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Alteration of Ribosomal Protein L6 in Gentamicin-Resistant Strains of *Escherichia coli*. Effects on Fidelity of Protein Synthesis†

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ABSTRACT: The effect of alterations in ribosomal protein L6 of gentamicin-resistant mutants of *Escherichia coli* on in vitro and in vivo polypeptide synthesis was studied. It was found that mutant ribosomes possess altered misreading properties by the following criteria. (1) Poly(U)-dependent incorporation of isoleucine or of a mixture of amino acids in the presence of misreading-inducing aminoglycosides like streptomycin, neomycin, or gentamicin is reduced in extracts from the mutants; this reduction of the misreading is a property of the 50S and not of the 30S subunit or the 100000*g* supernatant fraction. (2) Incorporation of L-histidine into the (normally histidine-less) coat protein from phage R17 or into a protein electrophoretically comigrating with the coat protein is stimulated by gentamicin in the case of wild-type but not with mutant ribosomes. Unlike streptomycin, gentamicin affects R17 RNA dependent polypeptide synthesis by wild-type ribosomes in a multiphasic way which supports the notion of multiple binding sites for the drug. Synthesis on mutant ribosomes on the other hand is monophasicly inhibited. The size classes of translation products made in the presence of gentamicin by wild-type ribosomes are very different from those obtained without antibiotic. (3) The leaky translation of the *argF40* amber codon [Rosset, R., & Gorini, L. (1969) *J. Mol. Biol. 39*, 95] is prevented or reduced by mutations in protein L6, and low concentrations of gentamicin or streptomycin antagonize this restriction, rendering the strains conditionally drug dependent. The different response of ribosomes with L6 or S12 (*strA*) mutations to aminoglycosides suggests that restriction of ribosomal ambiguity by these alterations occurs by different mechanisms and that the L6 alteration changes a parameter of the ribosome function which is involved in the codon recognition process.

In order to obtain information on the mode of action of the gentamicin group of aminoglycoside antibiotics we have previously undertaken an analysis of strains of *Escherichia coli* resistant to gentamicin sulfate (Buckel et al., 1977). It was found that mutants which are moderately resistant to this antibiotic possess at least two mutations which act cooperatively to express resistance. Whereas one class of these mutations interferes with the uptake of the drug by the bacteria (our unpublished results), the other one affects ribosomal properties (Buckel et al., 1977). For several strains an alteration of the 50S subunit protein L6 could be demonstrated. The L6 alteration confers an increase in the level of resistance to all misreading-inducing aminoglycosides, and its action is apparently independent from that of the classical mutations in the *strA* gene (structural gene for ribosomal protein S12) which determine resistance to streptomycin (Strigini & Gorini, 1970; Funatsu & Wittmann, 1972; Wittmann & Wittmann-Liebold, 1974).

The present study deals with the response to gentamicin in vitro of ribosomes from the L6 mutants. Evidence is presented which shows that these mutations can restrict translational ambiguity.

Materials and Methods

Bacterial Strains and Growth Conditions. Escherichia coli strains GE20-8, GS20-10, and GS50-15 are gentamicin-resistant derivatives (Buckel et al., 1977) of the parental strain A19 (thi⁻ met⁻ *rna* λ⁺) (Gesteland, 1966). They possess a mutationally altered ribosomal protein, L6, in addition to a second mutation interfering with gentamicin uptake (Buckel et al., 1977; our unpublished results). Strains TD3 and TD6 were constructed from mutant GS20-10 by P1 transduction; TD3 possesses the wild-type form and TD6 the mutant form of L6. In addition, both contain a streptomycin resistance mutation in ribosomal protein S12 (Buckel et al., 1977). Bacteria were grown at 37 °C either in 250-mL batches in 1-L Erlenmeyer flasks on rotatory incubators or in 10-L

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batches in a 15-L fermenter. The medium consisted of (per 1 L): 8 g of tryptone (Oxoid), 4 g of yeast extract (Oxoid), 8 g of NaCl, 7.3 g of $Na_2HPO_4 \cdot H_2O$, 2.93 g of KH_2PO_4, 2.0 g of NH_4Cl, and 2 g of glucose. The pH was adjusted to 7.4. Growth was monitored by absorption measurements at 546 nm (Eppendorf photometer) or at 420 nm (Bausch and Lomb Spectronic 70); at an A_{546} of 1.0 (flask cultures) or 2.0 (fermenter) growth was stopped by rapid chilling, and the bacteria were collected by centrifugation (4 °C), resuspended in TMNSH buffer (10 mM Tris-HCl, pH 7.5, 10 mM $Mg(OAc)_2$, 30 mM NH_4Cl, 6 mM 2-mercaptoethanol) containing 5 μg/mL of deoxyribonuclease I (purity degree I; Boehringer Mannheim; GmbH), and again sedimented by centrifugation (4 °C). The cell sediment was frozen in a dry ice–ethanol mixture and kept frozen at −70 °C until use.

