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Ribosomal RNA and protein mutants resistant to spectinomycin

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We have compared the influence of spectinomycin (Spc) on individual partial reactions during the elongation phase of translation *in vitro* by wild-type and mutant ribosomes. The data show that the antibiotic specifically inhibits the elongation factor G (EF-G) cycle supported by wild-type ribosomes. In addition, we have reproduced the *in vivo* Spc resistant phenotype of relevant ribosome mutants in our *in vitro* translation system. In particular, three mutants with alterations at position 1192 in 16S rRNA as well as an *rpsE* mutant with an alteration of protein S5 were analysed. All of these ribosomal mutants confer a degree of Spc resistance for the EF-G cycle *in vitro* that is correlated with the degree of growth rate resistance to the antibiotic in culture.

Key words: elongation factor G/ribosomes/spectinomycin

Introduction

Spectinomycin (Spc) is an aminoglycoside antibiotic that inhibits translation (Anderson *et al.*, 1967; Burns and Cundliffe, 1973; Gale *et al.*, 1981). The identification of Spc-resistant (SpcR) mutants with alterations either in ribosomal protein S5 (Bollen *et al.*, 1969), or at position 1192 of the 16S rRNA (Sigmund *et al.*, 1984; Makosky and Dahlberg, 1987) indicates that Spc's site of action is the ribosome. Nevertheless, the antibiotic and its associated resistance mutants have not received the attention that has been accorded its relative, streptomycin. One reason for this relative neglect is that the initial studies of the mode of action for Spc did not lead to a clear identification of the translation step that is inhibited by the antibiotic (Anderson *et al.*, 1967; Burns and Cundliffe, 1973; Gale *et al.*, 1981).

The existence of *SpcR* mutants with structural changes in either ribosomal protein or in rRNA prompted us to reinvestigate the mode of action of the antibiotic with the aid of improved *in vitro* techniques (Ehrenberg *et al.*, 1989a). A relatively straightforward pattern has emerged from these studies. Thus, we find a pronounced and selective influence of Spc on the interaction of elongation factor G (EF-G) with the wild-type ribosome. Furthermore, both a mutant variant of S5 and those of position 1192 in 16S rRNA protect to varying degrees the EF-G cycle from the inhibitory effect of Spc during polypeptide elongation.

Results

Inhibition of ribosomes

When all other components required for elongation are in excess, the rate of polypeptide synthesis is limited by the maximum rate of ribosome function. Figure 1 shows the influence of varying Spc concentrations on the elongation rates by such a ribosome-limited system containing wild-type or mutant ribosomes. Here we observe that maximum inhibition of wild-type ribosomes from strain 017 is reached at 10 μg/ml Spc where the elongation rate is reduced to 40% of its normal rate. In contrast, the ribosomes from strain UD10F3 (*rpsE14*) are unaffected by the same concentration of antibiotic.

Likewise, the wild-type ribosomes from strain US634 pKK 1192C (the wild-type) are inhibited nearly 2-fold by Spc. However, the ribosomes from US634 pKK1192U also are slightly inhibited (20%) by the antibiotic. As pointed out below, this effect of antibiotic on nominally resistant ribosomes can be attributed to the presence of a small fraction (~25%) of wild-type species in the ribosome population extracted from strain US634 pKK1192U.

A more detailed description of the response of wild-type and mutant ribosomes to Spc is summarized in Figure 2 and Table I. Here the translation system has been arranged with fixed ribosome levels and excess amounts of all other components except for EF-G, which is present in varying concentrations. Then the rates of elongation are measured as a function of the EF-G concentration in the presence and absence of Spc (100 μg/ml). As can be seen in Figure 2A, the influence of antibiotic on the maximum rate of ribosome function as well as on the association kinetics for EF-G with the ribosome are revealed in such a titration.

These same kinetic data can be replotted in the form of an Eadie–Hofstee plot (Figure 2B) in order to compare the influence of antibiotic on the k_{cat}, K_M and what we refer to as the R-factor (k_{cat}/K_M). The values for these kinetic parameters are summarized in Table I. Here, we observe that the k_{cat} values for both of the wild-type ribosomes are inhibited to 40% of their normal values by antibiotic (100 μg/ml). The three mutants at position 1192 of 16S rRNA are relatively resistant to Spc. They function at maximum rates between 69 and 84% of the uninhibited values. Similarly, the S5 mutant translates at ~90% of its normal maximum rate in the presence of 100 μg/ml Spc.

