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Codon-specific missense errors *in vivo*

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We have developed a simple method for measuring the missense substitution of amino acids at specified positions in proteins synthesized *in vivo*. We find that the frequency of cysteine substitution for the single arginine in *Escherichia coli* ribosomal protein L7/L12 is close to 10^{-3} for wild-type bacteria, decreases to 4×10^{-4} in streptomycin-resistant bacteria containing mutant S12 (*rpsL*), and is virtually unchanged in Ram bacteria containing mutant S4 (*rpsD*). We have also found that the frequency of the cysteine substitution for the single tryptophan in *E. coli* ribosomal protein S6 is $3-4 \times 10^{-3}$ for wild-type bacteria, decreases to 6×10^{-4} in streptomycin-resistant bacteria and is elevated to nearly 10^{-2} in Ram bacteria.

Key words: cysteine missense/ribosome mutants/translational errors

Introduction

Translational errors leading to the suppression of nonsense mutations have been studied in quite some detail in *Escherichia coli* (Gorini, 1971; Smith, 1979). Furthermore, relatively common mutants with alterations in ribosome structure have been shown to raise or lower the suppression frequencies for nonsense codons as well as other mutational defects both in the presence and absence of suppressor tRNA species (Gorini, 1971). One implication of these findings is that the error frequencies of translation are not the fixed properties of codons and anticodons, but must be viewed as part of the bacterial phenotype that is subject to selective pressures. One prerequisite to understanding how the error frequencies are selected is data concerning their distribution with respect to specific codons as well as to particular messenger contexts. Such data for codons other than the three that signal for termination are, to say the least, scarce.

The pioneer work in this field is that of Loftfield and Vanderjagt (1972), who studied the replacement of isoleucine by valine at a particular position in ovalbumin and estimated this missense error to be in the range of 3×10^{-4}. For *E. coli* flagellin, a similar average value was estimated for the cysteine missense error on an undefined collection of arginine codons (Edelmann and Gallant, 1977). In addition, the analysis of electrophoretic heterogeneity provides a way of measuring missense errors that lead to charge changes in proteins (O'Farrell, 1978; Parker *et al.*, 1978, 1980; Parker and Friesen, 1980). This procedure has been used recently to estimate a global missense error frequency which fits nicely into the range of $10^{-4}-10^{-3}$ (Ellis and Gallant, 1982).

The uniformity of these estimates for the missense frequencies in *E. coli* is deceptive. Thus, these estimates are based on averages of a number of different amino acid positions within a selected small number of proteins. In contrast, we know that the suppression frequency of a given nonsense codon can vary by orders of magnitude depending on the messenger context, and that different nonsense codons in the same context have widley different suppression frequencies (Fluck *et al.*, 1977; Bossi and Roth, 1980; Engelberg-Kulka *et al.*, 1981; Andersson *et al.*, 1982). Accordingly, we might suspect that the missense frequencies for the other 61 codons show the same large variations depending on codon and context. In order to determine whether or not this is so, measurements of the missense errors at single codons must be made.

In the present study we describe protein chemical methods for making estimates of missense error frequencies at defined positions in a polypeptide. Our estimates of one cysteine for arginine substitution and one cysteine for tryptophan substitution in two ribosomal proteins are in the neighbourhood of 10^{-3}, which places them somewhat higher than the average estimates calculated previously. In addition, comparison of these missense frequencies in bacteria with different mutant ribosome phenotypes suggests that the ribosome is the major site of these particular errors.

Results

Experimental design

Our experimental strategy depends on our ability to identify and purify a polypeptide with a nominal sequence that does not contain the amino acid responsible for the missense substitution under study. As shown below, the polypeptide can be either a whole protein or an excisable sequence within a whole protein. A second requirement of our method is the availability of a chemical reagent or enzyme that will specifically cleave the polypeptide at the particular amino acid position in the polypeptide at which the putative missense event occurs. Finally, the substituted polypeptide must be resistant to the cleavage reaction. When these conditions are met, a simple combination of radioisotopic measurements of polypeptides fractionated in polyacrylamide gels permits us to estimate a specific missense error rate at a particular position in a protein.

