 15.4% and 12.2% fragments (see Fig. 3) is within the *O* gene (M. Furth, unpublished data). The ability of the *Hin*dIII-*Sma* 4.5% fragment and the inability of the *Eco*RI 15.4% fragment to produce O′ and O proteins are consistent with the conclusion that both the O′ and O proteins are, in fact, encoded by the *O* gene.

(*c*) The [^{35}S]methionine-labeled proteins were extracted from the O′ and O protein bands, digested with trypsin, and analyzed by paper electrophoresis, followed by autoradiography. As can be seen in Fig. 4, the methionine-containing peptide patterns from both proteins were similar or identical. This experiment and the results obtained with *O* amber mutant DNA clearly show that the proteins O′ and O are chemically related and that both are encoded by the *O* gene. In addition, the fact that *Oam*125 (Fig. 1, slots 11–14) produces a single species of amber fragment suggests that the chemical difference (responsible for the gel electrophoretic mobility difference) between O′ and O proteins occurs distal to the site of the amber mutation, i.e., it resides in the carboxy-terminal parts of the proteins. If the chemical difference were at the NH_2-terminal, the presence of the *Oam*125 mutation would have produced two amber fragments corresponding to the O′ and O proteins, and these could have been separated on the gel system used.

(*d*) The experiments described above suggested that the O protein is a major *O* gene product and the O′ protein is a "read-through" protein produced by translation of a chain termination codon (presumably the "leaky" UGA codon) because of an inherent "ambiguity" of wild-type ribosomes in translation, and suggested that Str-R ribosomes apparently re-

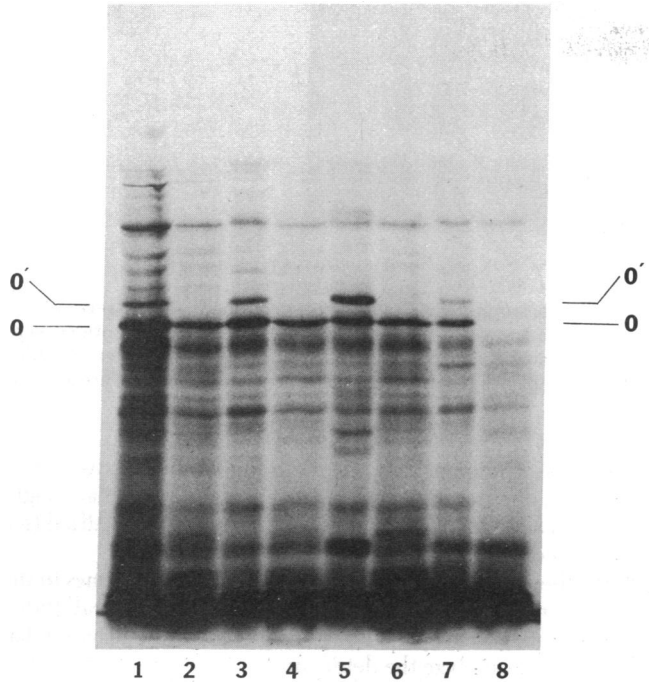

FIG. 2. Effects of amber and UGA suppressor tRNA on the synthesis of the *O* gene read-through protein (O′ protein). The λ-DNA-directed *in vitro* protein synthesizing system received Su° tRNA (slots 1 and 2), amber suppressor tRNA (slots 3 and 4), and UGA suppressor tRNA (slots 5 and 6). Ribosomes were either Str-S PR-C600 ribosomes (slots 1, 3, and 5) or Str-R PR-SM3 ribosomes (slots 2, 4, and 6). Samples in slots 7 and 8 were from the same system with Str-S PR-C600 ribosomes except that proteins were synthesized using DNA from λ*Oam*29 as a template in the presence of amber suppressor tRNA (slot 7) or Su° tRNA (slot 8). Analysis was done as in Fig. 1, except that the polyacrylamide concentration used in the electrophoresis was 12%.