The influence of the antibiotic on the effective association kinetics for the EF-G–ribosome complex (the R-factor) is comparable to its influence on the k_{cat} value (see Table I). This suggests that the association of EF-G and ribosomes may be disturbed by Spc. In contrast, when the ribosome limited assay is performed with a constant excess of EF-G and a varying concentration of EF-Tu, a somewhat different pattern is observed (Table II). Here, as expected, the same sorts of effects of antibiotic on the k_{cat} value are observed, but the R-factor (k_{cat}/K_M) describing the association kinetics

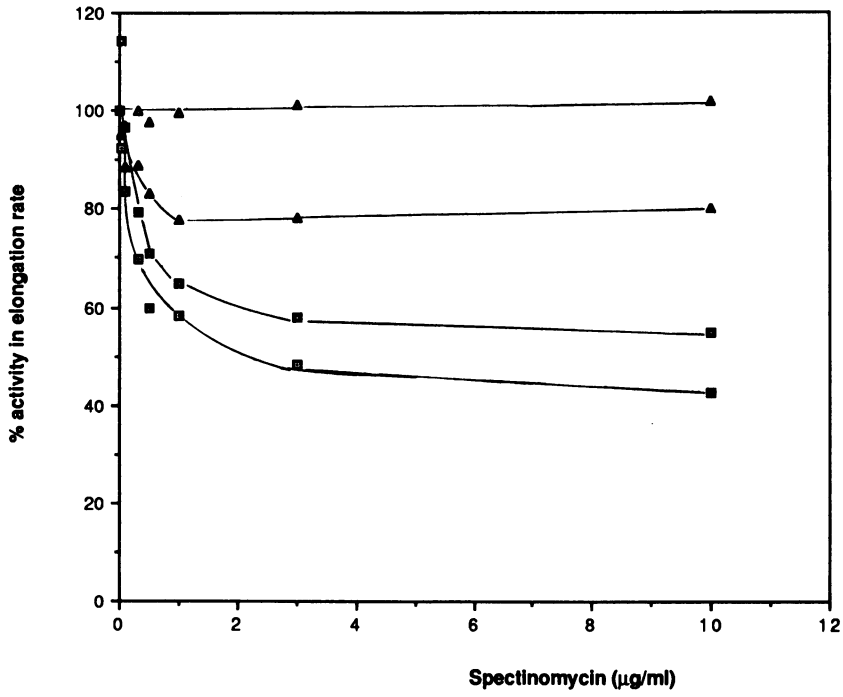

Fig. 1. Inhibition under ribosome-limited conditions of elongation rate at varying concentrations of spectinomycin with 10 pmol active ribosomes in 100 μl as follows. (□) 017 (wt), (■) US634 pKK 1192C (wt), (▲) US634 pKK 1192U (containing 75% U) and (△) UD10F3 (*rps*E14) ribosomes. The ribosomal elongation rates (Phe incorporation/s/active ribosomes), measured in the absence of the antibiotic, are taken as 100% and correspond to 5.07 s^{-1}, 3.15 s^{-1}, 3.91 s^{-1} and 4.18 s^{-1} respectively.

of EF-Tu and ribosomes is less responsive to antibiotic than is the R-factor describing the EF-G–ribosome interaction (i.e. 25% versus 60% inhibition of wild-type). Taken together, the data in Tables I and II suggest that the functional association of EF-G and ribosomes is more sensitive to the drug than that of EF-Tu and ribosomes.

Inhibition of the EF-G cycle

Spc titrations have been carried out for the EF-G cycle in translating systems containing ribosome with each of the four variants of 16S rRNA at position 1192 (Figure 3). This experiment is analogous to that in Figure 1, but here the rate limiting component in the system is EF-G. We observe nearly a 10-fold inhibition of the rate of EF-G function with wild-type ribosomes (1192 C) and roughly a 5-fold inhibition of the EF-G rate with the 1192 U as well as 1192 G variant in the presence of antibiotic.