Preparation of S30 Extracts, Ribosomes, Ribosomal Subunits, and Crude Initiation Factors. For the preparation of S30 extracts the cells were broken by grinding with twice the amount of alumina or, alternatively, they were resuspended in TMNSH buffer (1 mL of buffer per g wet weight) and passed through a French press cell at 6000 psi. Cell debris was removed by two consecutive centrifugations, first for 10 min at 10000*g* and then for 30 min at 30000*g*. The upper four-fifths of the supernatant of the last centrifugation step was dialyzed overnight against TMNSH buffer and used as S30 extract.

70S ribosomes were prepared from the S30 extracts by three consecutive centrifugations for 180 min at 48 000 rpm (2 °C) in a 75 Ti rotor. The upper two-thirds of the supernatant of the first centrifugation step was kept frozen at −70 °C and used as the S100 fraction. The ribosomes were resuspended in TMNSH, freed from aggregates by a brief centrifugation at 30000*g*, frozen in liquid nitrogen, and stored at −70 °C. Ribosomal subunits were prepared by sucrose density gradient centrifugation in a SW27 rotor as previously described (Wittmann et al., 1974). After separation, the Mg^{2+} concentration was adjusted to 10 mM and the subunits were sedimented by centrifugation at 42 000 rpm for 13 h (Ti 75). The sediment was resuspended in TMNSH buffer and the suspension stored frozen (−70 °C) until used.

Preparation of a crude initiation factor fraction was carried out as described by Dubnoff & Maitra (1971) but with omission of the ammonium sulfate precipitation step.

Determination of protein concentration was performed with the Folin phenol procedure (Lowry et al., 1951) employing bovine serum albumin as standard protein. The ribosome concentration was measured spectrophotometrically; an A_{260} value of 15 was taken for a concentration of 1 mg/mL.

R17 RNA Dependent in Vitro Polypeptide Synthesis. The basic procedure used for the determination of R17 RNA dependent in vitro polypeptide synthesis was as described by Funatsu et al. (1972). L-[^{14}C]Valine was present in the incubation mixture at 30 μM and at a specific radioactivity of 55 μCi/μmol; the unlabeled amino acids were present at 0.135 mM each.

When the R17-directed incorporation of a mixture of radioactively labeled amino acids was measured, each assay received 0.5 μCi of a mixture (New England Nuclear, NEN445) of 15 ^{14}C-labeled amino acids. The final concentration of all amino acids was 0.135 mM except for valine, which was again present at 30 μM.

For the determination of the incorporation of L-histidine into the R17 coat protein fraction the incubation was carried out in the presence of L-[^{14}C]valine and L-[^{3}H]histidine. The L-[^{14}C]valine concentration was 30 μM and the specific radioactivity 55 μCi/μmol. The final concentration of L-histidine was 2.5 μM and the specific radioactivity 1863 μCi/μmol. The incubation (30 min at 37 °C) was terminated by chilling in ice, the protein was precipitated by the addition of 10 mL of 100% acetone, and the resulting precipitate was collected by centrifugation at 10000*g* for 10 min. The sediment was dried in vacuo and solubilized by incubating for 3 min at 95 °C in sample buffer (Laemmli & Favre, 1973). The 40-μL aliquots were then applied to a 10–25% exponential gradient sodium dodecyl sulfate–polyacrylamide slab gel. Electrophoresis was carried out at 20 mA and at room temperature until the bromophenol blue marker reached the bottom of the gel. The gels were soaked in 20 mL of 50% trichloroacetic acid for 30 min and then washed twice in deionized water for 30 min each. They were stained with 0.2% of Coomassie Brillant Blue R-250 in 30% methanol and 10% acetic acid, destained by diffusion, and further processed for fluorography as described by Bonner & Laskey (1974) and Laskey & Mills (1975). The bands corresponding to the R17 coat protein as indicated by the fluorograph were cut from the gel and processed in a Packard Sample Oxidizer 306. The ^{3}H and ^{14}C radioactivities were determined in a liquid scintillation spectrometer using Carbosorb II and Permafluor (for ^{14}C) and Monophase 40 (for ^{3}H) as scintillation fluids.

In Vitro Measurement of Aminoglycoside-Induced Translational Misreading. Two test systems were employed for the direct determination of in vitro translational ambiguity. The first consists of the measurement of poly(uridylic acid) [poly(U)]-dependent incorporation of isoleucine into hot trichloroacetic acid precipitable material as described by Rosset & Gorini (1969). Mg^{2+} concentration was 18 mM unless otherwise stated.

In the second assay system "total misreading" was determined according to Davies & Davis (1968). In this case the poly(U)-coded incorporation of radioactivity into hot trichloroacetic acid insoluble material was measured from a mixture of 15 amino acids (Davis et al., 1974) under conditions where the incorporation of the correct amino acid is quenched. The composition of the test system is that described above (Rosset & Gorini, 1969; Piepersberg et al., 1975) except that each sample received 0.5 μCi of a mixture of 15 ^{14}C-labeled amino acids (New England Nuclear, NEN445) and that the assays also contained 20 mM unlabeled L-phenylalanine and L-leucine (Davies & Davis, 1968); no other unlabeled amino acids were added.