strict this translational ambiguity. In order to test this interpretation, unfractionated preparations of amber (UAG) suppressor and UGA suppressor tRNA were added to the standard λ-DNA-directed *in vitro* systems. As can be seen in Fig. 2, addition of UGA suppressor tRNA to the system with wild-type ribosomes caused a marked increase in the amount of O′ and a large decrease in the amount of O protein (slot 5) relative to the control which received Su° tRNA (slot 1). In contrast, addition of amber suppressor tRNA did not cause any significant effect (slot 3), even though the same suppressor tRNA preparation was effective in suppressing the *am*29 mutation in gene *O* (slot 7). Calculation showed that the degrees of read-through were about 13%, 17%, and 56% in the presence of Su° tRNA, amber suppressor tRNA, and UGA suppressor tRNA, respectively, in this experiment (Table 1). In contrast, in the system containing the Str-R PR-SM3 ribosomes, the addition of neither UGA suppressor tRNA (slot 6) nor amber suppressor tRNA (slot 4) caused the synthesis of O′ protein. We conclude that the natural termination codon for *O* gene messenger RNA is probably UGA, that O′ protein is synthesized as a result of read-through of this termination signal by the parent ribosomes, and that the mutational alteration in PR-SM3 ribosomes prevents this read-through.

Str-R mutations restrict read-through as well as suppression of amber mutations *in vitro*

Extensive *in vivo* studies done by Gorini and his co-workers (2, 3) demonstrated that Str-R mutations restrict suppression of amber mutations by suppressor tRNA. We have now confirmed this conclusion in our *in vitro* experiments. For example, in the experiment shown in Fig. 1 (slots 13 and 14), the degree of suppression by amber suppressor tRNA was about 60% in the presence of the parent PR-C600 ribosomes, while it was about 10% in the presence of the mutant PR-SM3 ribosomes (Table 1).

Restriction of read-through of the *O* gene chain termination signal by Str-R ribosomes appeared analogous to the restriction of amber mutations by Str-R mutations. The results of two further experiments support this general conclusion. First, the same phenomenon of restriction of *O* gene read-through was observed when a λ-mRNA-directed protein synthesizing system was used (Fig. 5); λ mRNA directed the synthesis of both O′ and O proteins with the parent ribosomes (Fig. 5a, slot 1), but only that of O with the mutant PR-SM3 ribosomes (Fig. 5a, slot 2). Thus, as expected, the effect of the Str-R mutation occurs at the level of translation, not transcription.

Second, experiments have been done using ribosomes from two other Str-R mutants having *strA* alleles characterized by Gorini: one with the *strA1* allele, which restricts suppression of amber mutations most strongly, and the other with the *strA2* allele, which shows only a moderate restriction (2, 3). Fig. 5b