In order to explore these effects in more detail we have carried out ribosome titrations of the translation system with a fixed concentration of EF-G in the presence and absence of 100 μg/ml Spc. The data were analysed with the aid of Eadie–Hofstee plots and the relevant kinetic parameters are summarized in Table III. We find that the k_{cat} of the EF-G cycle supported by both wild-type ribosomes is depressed > 10-fold by the antibiotic. The S5 variant (*rps*E14) is quite resistant to the effects of Spc and supports an EF-G k_{cat} in 100 μg/ml Spc that is 70% of the normal rate. The rRNA mutants are not nearly as resistant; thus, the U and G variants of 16S rRNA position 1192 support only 25% of the normal EF-G k_{cat} in the presence of Spc. This greater sensitivity to antibiotic for the RNA mutants is only party due to the presence of wild-type species in the relevant ribosome preparations (see below).

Corresponding kinetic data for the EF-Tu cycle were obtained from the analogous ribosome titrations of a translation system with a fixed EF-Tu concentration. The data, summarized in Table IV are not as complete as that for EF-G because the most important point established by these titrations is that the k_{cat} of the EF-Tu is insensitive to Spc at 100 μg/ml even when only wild-type ribosomes are present.

Discussion

One of the practical problems that we meet in the study of rRNA mutants is the heterogeneity of the ribosomes produced by bacteria carrying a variant form of rRNA on a plasmid. We have measured the fraction of wild-type 16S rRNA in our nominally mutant preparations and we have used this information to calculate hypothetical kinetic values for pure mutant ribosomes as described in Materials and methods. Some of these calculations are presented in Table III from which it can be seen that the corrections have only a marginal influence on our results because in all cases we have been using preparations in which most of the ribosomes are mutant (60–80%).

There is little doubt that the EF-G cycle is the most vulnerable step in our elongation assay in the presence of Spc. Thus we observe little influence of the antibiotic on the EF-Tu ribosome interaction, which is in stark contrast to the profound inhibition of the EF-G cycle caused by the antibiotic. Furthermore, when the ribosomes are saturated with elongation factors, we obtain only a modest inhibition of elongation. This result suggests, among other things, that an elongation factor-independent function of the ribosome, such as peptide bond formation, is not the main target of the antibiotic.