Determination of in Vivo Translational Ambiguity. As a measure for translational ambiguity in vivo the ability of the L6 mutations to influence the reading of the leaky *argF40* amber codon (Rosset & Gorini, 1969) was investigated. For this purpose the mutant L6 alleles were transferred by P1 transduction (Piepersberg et al., 1975) into a strain containing the *argF40 argR11* genotype (Rosset & Gorini, 1969). Thirty recombinants of the crosses with each L6 mutant were purified and analyzed for the inheritance of low-level gentamicin resistance (Buckel et al., 1977). Two transductants with this property from each cross were then tested by two-dimensional polyacrylamide gel electrophoresis (Kaltschmidt & Wittmann, 1970) to ascertain that they had obtained the altered L6 from the parental donor strains. In each case a positive correlation was observed. The two transductants were then compared together with control strains for their arginine phenotype on minimal medium plates containing streptomycin sulfate in one of the following concentrations (μg/mL): 0, 2, 5, 10, 50, 100, 200, and 500. The identical screening procedure was carried out on media containing gentamicin sulfate at 0, 0.5, 1, 2, 5,

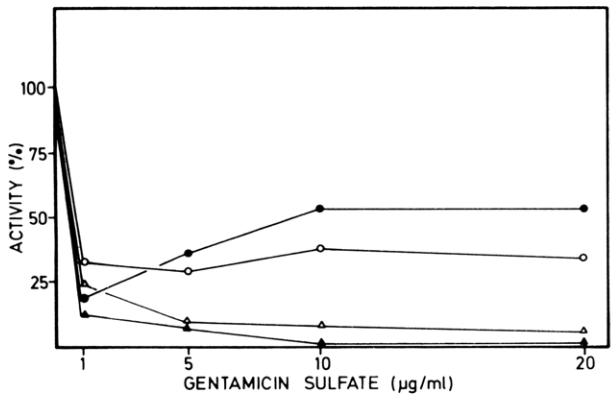

FIGURE 1: Effect of gentamicin on R17 RNA dependent in vitro incorporation of L-[^{14}C]valine (○, △) or of a mixture of 15 ^{14}C-labeled amino acids (●, ▲) into trichloroacetic acid insoluble material by ribosomes (1.7A_{260} units) and S100 from strains A19 (○, ●) and GS50-15 (△, ▲). The 100% values for [^{14}C]valine incorporation are 32 700 cpm for strain A19 and 21 300 cpm for GS50-15; those for the incorporation of the amino acid mixture are 19 500 cpm for strain A19 and 13 050 for GS50-15.

10, 20, 50, and 100 μg/mL. Plates were incubated at 37 °C for 4 days.

Chemicals. L-[U-^{14}C]Valine and L-[2.5-^{3}H]histidine were purchased from the Radiochemical Centre, Amersham. The mixture of uniformly labeled ^{14}C amino acids (NEN445) was obtained from New England Nuclear. Gentamicin sulfate was from Sigma Chemicals and consists of the components C_1, C_{1a}, and C_2. Streptomycin sulfate and neomycin sulfate were from Serva, Heidelberg.

Results

Effect of Gentamicin on the R17 Phage RNA Dependent Protein Synthesis. Figure 1 shows the effect of different concentrations of gentamicin sulfate on R17 RNA coded protein synthesis by ribosomes and 100000*g* supernatant (S100) from the parental strain A19 and from mutant GS50-15 (altered L6 protein). Unlike streptomycin (Modolell & Davis, 1968) gentamicin does not completely inhibit [^{14}C]valine incorporation into hot trichloroacetic acid precipitable material by wild-type ribosomes. After an initial phase of inhibition of amino acid incorporation at antibiotic concentrations lower than 1 μg/mL, the level of [^{14}C]valine incorporation activity stays constant or even rises. At gentamicin concentrations above 200 μg/mL this residual incorporation activity is abolished again (not shown). The biphasic nature of the incorporation is particularly pronounced when 15 of the 20 amino acids were radioactively labeled (Figure 1). The different magnitude of the second phase of incorporation, depending on whether incorporation of a single amino acid or of 15 of them is followed, suggests that misreading occurs in this range of antibiotic concentration since, in the case of the synthesis of "correct" peptides, one should not observe any difference in the relative inhibition level.

The distinct biphasic nature could be plausibly explained by the assumption that more than one drug molecule binds to the ribosome. At low gentamicin concentration binding to high affinity sites could be responsible for the inhibitory phase; further increase of the drug concentration might lead to saturation of other (low affinity) sites, and this could cause the release of inhibition.

Ribosomes from the mutant GS50-15 seem to be more sensitive to inhibition by the drug than those from the wild strain. Incorporation of [^{14}C]valine and of 15 ^{14}C-labeled amino acids is inhibited almost completely; no second phase of incorporation appears at higher drug concentrations.