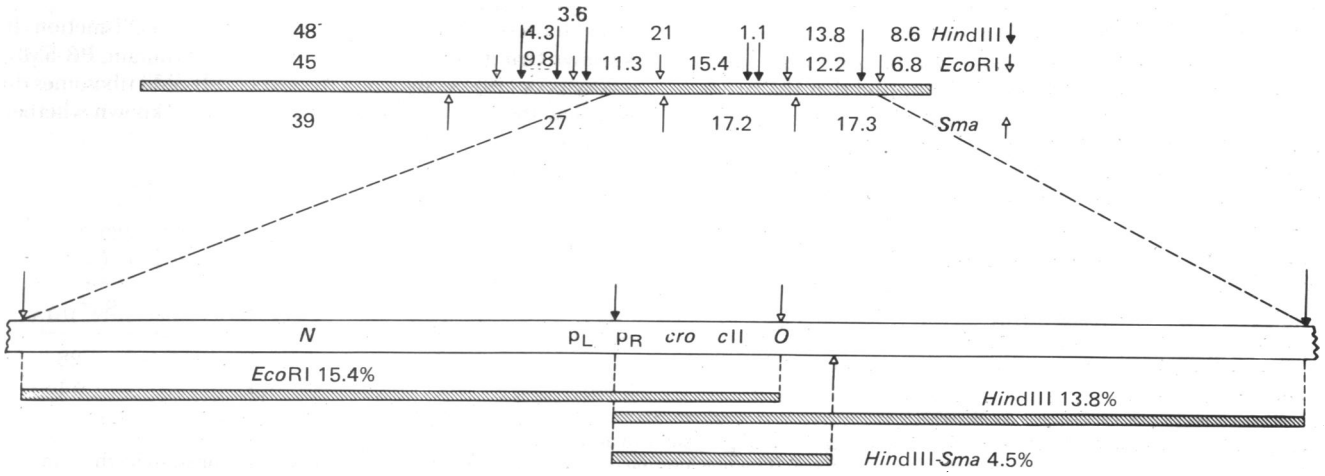

FIG. 3. Cleavage sites of the restriction enzymes *Eco*RI, *Hin*dIII, and *Sma* in the λ genome. The open arrows above the genomes indicate positions of *Eco*RI sites and the distances between these are given in the lower line above the genome. *Hin*dIII sites are indicated by the solid arrows above the genome, and the distances between them are given in the upper line above the genome. *Sma* sites are indicated below the genome. Distances are given in %-λ units. The map is taken from ref. 21. Three DNA fragments used in the experiment described in Fig. 1 are shown.

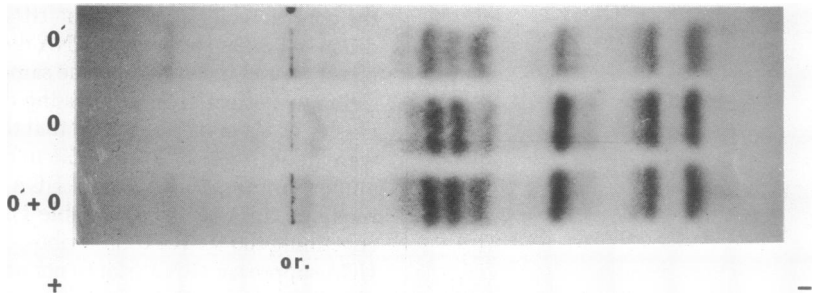

FIG. 4. Autoradiogram of methionine-containing peptides separated by paper electrophoresis. Radioactive proteins O and O′ were cut out from a 10% polyacrylamide gel in which they were well separated, and digested with trypsin. The [^{35}S]methionine-containing peptides were then separated by paper electrophoresis on Whatman 3MM at pH 3.6, using a H_2O/acetic acid/pyridine (90/10/1 vol/vol/vol) buffer. Samples were analyzed either alone (O, O′) or as a mixture of equal amounts of ^{35}S-labeled peptides (O′ + O). The origins where samples were applied are indicated by or.

demonstrates that ribosomes from the *str*^{s} isogenic strain synthesized the read-through O′ protein (slot 1), whereas *strA1* ribosomes failed to synthesize detectable O′ protein (slot 3), similar to the case with Str-R PR-SM3 ribosomes. The *strA2* ribosomes showed greatly reduced, but detectable synthesis of O′ protein (slot 2). Thus, there is a correlation between the degree of restriction of suppression of amber mutations and the extent of restriction of read-through of the chain termination codon.

Restriction of synthesis of Qβ coat read-through protein

It is known that Qβ phage particles contain three capsid proteins. One of them is a product resulting from read-through of the coat protein chain termination codon (15–17). The relative amount of this read-through protein is increased when Qβ phage is grown in cells carrying a UGA suppressor gene (15). Our *in vitro* system containing Str-S ribosomes synthesized both the coat and read-through proteins when Qβ RNA was used as a template. Addition of UGA suppressor tRNA caused a marked increase in the relative amount of the read-through protein (Table 1), confirming the previous results. Use of Str-R PR-SM3 mutant ribosomes abolished the synthesis of read-through protein both in the absence and presence of UGA suppressor tRNA (Table 1).