We take as an example the missense event consisting of a

Table I. Strains of *E. coli* K12 used in this study

Strain	Relevant genotype	Reference/Source
017	*rpsD*$^+$, *rpsL*$^+$	Olsson and Isaksson (1979) Andersson *et al.* (1982)
UK 235[a] (017-141)	As 017 but *rpsL*	Derived from 017 (Kaj Bohman)
UE 144[b] (EA 4.0.0)	ArgH, D(*tonB-trpA,B*)	This laboratory
UE 145[b] (EA 4.0.2.)	As UE 144 but *rpsD*	Derived from UE 144 (L. Isaksson)

[a]UK 235 was obtained by P1 transduction from a spontaneous derivative of UD111 (Andersson *et al.*, 1982) resistant to 500 μg/ml streptomycin.
[b]UE 144 and UE 145 are derivatives of KL 37 (E. Murgola).

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Table II. Errors in wild-type strain (017)

Protein or peptide	Molar ratio Cys/Leu[a]	Experimental d.p.m. ratio $^{35}S:^{3}H$[b]	d.p.m. ratio normalized to 10 leucines[c]	Mol ^{35}S per mol polypeptide x 10^{3} [e]	Specific misincorporation x 10^{3} [f]
L10	1:15	436 900:28 570	22.94[d] Average		
L11	1:7	461 600:14 600	22.13[d] 22.8		
S4	1:20	249 940:23 350	21.34[d]		
S8	1:9	83 100:3030	24.68[d]		
S6	x:5	587:1601	0.183	8.0	
S6p1	x:4	169:1370	0.049	2.2	8.0 − (2.2 + 1.8) = 4.0
S6p2	x:1	112:266	0.042	1.8	
L7/L12	x:8	179:1089	0.132	5.8	
L7/12p1	x:2	68:250	0.055	2.4	5.8 − (2.4 + 2.1) = 1.3
L7/L12p2	x:6	9:111	0.048	2.1	

Misreading frequencies in 017 (*rpsL*$^{+}$, *rpsD*$^{+}$).

[a]Molar ratios of cysteine to leucine in the proteins and peptides calculated from the published sequences (Wittmann, 1982; Wittmann *et al.*, 1980). 'x' is the misincorporation under investigation.

[b]Ratios of radioactivities expressed in d.p.m. ($^{35}S/^{3}H$) detected in the polypeptides isolated from a peptide gel and processed as described in Materials and methods. Background values of 50 d.p.m. for ^{35}S and 20 d.p.m. for ^{3}H have been subtracted.

[c]The ratios listed in (b) have been calculated and converted to a ratio of the number of cysteines per 10 leucines for standardization of our calculations.

[d]Shows the normalized specific activity of cysteine. The ratio for 1 cysteine to 10 leucines is calculated as the average of the values obtained from the four standard proteins that contain known amounts of cysteine.

[e]Molar amounts of cysteine in polypeptides that do not contain this amino acid in their normal sequence. These amounts are obtained by dividing the normalized values listed in (c) for each such polypeptide by the normalized specific activity of cysteine in (d). Thus these figures represent the total misreading frequencies in the different polypeptides.

[f]The difference between the cysteine amounts found in the peptides and those found in the parent protein before cleavage reflects the specific misincorporation at the cleavage position. (For further details see Results.)

replacement by cysteine of the single arginine in the protein L7/L12 from the *E. coli* ribosome. The nominal sequence of this protein contains no cysteine (Terhorst *et al.*, 1973; Post *et al.*, 1979). Therefore, when a methionine-requiring strain of *E. coli* is grown in the presence of $^{35}SO_4^{2-}$ and excess unlabelled methionine, the sulphur label incorporated into L7/L12 should represent missense incorporation of cysteine. The problem then is to identify the positions at which the cysteine has been incorporated; this is where the cleavage reaction comes in.