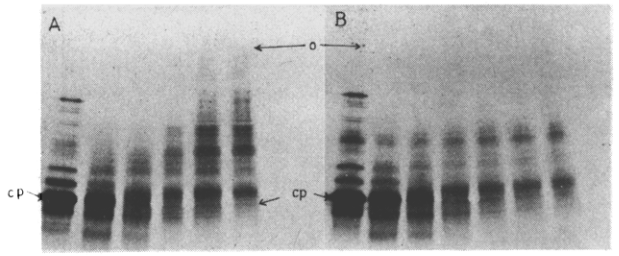

FIGURE 2: Fluorographs of the radioactive products made in the R17 system by ribosomes from strains T/BM-24 (A) (wild type) and T/GS50-15/5 (B) which contains the L6 mutation of strain GS50-15 in an otherwise wild-type genetic background. The assays contained the following concentrations of gentamicin sulfate. (A) From left to right: 0, 0.2, 0.5, 2, 10, 50 μg/mL. (B) 0, 0.2, 0.5, 2, 10, 20, 50 μg/mL. The radioactivity of the samples incubated in the absence of the antibiotic were 17 068 cpm for (A) and 17 680 cpm for (B). The arrows indicate the position of the gel origin (o) and of the coat protein band (cp).

Essentially the same results were obtained when extracts from strains GE20-8 and GS20-10 (two other L6 mutants) were used (not shown).

The fidelity with which R17 RNA is translated by wild-type and mutant ribosomes in the presence of gentamicin is strikingly revealed by autoradiographic analysis of the products. Figure 2A shows the autoradiogram of an SDS slab gel electrophoretic separation of the R17 products synthesized in vitro on wild-type ribosomes and Figure 2B shows those made on mutant ribosomes. The staining of marker protein on the gels indicates that the major radioactive product migrates with coat protein (indicated by arrow). The synthetase and maturation proteins have not been identified among the other bands; some of them may represent imperfectly translated peptides. Proteins of distinct size classes appear (besides a general "smear" of radioactivity) which are not visible in the control. In comparison, most of the products synthesized on mutant L6 ribosomes are the same size as those present in the control; gentamicin seems to decrease their amount. In controls without R17 RNA the addition of gentamicin did not cause the synthesis of any polypeptides visible in the autoradiograms.

The coat protein of phage R17 does not contain any L-histidine residues (Weber & Konigsberg, 1975). The incorporation of L-histidine into a polypeptide migrating to the coat protein position, therefore, is a result of the false incorporation of an amino acid into a defined protein, namely coat protein, or the synthesis of a protein normally containing histidine of different size. Figure 3 shows the results of experiments in which incorporation of L-[^{14}C]valine and L-[^{3}H]histidine into the coat protein band was measured in one and the same assay sample. Gentamicin causes a considerable incorporation of L-histidine (about 1 histidine residue per 37 valine residues) into polypeptides of the size of coat protein when wild-type A19 ribosomes are used but not with those from the L6 mutant GS50-15.

Milanesi & Ciferi (1966) have reported that gentamicin resistance in a mutant of *E. coli* resistant to 20 μg/mL of the drug appears to be a property of the S100 fraction. The result of an experiment in which R17 RNA dependent protein synthesis was measured with mutant ribosomes and wild-type S100 (and vice versa) clearly demonstrated that in the case of our L6 mutants the altered sensitivity to gentamicin is a property of the ribosomal and not of the S100 fraction (experiment not shown).

Effect of Gentamicin Sulfate on the Poly(uridylic acid) Coded Polypeptide Synthesis. In order to investigate whether the L6 mutations indeed alter the translational fidelity, the

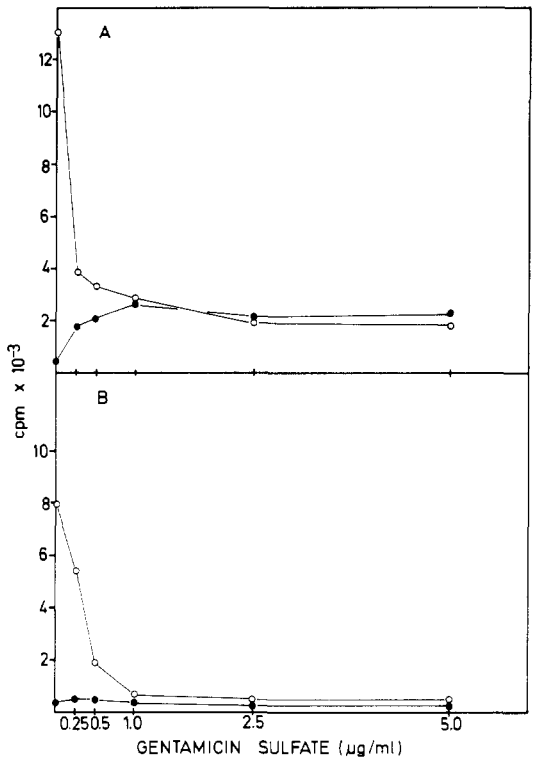

FIGURE 3: Effect of gentamicin on the in vitro incorporation of L-[^{14}C]valine (○) and L-[^{3}H]histidine (●) into R17 coat protein. (A) Ribosomes and S100 from strain A19. (B) Ribosomes and S100 from strain GS50-15. Equal-sized and analogous areas were cut out from the electropherogram in all cases.