The modest inhibition of the elongation rate we observed

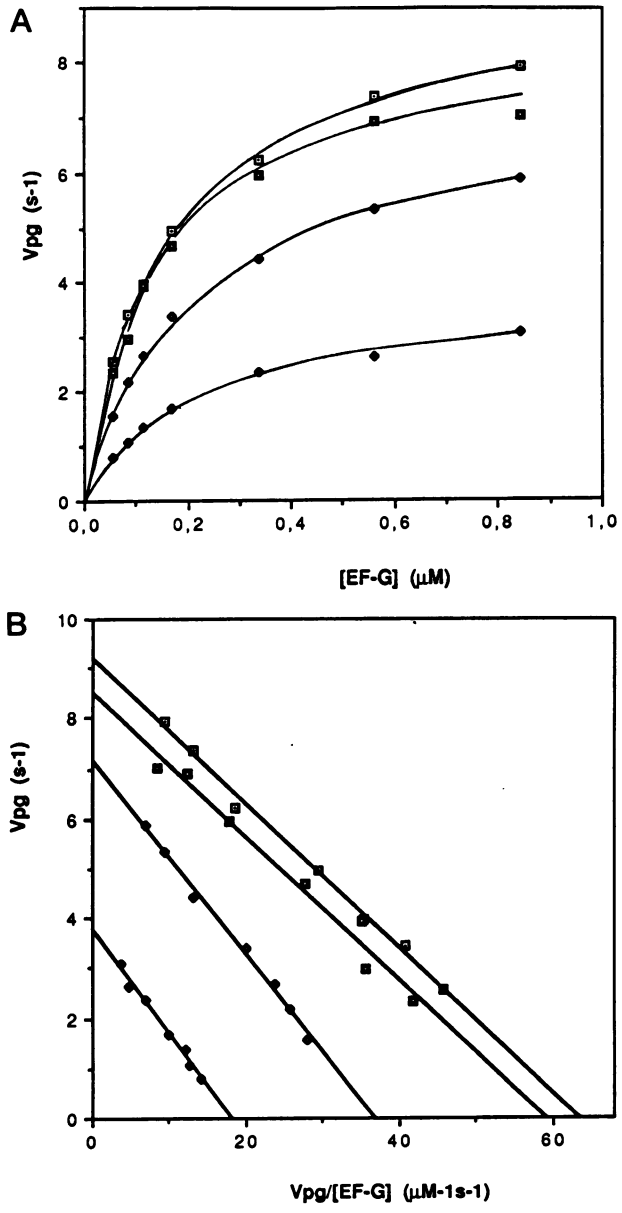

Fig. 2. EF-G titrations at fixed ribosome concentrations (Materials and methods) in the absence and presence of 100 μg/ml spectinomycin (Spc). A representative experiment for the data is summarized in Table I. The rate of poly(Phe) synthesis per active ribosome (Vpg) is plotted versus [EF-G] or Vpg/[EF-G] respectively in linear (**A**) and in Eadie–Hofstee (**B**) plots. 1192C (wt) (□), 1192U (■), 1192C + Spc (◆), 1192U + Spc (◇).

when ribosomes are rate limiting *in vitro* is not commensurate with the strong inhibition of the wild-type bacterial growth rate by the antibiotic. On the other hand, when EF-G is rate limiting *in vitro* not only is the inhibition of translation marked, but there is a quantitative similarity between the individual response of the different rRNA variants to titration with Spc and that seen for the growth rates of the mutants at different antibiotic concentrations (Figure 2 and Makosky and Dahlberg, 1987). These observations are consistent with the interpretation that the mutant ribosomes are not kinetically saturated by EF-G *in vivo* and, as a consequence, the maximum rate of EF-G function limits translation by the mutant bacteria in the presence of Spc. Although it is too early to be certain, we

Table I. Kinetic parameters for the ribosomal elongation cycle at varying concentrations of EF-G

Ribosomes	k_{cat} (s^{-1})		K_M (μM)		k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	
	−spc	+spc	−spc	+spc	−spc	+spc
US634 pKK1192C (wt)	9.2	3.8	0.14	0.21	64	18
US634 pKK1192A	8.7	6.0	0.16	0.22	55	28
US634 pKK1192U	8.5	7.2	0.14	0.20	59	37
US634 pKK1192G	8.4	6.8	0.14	0.18	59	37
017 (wt) UD10F3	5.7	2.4	0.13	0.15	43	16
(*rps*E14)	4.5	4.1	0.12	0.14	36	29

Data were obtained from EF-G titrations at fixed (0.1 μM active) ribosome concentrations (Materials and methods) in the absence and presence of 100 μg/ml spectinomycin (Spc). The rate of poly(Phe) synthesis per active ribosome (Vpg) is plotted versus Vpg/EF-G in the Eadie–Hofstee plots. The intercept at the ordinate is the k_{cat} for the ribosome cycle (p) and the intercept at the abscissa is the effective association rate constant (Rpg) of EF-G to ribosomes (k_{cat}/K_M).

Table II. Kinetic parameters for the ribosomal elongation cycle at varying EF-TU concentrations in the absence and presence of 100 μg/ml spectinomycin (Spc)

Ribosomes	k_{cat} (s^{-1})		K_M (μM)		k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	
	−spc	+spc	−spc	+spc	−spc	+spc
US634 pKK1192C (wt)	12	3.7	0.60	0.25	20	15
US634 pKK1192U	12	8.3	0.73	0.55	17	15
UD10F3 (*rps*E14)	11	9.4	0.57	0.53	20	18

Data were obtained from EF-TU titrations at fixed (0.1 μM active) ribosome concentrations (Materials and methods). The kinetic constants were calculated from Eadie–Hofstee plots where the rate of poly(Phe) synthesis per active ribosome(Vpt) is plotted versus Vpt/EF-Tu. The intercept at the ordinate is k_{cat} for the ribosome cycle (p) and the intercept at the abcissa is k_{cat}/K_M, the effective association rate of ternary complex to the ribosome (Rpt).

suspect that this is also so in the absence of Spc. Indeed, there is already evidence that EF-Tu ternary complex concentrations normally limit translation *in vitro* (Kurland and Ehrenberg, 1987).

Unfortunately, we have failed so far to detect an influence on EF-G function of the *SpcR* ribosome mutants in the absence of antibiotic. Strictly speaking, this means that our data show that S5 and position 1192 in 16S rRNA influence EF-G function only in the presence of Spc. Nevertheless, it would be a genuine surprise if it turns out that these same ribosomal domains are not normally involved in EF-G function.