DISCUSSION AND CONCLUSIONS

Although the function of the λ*O* gene has been extensively studied, its protein product has not been observed before. Among the proteins synthesized *in vitro* in the present λ-DNA-directed system, two radioactive proteins were identified as *O* gene products. It was demonstrated that the larger one (O′ protein) is a read-through protein and is produced with wild-type ribosomes, but not with Str-R mutant ribosomes. Similarly, a radioactive protein (approximate molecular weight 13,000) coded for by the *c*II gene has also been identified (see Fig. 1 and its legend).

Synthesis of proteins coded for by the *O* or *c*II genes in the present *in vitro* system may be useful for physiological studies of the λ phage life cycle. For example, the present system has been used to analyze the determinants of specificity by which the *O* gene controls λ replication (M. Furth and J. Yates, unpublished data).

Both O (and O′) and cII proteins are synthesized using the *Hin*dIII-*Sma* 4.5% DNA fragment as a template. This small DNA fragment carries only three known genes (*cro*, *c*II, and *O*) besides the p_R and p_{re} promoters, and probably does not carry the entire *P* gene. A fourth major protein product synthesized *in vitro* in the presence of the *Hin*dIII-*Sma* 4.5% fragment [compare Fig. 1, slots 5, 6 (and 15, 16); and other experiments not shown] has an approximate molecular weight of 8500, which is similar to the value (9000) for a protein recently purified and believed to be the *cro* gene product (18). Further studies will be required to find out whether or not the unidentified protein with the molecular weight of 8500 is encoded by the *cro* gene.

The significance of the read-through translation of the λ *O* gene mRNA is not clear. Synthesis of the Qβ coat read-through protein takes place *in vivo*, and its role in the *in vitro* reconstitution of infectious phage particles has been demonstrated (17). It is, therefore, possible that synthesis of the *O* gene read-through protein also takes place *in vivo* and that the read-through protein has some unknown physiological function. It is known that growth of phage λ in the Str-R mutant, PR-SM3, is very poor (19). As shown in this paper, PR-SM3 ribosomes do not allow the read-through. However, it is not known whether

Table 1. Restriction of amber suppression and read-through translation *in vitro* by Str-R mutations

Ribosomes	Suppression of *Oam*125 with Su^+_{UAG}*	Read-through of *O* gene (%)†			Read-through of Qβ coat gene (%)†	
		Su°	Su^+_{UAG}	Su^+_{UGA}	Su°	Su^+_{UGA}
PR-C600	60	13	17	56	6	28
PR-SM3	10	<1	<1	<1	<1	<1

* Calculated as [O + O′]/[O + O′ + A] from results shown in Fig. 1, slots 13 and 14.

† Calculated as [O′]/[O + O′] from results shown in Fig. 2. The methionine contents of O, O′, and A proteins were assumed to be the same. Qβ coat and read-through coat proteins were synthesized *in vitro* using [^{3}H]lysine and analyzed on 15% polyacrylamide/sodium dodecyl sulfate gels by fluorography (22). Degree of read-through Qβ coat gene was calculated in the same manner as for the λ O gene. Relative molar amounts of coat and read-through proteins were determined assuming lysine contents of 7 and 20 residues per mole, respectively (16). Su^+_{UAG}: amber suppressor tRNA; Su^+_{UGA}: UGA suppressor tRNA.