Table I: Total Misreading of Poly(U) by Homologous and Heterologous Combinations of 30S and 50S Subunits from Strains A19 and GS20-10

poly(U)	30S[b]	50S[b]	GM[a]	cmp
+	A19	A19	−	5145
−	A19	A19	−	2928
+	A19	A19	+	49960
−	A19	A19	+	8239
+	GS20-10	GS20-10	−	4969
−	GS20-10	GS20-10	−	2503
+	GS20-10	GS20-10	+	13425
−	GS20-10	GS20-10	+	4989
+	A19	GS20-10	−	4532
−	A19	GS20-10	−	2793
+	A19	GS20-10	+	12039
−	A19	GS20-10	+	4142
+	GS20-10	A19	−	6419
−	GS20-10	A19	−	3116
+	GS20-10	A19	+	39850
−	GS20-10	A19	+	6680

[a] GM: gentamicin sulfate, 5 μg/mL final concentration.
[b] Concentrations of the subunits used were 5 A_{260} units of 30S and 8 A_{260} units of 50S. S100 was from wild-type strain A19 throughout.

misreading properties of mutant and wild-type 30S and 50S ribosomal subunits were determined in the "total misreading" assay (Table I). It is evident that misreading is reduced whenever 50S subunits from the mutant are used in homologous or heterologous combination with the 30S subunit. The experiment therefore indicates that gentamicin-induced ribosomal ambiguity is reduced on the mutant ribosomes and that the 50S subunit is responsible for it. The same result was obtained with 30S and 50S subunits from mutant GS50-15 (not shown).

In total misreading experiments it is difficult to differentiate between an actual decrease of misreading and an inhibition

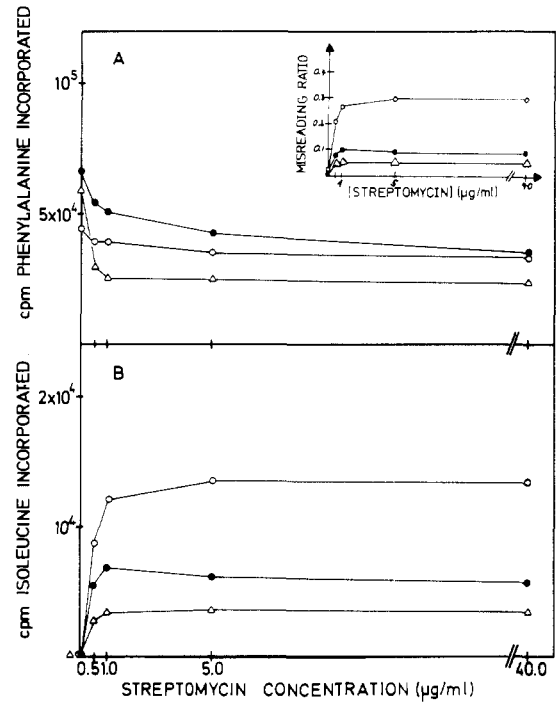

FIGURE 4: Effect of streptomycin sulfate on the poly(U)-dependent incorporation of phenylalanine (A) and isoleucine (B) by S30 extracts from strains A19 (○), GE20-8 (●), and GS50-15 (△). Mg^{2+} was 18 mM. The inset figure shows the misreading ratio calculated as cpm of isoleucine incorporated/cpm of phenylalanine incorporated, both measured in the presence of the respective drug concentration.

of polypeptide synthesis. Therefore, in additional experiments the frequency was determined with which a single amino acid, namely phenylalanine, is replaced by another one, isoleucine, in the poly(U)-directed in vitro system (Rosset & Gorini, 1969) in the presence of an aminoglycoside. Streptomycin was chosen for these studies since it inhibits amino acid incorporation less than gentamicin (see below); as a result the evaluation of the misreading effect is facilitated. The results of a representative experiment are shown in Figure 4 in terms of the absolute incorporation values and in the inset figure as the misreading ratios, i.e., the incorporation of isoleucine relative to that of phenylalanine in the presence of the drug. The plots show that the streptomycin-induced incorporation of isoleucine into polypeptides is lower on mutant ribosomes compared with the wild type.

The absolute level of misreading is highly dependent on the tRNA concentration present in the test system (results not shown). Highest misreading occurs at low tRNA concentration, both with mutant and wild-type ribosomes, a result previously reported by Pestka et al. (1965) and Davies et al. (1965) and interpreted in terms of a less-effective competition of the binding of the false aminoacyl-tRNA by the correct one to the ribosome at low tRNA concentration.