Materials and methods

Chemicals

^3H- and ^{14}C-labelled amino acids were obtained from Amersham (UK). ^{35}S-labelled dATP was a product of NEN. Non-labelled amino acids,

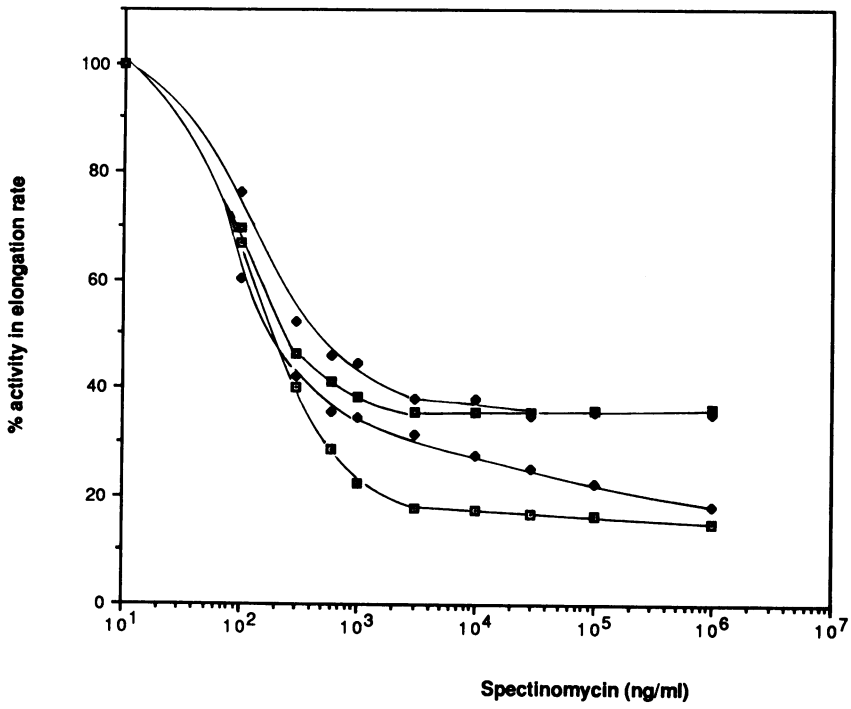

Fig. 3. The inhibition of the elongation rate with EF-G limiting concentrations. The 1192 variants of US634 ribosomes were all present in 100 μl at 70 pmol with rate limiting EF-G (3 pmol) as follows: (□) 1192C (wt), (◆) 1192A (62.8% A), (■) 1192U (66.9% U) and (◇) 1192G (80.7% G). The ribosomal elongation rates (Phe incorporation/s/active ribosomes) measured in the absence of the antibiotic are taken as 100% and correspond to 0.93 s^{-1}, 1.23 s^{-1}, 0.99 s^{-1} respectively.

Table III. Kinetic parameters for the EF-G cycle

Ribosomes	k_{cat} (s^{-1})		K_M (μM)		k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	
	−spc	+spc	−spc	+spc	−spc	+spc
US634 pKK1192C (wt)	48	4.6	0.77	0.14	63	32
UD634 pKK1192A	46	8.5 (13)	0.79	0.21 (0.28)	58	41 (46)
US634 pKK1192U	53	13 (31)	0.92	0.29 (0.61)	58	45 (58)
US634 pKK1192G	52	12 (16)	0.79	0.23 (0.27)	66	53 (56)
O17 (wt)	78	6.4	1.59	0.33	49	20
rpsE14	59	42	1.20	0.99	49	43

Data were obtained from titrations with ribosome mutants at a fixed concentration of EF-G (0.03 μM) (Materials and methods) in the presence and absence of 100 μg/ml spectinomycin (Spc). The rate of poly(Phe) synthesis per EF-G (Vg) is plotted versus Vg/active ribosomes in Eadie–Hofstee plots. The intercept at the ordinate is k_{cat} for the EF-G cycle (g) and the abscissa is the effective association rate constant (Rgp) of EF-G to ribosomes (k_{cat}/K_M). The numbers in parenthesis are calculated for mutant ribosome mixtures containing 62.8% 1192A, 66.9% 1192U and 80.7% 1192G respectively, as described in Materials and methods.

phosphoenol pyruvate (PEP), GTP, putrescine, spermidine, myokinase (EC 2.7.4.3) were purchased from Sigma (St. Louis). Pyruvate kinase (EC 2.7.1.40.) is a product of Boehringer (Mannheim). Polyuridylic acid and ATP were obtained from Pharmacia (Uppsala). Spectinomycin is a product of Upjohn Company (Kalamazoo, Michigan).