First, we measure the total missense incorporation at all positions in L7/L12. Next, we cleave the protein with the protease from mouse submaxillary gland which is specific for the arginine residue (Schenkein *et al.*, 1977). This will yield two polypeptides: L7/L12 residues 1−73 (p1) and 74−120 (p2), respectively. Since the enzyme does not cleave at a cysteine, the cysteine incorporation into the two peptide cleavage products informs us of the missense incorporation at all positions except that corresponding to the single arginine in the nominal sequence. Hence, we obtain the specific arginine replacement rate by cysteine simply by subtracting the cysteine incorporation in the two peptide cleavage products from the cysteine incorporation in the intact L7/L12. As discussed below, we use a double-label protocol. This has two advantages: quantitative cleavage as well as total recovery of the peptides is unnecessary, and only the missense substitution by the labelled amino acid is recorded.

Cysteine for arginine in L7/L12

The bacteria (Table I) were grown in a medium containing unlabelled methionine, $^{35}SO_4^{2-}$ and [^{3}H]leucine. The latter label was included so that we could measure the relative amounts of a given polypeptide of a known amino acid sequence; i.e., the [^{3}H]leucine serves as an internal standard.

We know from the work of others that proteins S4, S2, S8, L10 and L11 contain leucine and cysteine in molar ratios corresponding to 20:1, 21:1, 9:1, 15:1 and 7:1, respectively (Wittmann, 1982; Wittmann *et al.*, 1980). Therefore, these proteins which are easily purified electrophoretically, were isolated and their ratios of [^{3}H]leucine to [^{35}S]cysteine were measured to provide the ratio of their relative specific activities in proteins (Table II). This could then be used to measure the molar amounts of cysteine incorporated into polypeptides containing known amounts of leucine.

One advantage of the double-label technique we use is that total recovery of the polypeptides is not required. Thus, we measure the [^{35}S]cysteine as well as the [^{3}H]leucine in a test sample of a peptide with a known nominal sequence. The [^{3}H]leucine content of the sample tells us how many mols of polypeptide are present. The ratio of [^{35}S]cysteine to [^{3}H]-leucine obtained from the four standard proteins (S4, S8, S10 and L11) can now be used together with the [^{35}S]cysteine content of the test sample to calculate the molar amounts of cysteine incorporated per mol of test polypeptide.

The ribosomes and the L7/L12 were isolated from the labelled bacteria as described in Materials and methods. The estimate of the total cysteine missense error for L7/L12 was 5.8×10^{-3} per protein molecule (Table II). After cleavage of the protein with the arginine-specific enzyme, the resulting two peptides (Figure 1) were isolated and their isotopic content measured. This corresponded to a missense error ratio of 4.5×10^{-3} summed over all the positions except that corresponding to the arginine position (Table II). The difference between these figures suggests that the cysteine substitution rate for arginine in this protein is 1.3×10^{-3}.

Since this estimate of the missense frequency is obtained as

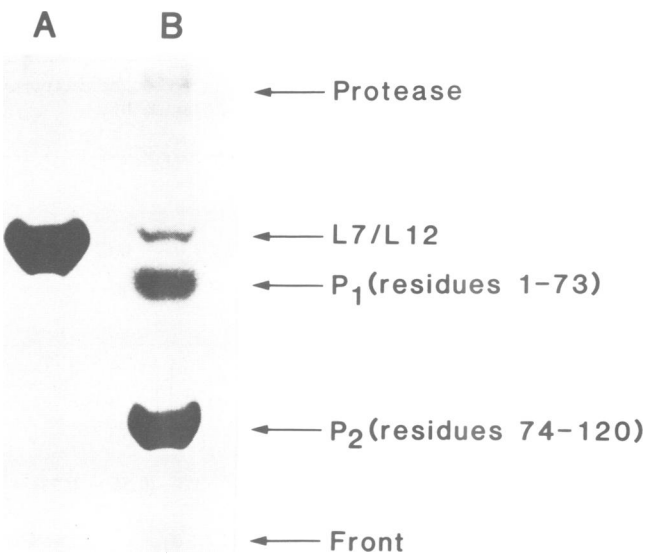

Fig. 1. Peptide gel pattern of ribosomal protein L7/L12. **(A)** Before cleavage, and **(B)** after specific cleavage at arginine 73 (see Materials and methods).