Effect of Gentamicin, Neomycin, and Streptomycin on Misreading by Ribosomes from Streptomycin-Resistant and Gentamicin-Resistant Mutants. The results described so far indicate that alterations of a protein of the 50S ribosomal subunit, namely of protein L6, increase the fidelity of protein synthesis. This property, and also the altered in vivo response to an aminoglycoside antibiotic, has, until now, been described only with mutations in proteins of the small ribosomal subunit (Wittmann & Wittmann-Liebold, 1974). It was therefore important to determine whether the classical *strA* mutations in ribosomal protein S12 can restrict the translational ambiguity imposed by gentamicin. To this end, the effect of

Table II. Effect of L6 Mutations on Translational Ambiguity in Vivo

strain	genotype	arginine phenotype[a] without drug	arginine phenotype[a] with GM[b]	arginine phenotype[a] with SM[b]	in vitro misreading[c] without SM	in vitro misreading[c] with SM
T-BM-24	*argF40 argR11*	±	+ (2–10)	+	1.05 (3.50)	6.94 (19.20)
T-GS20-10/8	*argF40 argR11 rplF10*	∓	+ (2–20)	nt	nt	nt
T-GS50-15/5	*argF40 argR11 rplF15*	–	+ (20–50)	+ (20–50)	0.96 (1.53)	2.06 (4.90)
T-GE20-8/1	*argF40 argR11 rplF8*	–	+ (10–20)	+ (20–50)	0.84 (1.48)	0.76 (1.78)

[a] "+" means full growth on plates overnight, "±" means full growth, "∓" means very poor growth within 4 days at 37 °C, and "–" means no growth within 4 days. [b] Growth in the presence of gentamicin sulfate (GM) or streptomycin sulfate (SM) at the concentrations (μg/mL) given in parentheses; nt = not tested. [c] cpm of [^{14}C]isoleucine incorporation/cpm of [^{14}C]phenylalanine incorporation × 100, measured in the absence or presence of 20 μg/mL of streptomycin sulfate (SM); Mg^{2+} concentration was 14 mM or 20 mM (in parentheses).

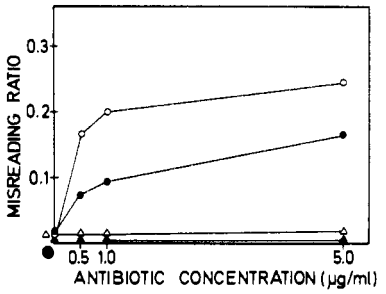

FIGURE 5: Effect of gentamicin sulfate (○, ●) and streptomycin sulfate (△, ▲) on misreading of poly(U) by ribosomes from strains TD3 (○, △) (*strA*^{R} and *rplF*$^+$) and TD6 (●,▲) (*strA*^{R} and *rplF10*). Misreading ratios are calculated according to Figure 4.

different concentrations of the aminoglycosides gentamicin, neomycin, and streptomycin on the poly(U)-dependent incorporation of isoleucine by extracts from the *strA1* (S12) mutant Sm10 (Funatsu & Wittmann, 1972) and the L6 mutants GE20-8 and GS50-15 was studied. It was found that gentamicin and neomycin are able to induce translational ambiguity in all three strains, though to a different degree. In the two L6 mutants used misreading is less than in the streptomycin-resistant strain Sm10. On the other hand, as expected (Rosset & Gorini, 1969; Piepersberg et al., 1975), streptomycin does not cause any increased misreading by Sm10 ribosomes but does induce moderate isoleucine incorporation by extracts from the L6 mutants (experiments not shown). Taken together, the results of these experiments imply that the L6 mutations can generally but only partially counteract the derestriction of translational ambiguity caused by three different aminoglycosides whereas the *strA1* mutation specifically prevents streptomycin-induced misreading in this test system.

The results of these experiments were corroborated by an investigation of ribosomes from strains TD3 and TD6; both strains possess the same streptomycin resistance (*strA*) marker but TD6 carries the mutant L6 protein (from strain GS20-10) whereas TD3 possesses the wild-type L6 form (Buckel et al., 1977). Figure 5 demonstrates that ribosomes from both strains are equally "resistant" to the ambiguity effect of streptomycin; gentamicin, on the other hand, has a much higher effect on misreading by ribosomes from strain TD3 than by those from strain TD6. It is clear from these results that the ability of the L6 mutation to restrict misreading in vitro is independent of the presence or absence of a streptomycin resistance mutation in ribosomal protein S12.