Strains and plasmids

The wild-type *Escherichia coli* strain 017 (Olsson and Isaksson, 1979) and its spectinomycin resistant (*rps*E14) derivative UD10F3 are described by Andersson *et al.* (1986). The *E.coli* wild-type strain US634 [*ara argE(UAG)* Δ (*lacproB*) *nalA recA srl thi*] is a derivative of XAc (Coulondre and Miller, 1977). It was constructed and kindly provided by M.Rydén and has been used as the plasmid host.

pKK3535 (Brosius *et al.*, 1981), containing the entire *rrnB* operon of *E.coli* in pBR322 and mutant plasmids containing A, G or U in place of C at residue 1192 in 16S rRNA in pKK3535 have been described earlier (Makosky and Dahlberg, 1987). Purification and transformation of plasmids into US634 were performed as described by Maniatis *et al.* (1982). Following transformation colonies containing wild-type and 1192 mutant plasmids were

Table IV. Kinetic parameters for the EF-Tu cycle

Ribosomes	k_{cat} (s^{-1})		K_M (μM)		k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	
	−spc	+spc	−spc	+spc	−spc	+spc
US634 pKK1192C (wt)	19	19	1.3	1.8	15	10
US634 pKK1192U	20	19	1.6	1.7	12	12

Data were obtained from ribosome titrations at fixed EF-Tu concentration (0.1 μM) (Materials and methods) with or without 100 μg/ml spectinomycin (Spc). Kinetic constants were determined from Eadie–Hofstee plots where the rate of poly(Phe) synthesis per EF-Tu (Vt) is plotted versus Vt/active ribosome. The intercept at the ordinate is k_{cat} for the EF-Tu cycle (t) and the intercept at the abscissa (k_{cat}/K_M) is the effective association constant of ternary complex to ribosomes (Rtp).

grown on agar plates containing 200 μg/ml ampicillin and 50 μg/ml spectinomycin; they were restreaked repeatedly prior to preparing ribosomes.

Purifications and preparations

The cells were grown in LB medium supplemented with 0.4% glucose. 200 μg/ml ampicillin was added for plasmid containing strains. The cells were harvested in late exponential phase. They were washed with polymix buffer (Jelenc and Kurland, 1979): 5 mM magnesium acetate, 5 mM potassium phosphate (pH 7.3), 95 mM potassium chloride, 5 mM ammonium chloride, 0.5 mM calcium chloride, 1 mM spermidine, 8 mM putrescine and 1 mM 1,4-dithioerythritol and were stored at −80°C prior to use.

Ribosomes were purified according to Jelenc (1980): following 35% ammonium sulfate precipitation, the supernatant of the cell lysate was passed through a Sephacryl S-300 column (Pharmacia) in polymix buffer containing 500 mM ammonium chloride. The ribosome peak was pooled and precipitated with 10% PEG 6000, dissolved and dialysed extensively in polymix buffer before storage at −80°C.

Purification of the elongation factors EF-Tu, EF-Ts and EF-G as well as Phe-tRNA synthetase have been described by Ehrenberg *et al.* (1989a) and in the references therein. The tRNA bulk preparations were made from *E.coli* MRE 600 cells and purified on DEAE−celluose (DE-52, Whatman) according to Kelmers *et al.* (1971); $tRNA^{Phe}$ was partialy purified on BD cellulose (Boehringer) according to Gillam *et al.* (1967). N-Acetyl-Phe-$tRNA^{Phe}$ was prepared as described by Wagner *et al.* (1982).