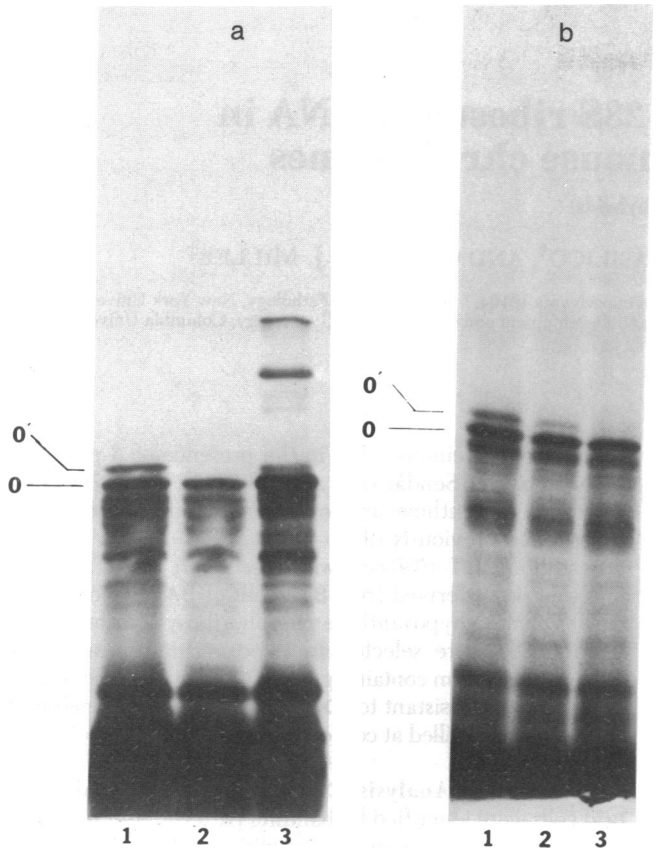

FIG. 5. Translation of λ mRNA *in vitro* and effects of ribosomes from various Str-R mutants. All of the standard assay components except ribosomes were mixed and incubated at 37° for 20 min to allow transcription of λ DNA to occur. DNase was then added (5 μg/ml). After 5 min at 37°, the ribosomes were added as indicated below and incubation was continued for 60 min. The samples were analyzed as in Fig. 1, except that a 10–20% linear gradient of polyacrylamide was used in electrophoresis. Experiments (a) and (b) were done independently. (a) Slot 1, PR-C600 ribosomes; slot 2, PR-SM3 ribosomes; slot 3, PR-C600 ribosomes, but using the standard DNA-dependent "coupled" system. (b) Slot 1, ribosomes from strain L44 (*str*^{s}); slot 2, ribosomes from strain L44-2 (*strA2*); slot 3, ribosomes from strain L44-401 (*strA1*). Two controls were done simultaneously. One was without DNA, the second was with DNase added before the first incubation. In both cases, no significant synthesis of radioactive proteins was observed (except for one weak band at the bottom of the gel), indicating that in this "uncoupled" system proteins were synthesized using λ mRNA as a template and that this λ mRNA was synthesized during the first incubation.

there is any relation between restriction of read-through of *O* gene mRNA and the poor growth of phage λ.

We have demonstrated that the mutational alteration in the PR-SM3 ribosomes abolished read-through translation of λ *O* gene mRNA and *Qβ* coat gene. An alteration in the 30S ribosomal protein S12, the protein coded for by the *strA* gene (1), must be responsible for this effect on read-through (cf. ref. 4). The simplest model to explain the observed restriction of read-through is to assume that there is a competition between the protein chain release factor and one or more aminoacyl tRNAs which misread a chain termination codon at the ribosome "A" (acceptor) site, and that various alterations in S12 caused by Str-R mutations decrease the efficiency of misreading of termination codon(s) by aminoacyl tRNA, leading to restriction of read-through to various extents. It should be noted that a decrease in the degree of misreading in general, as well as a decrease in the efficiency of suppression, by Str-R mutations is known (2), and an important role of the protein S12 in determining the efficiency and/or specificity of codon-dependent tRNA binding to ribosomes has been postulated by various workers (2, 20, 23). Our explanation for the observed effects of Str-R mutations on read-through is in accord with this previous notion. As described in the present work, *strA* alleles display correlation between the strength of restriction of suppression (2) [as well as on the rate of peptide chain elongation (23)] and the strength of restriction of read-through translation. Such a correlation gives further support to the present model to explain the restriction of read-through by Str-R mutations.

We have demonstrated restriction of suppression of amber mutations *in vitro* and read-through of a chain termination codon as a result of Str-R mutations. Availability of such an *in vitro* system has now made it possible to study the molecular mechanisms involved in the role of ribosome structure in the codon-dependent selection of aminoacyl tRNAs as well as other protein factors such as release factors.

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