Effect of the L6 Mutations on Translational Ambiguity in Vivo. All the tests for translational ambiguity employed so far have demonstrated that the L6 mutations are able to restrict ambiguity in vitro. In order to study whether this also occurs in vivo we have employed the *argF40 argR11* mutant combination described by Rosset & Gorini (1969) which measures the efficiency by which a leaky amber codon (*argF40*) is translated by ribosomes in vivo. The L6 mutations were transferred into the *argF40 argR11* genetic background by P1 transduction (Piepersberg et al., 1975). The resulting transductants were tested for their arginine phenotype in the absence and in the presence of different concentrations of streptomycin and gentamicin. Table II shows that all three L6 mutations restrict the translation of the leaky *argF40* mutation, those from strains GE20-8 and GS50-15 completely, that from mutant GS20-10 only partially. The leakiness or restriction of translation can be overcome by the incorporation into the medium of either gentamicin or streptomycin at the concentration range indicated in Table II, i.e., the strains are conditionally gentamicin or streptomycin dependent. Concomitant determination of the misreading level in extracts of these strains by the poly(U)-dependent isoleucine incorporation (Rosset & Gorini, 1969; Piepersberg et al., 1975) demonstrates (Table II) that this restriction is also paralleled by reduced error frequency in vitro.

Discussion

We have previously shown that mutants of laboratory strains of *E. coli* which have become moderately resistant to the gentamicin group of aminoglycosides can be isolated by stepwise exposure to increasing concentrations of the drug (Buckel et al., 1977). The resistance generated is not specific for the gentamicins but generally decreases the sensitivity to all misreading-inducing aminoglycosides. Genetic and biochemical analysis revealed that two mechanisms are almost exclusively involved in this process; they cooperatively increase the minimal inhibitory concentrations. One is a mutation interfering with the uptake of the drug; commonly, this defect is introduced upon the first exposure step (unpublished results). The detailed biochemical analysis of the mechanisms by which aminoglycosides are transported into Gram-negative bacteria reveals that many different sites can be altered in order to introduce a block into the transport process (Bryan & van den Elzen, 1977; Thorbjarnardóttir et al., 1978).

The second mutation is ribosomal in nature, thereby altering the target of the antibiotic. The ribosomal alteration of three of the resistant strains was identified as a mutationally changed ribosomal protein, L6. Transduction and reversion analysis revealed a clear correlation between the alteration of protein L6 and gentamicin resistance. A thorough genetic analysis of mutants of *E. coli* resistant to kanamycin by Eggertsson and co-workers (Thorbjarnardóttir et al., 1978) has shown that the properties of mutants obtained with this antibiotic are very similar to those found in the case of the gentamicin-resistant strains: mutations affecting drug transport and ribosomes cooperate to increase resistance. In the kanamycin-resistant mutants, however, different ribosomal proteins than L6 may be altered.

It was the principal aim of this study to investigate whether the alteration of ribosomal protein L6 changes the response of the ribosome to the antibiotic. Wild-type ribosomes were shown to be affected by gentamicin in their R17 RNA dependent protein synthesis activity in a different way than by streptomycin; this is in accordance with results reported by Davies et al. (1969) (for review, see Pestka, 1977). Whereas streptomycin causes a complete cessation of amino acid incorporation, gentamicin, after an inhibitory phase at low concentrations, stimulates incorporation at higher concentrations. Any one of the following mechanisms could be responsible for this finding: (a) gentamicin is a less potent blocker of initiation than streptomycin; (b) gentamicin reverses the blockade of ribosomes caused by antibiotic molecules bound at other ribosomal sites; and (c) gentamicin preferentially induces "overreading" of termination signals, thus causing the ribosome to remain associated with mRNA for a longer time. The analysis of the products made in the presence of the antibiotic at any rate shows that under this condition almost no polypeptides of the correct size are made in the wild-type system, which could be the result of each or both of mechanisms b and c. The multiphasic effect of gentamicin on the R17 RNA directed protein synthesis has been analyzed in detail by Tai & Davis (1979), and a similar conclusion was reached by them. The screening of a number of aminoglycosides for their ability to affect protein synthesis in a multiphasic way revealed that they differ greatly in this property (Zierhut, Piepersberg, & Böck, unpublished results).

In this connection it has to be emphasized that the multiphasic inhibition supports the conclusion reached by Davies & Davis (1968) in their comparative analysis of the misreading effects of different aminoglycosides, namely, that gentamicin binds to at least two sites at the ribosome. They have assumed that one site might be responsible for inhibition of chain elongation and another for the induction of misreading. The result that mutant L6 ribosomes completely lack the secondary incorporation phase might reflect the loss of the gentamicin binding site responsible for the induction of misreading.