Quantitation of mutant rRNA in purified ribosomes

Purified ribosomes were diluted in TE buffer (10 mM Tris−HCl pH 7.4, 1 mM EDTA pH 8.0), then they were extracted four times with phenol and precipitated with ethanol. After washing with 70% ethanol the rRNA pellet was dissolved in TE buffer and sequenced. A deoxyoligonucleotide primer complementary to nucleotides 1240−1224 was used for primer extension by AMV reverse transcriptase (Promega) as described by Moazed *et al.* (1986). Sequencing was done using the dideoxy method of Sanger *et al.* (1977) with ^{35}S[dATP]. The products of the reaction were separated electrophoretically on 8% polyacrylamide gels containing 8 M urea in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA pH 8.0) for 90 min at 2500 V at 58°C. After 24 h exposure, the autoradiograms of the gels were scanned with a dual wavelength TLC scanner (CS-930, Shimadzu). The densities of the bands around position 1192 were used to quantitate the percentages of wild-type and mutant rRNA in the following way: the wild-type base at position 1192 is a C, and therefore the C-lanes were scanned for both wild-type and mutants. The 1192 C peak areas for mutant and wild-type were first normalized to neighbouring C peak areas other than position 1192 in order to compensate for differences in the amount of sample applied. The ratio between the normalized wild-type 1192 C-peak area and the normalized mutant 1192 C-peak area was then calculated.

Determination of the kinetic parameters of RNA mutants

The 16S rRNA mutant ribosomes studied in this work were in fact a mixture of ribosomes with mutant and wild-type 16S rRNA. By sequence analysis, as described above, the fractions of wild-type and mutant were determined in all of the ribosome preparations. This enabled us to estimate kinetic parameters for the mutant and wild-type ribosomes. The calculations described below are made for EF-G cycle experiments and an analogous treatment can be used for other translation steps. The rate of poly(Phe) synthesis (J) is given by:

$$J = \frac{G_o R_o [[x\, k_{cat1}/K_{M1}] + [1 - x]k_{cat2}/K_{M2}]}{1 + R_o [[x/K_{M1}] + [1 - x]/K_{M2}]} \quad (1)$$

G_o is the total amount of EF-G. R_o is the total concentration of elongating ribosomes out of which xR_o are mutant and $(1 - x)R_o$ wild-type. k_{cat1} and k_{cat2} are the maximal EF-G cycling rates for mutant and wild-type ribosomes, respectively and K_{M1} and K_{M2} are the corresponding K_M values. The EF-G cycling rate formally obeys Michaelis−Menten kinetics if R_o is taken as substrate:

$$J = \frac{G_o R_o k_{cat}/K_M}{1 + [R_o/K_M]} \quad (2)$$

In the experiment with both mutant and wild-type ribosomes present, we determine the phenomenological k_{cat} and K_M values in equation 2 and we use these together with k_{cat2} and K_{M2}, obtained from another, independent, experiment performed on wild-type ribosomes, to calculate the mutant parameters k_{cat1} and K_{M1}:

$$k_{cat1}/K_{M1} = [(k_{cat}/K_M) - (1 - x)k_{cat2}/K_{M2}]/x \quad (3a)$$

$$K_{M1} = x/[(1/K_M) - (1 - x)/K_{M2}] \quad (3b)$$

Equations 3a and b can be derived by identifying the parameters in equation 1 with those in equation 2, provided that the elongating fraction is the same among mutant and wild type ribosomes.

Assays

A poly(U) directed poly(Phe) synthesizing system optimized for rate and accuracy (Wagner *et al.*, 1982) was used throughout the work. Determination of ribosomal elongation rates as a function of varying concentrations of EF-Tu or of EF-G were performed under burst conditions as in Wagner *et al.* (1982). Measurements of cycle times for EF-Tu and EF-G were performed as ribosome titrations at limiting factor concentrations (Bilgin *et al.*, 1988). These assays have been summarized in detail by Ehrenberg *et al.* (1989a). When added, 100 μg/ml spectinomycin was present, unless otherwise stated. The concentration of active EF-G was determined in a poly(Phe) synthesis assay at increasing concentrations of ribosomes in the presence of 200 μM fusidic acid as described by Bilgin *et al.* (1988). The concentration of active EF-Tu was determined by a nucleotide exchange method as described by Ehrenberg *et al.* (1989a,b). The concentration of active ribosomes was determined using N-Acetyl[^{3}H]Phe-$tRNA^{Phe}$ to initiate the poly(Phe) chains as described by Wagner *et al.* (1982). In all poly(Phe) synthesis assays, the reaction times were chosen so that the length of poly(Phe) chains is between 16 and 20 amino acids.

Acknowledgements

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