The in vitro and in vivo data presented establish that the L6 mutations of the gentamicin-resistant mutants predominantly seem to alter the misreading response of the respective ribosomes. The restriction of translational ambiguity exerted is different in its strength for the three mutants studied. Whereas the mutation of strain GS20-10 cannot completely prevent the leaky translation of the *argF40* amber codon, the mutations of strains GS50-15 and GE20-8 can restrict this ambiguity to an extent which does not allow growth without arginine any longer. It is interesting in this connection that the alterations in protein L6 of the latter two strains seem to be very similar to each other and different from that of GS20-10 as judged by carboxymethylcellulose column chromatography (Buckel et al., 1977). The in vivo restrictive effect may be overcome by the addition of certain concentrations of either streptomycin or gentamicin to the medium. On minimal medium *argF40* strains which carry one of the L6 mutations are thus conditionally streptomycin or gentamicin dependent. The strength of restriction, therefore, is moderate and resembles that of the *strA40* mutations of ribosomal protein S12 (Rosset & Gorini, 1969). The finding of an alteration of a 50S subunit protein in gentamicin-resistant strains is unusual since aminoglycoside resistance mutations at the ribosomal level have always been correlated with a mutation in a 30S subunit protein (for review see Benveniste & Davies, 1973; Gorini, 1974; Wittmann and Wittmann-Liebold, 1974). Especially, however, the observation that a 50S subunit protein can influence the fidelity of translation is striking. One possible explanation for this finding could be that the L6 mutation does not affect translational fidelity per se but via pleiotropic 30S–50S ribosome interactions (Apirion & Saltzman, 1974; reviewed by Pestka, 1977). This possibility, however, seems improbable because the L6 mutation exerts its effect independent of whether or not the 30S subunit harbors a restrictive *strA* mutation (Figure 5).

The L6 mutations generally reduce the misreading induced by different aminoglycosides including also streptomycin; since streptomycin is known to bind in a 1:1 stoichiometric ratio to the 30S subunit (Chang & Flaks, 1972; Schreiner & Nierhaus, 1973; Lando et al., 1976), the results may implicate the L6 mutations causing a kinetic (Ninio, 1974; Hopfield, 1974) or a conformational (Kurland et al., 1975) alteration of the ribosome, thereby changing parameters which might be directly participating in the codon recognition process. In this connection it is interesting that protein L6 has been shown to be part of the aminoacyl-tRNA binding site and to be associated with the EF-G and EF-Tu binding site (Pongs et al., 1974).

In contrast to the altered misreading response, the inhibitory effects on polypeptide synthesis by gentamicin are not alleviated in the mutants; in fact, mutant ribosomes are apparently more sensitive to the antibiotic than those from the wild type. A definite answer to the question why the L6 mutations increase resistance in vivo cannot be given at this time. Two main possibilities can be seen. First, the potency of the aminoglycosides to cause misreading might be involved in their bactericidal activity (Gorini, 1974). If there is such a connection and, in addition, if the bactericidal effect and not the inhibitory effect determines the minimal "inhibitory" concentration, it could well be that a decrease in ambiguity results in the increase of drug resistance as is observed (Buckel et al., 1977). On the other hand, we could observe recently that the L6 mutation alone drastically impairs gentamicin uptake, a finding which is in accordance with the hypothesis that ribosomes play an essential role in aminoglycoside transport (Bryan & van den Elzen, 1977). A similar effect was reported by Thorbjarnardóttir et al. (1978) for kanamycin-resistant strains. Both of these mechanisms—the purely ribosomal one or the connection between the alteration of the target site and drug uptake—can explain why resistance to all misreading-inducing aminoglycosides is increased (Buckel et al., 1977).

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Triphasic Concentration Effects of Gentamicin on Activity and Misreading in Protein Synthesis†

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ABSTRACT: Gentamicin is shown to exert a triphasic concentration effect on peptide synthesis in vitro with natural messengers. Low concentrations (up to 2 μM) caused slowing and a decrease in total synthesis, but little misreading (assayed with extracts lacking Glu-tRNA); the inhibition was greater with an initiating system (with phage RNA as messenger) than with pure chain elongation on purified endogenous polysomes of *Escherichia coli*. Moderate concentrations (up to 100 μM) slowed synthesis less, markedly increased its duration in the noninitiating system, and strongly stimulated misreading; at optimal concentrations total synthesis was even greater than normal. Moreover, with phage RNA these concentrations increased the synthesis of large polypeptides. We conclude that binding of gentamicin to its first site causes inhibition but little misreading; binding to additional site(s) partly reverses the inhibition by first-site binding and markedly stimulates misreading, and the misreading appears to favor "readthrough" of termination codons. In the third phase (>100 μM) synthesis is slowed again but the pattern of misreading does not appear to be altered; this effect need not involve a specific further action on the ribosome.

Among the aminoglycoside antibiotics that inhibit protein synthesis and can also cause extensive misreading (Davies et al., 1965; reviewed by Pestka, 1977), streptomycin (Str)[1] has been studied most extensively. Its blockade of initiating ribosomes and its slowing and misreading of ribosomes already engaged in chain elongation are identical in their concentration range (reviewed by Davis et al., 1974; Wallace et al., 1978). These actions are consistent with the evidence that ribosomes

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[1] Abbreviations used: IF, initiation factors; Gm, gentamicin C mixtures containing C_1, C_{1a}, and C_2; Str, streptomycin; $NaDodSO_4$-gel electrophoresis, polyacrylamide gel electrophoresis containing 0.1% sodium dodecyl sulfate in 0.1 M phosphate buffer; ts, temperature sensitive.

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