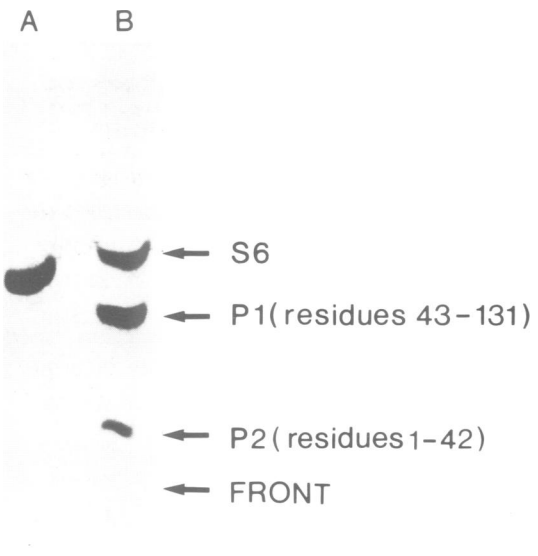

Fig. 2. Peptide gel pattern of ribosomal protein S6. **(A)** Before cleavage, and **(B)** after cleavage at the single tryptophan residue with BNPS-skatole (see Materials and methods).

the difference between two large numbers (Table II), it might be subject to a relatively large error, which should be reflected in the variation of the estimate obtained from independent experiments. In fact, we have repeated this experiment on four separate occasions with L7/L12 extracted either from 70S ribosomes or from 50S ribosomal subunits. We found that our estimates for the cysteine-arginine substitution rate varied between 1.1 and 2.0 x 10^{-3} (see also Table IV). Such scatter in our results suggests that this missense frequency is of the order of 1.5 ± 0.5 x 10^{-3}.

The relatively high incorporation of cysteine into the two cleavage products of L7/L12, p1 and p2 (Figure 1), could be due to the missense substitution of cysteine for amino acids other than arginine. There is, however, an additional explanation for this observation. Thus, we have assumed so far that there is no incorporation of ^{35}S into methionine because we have used a methionine-requiring strain and cultured it in the presence of unlabelled methionine. Nevertheless, L7/L12 contains three methionines. If these contained as little as one thousandth the ^{35}S content of cysteine, they would contribute enough radioactivity to L7/L12 p1 to account for its unexpectedly high ^{35}S content. Indeed we have isolated the methionine from the experimental cultures and we have found, as did Edelmann and Gallant (1977) in a similar experiment, that there is a leakage of ^{35}S into this amino acid that corresponds to approximately one thousandth the level of cysteine (not shown).

If a low background of the ^{35}S label in methionine is responsible for some part of the ^{35}S label in L7/L12 p1, the cleavage of L7/L12 to release a peptide containing the arginine, but lacking methionine, should remove this part of the background. Therefore, we have used V8 protease from *Staphylococcus aureus* (see Materials and methods) to isolate the appropriate peptide from L7/L12 corresponding to residues 56–82; this contains the arginine position but no methionine (Terhorst *et al.*, 1973). The total ^{35}S content of this peptide yielded a missense substitution frequency of cysteine for arginine corresponding to 1.5 x 10^{-3} assuming that all the label was incorporated at a single position.

Cysteine substitution for tryptophan in S6

The ribosomal protein S6 contains a single tryptophan in its sequence and no cysteine (Hitz *et al.*, 1975). The protein can be cleaved at the tryptophan with the reagent BNPS-skatole (Fontana, 1972) to yield two peptides S6 p1 and S6 p2 (Figure 2). Accordingly, we can use the same strategy as described above to estimate the cysteine missense substitution for tryptophan in S6. Indeed, we have used the same labelled culture as well as the corresponding cysteine/leucine radioactivity ratios (Table II). Two conclusions can be drawn from the data for S6 and the cleavage products summarized in Table II. First, roughly one-half of the ^{35}S label incorporated into the intact protein is found at the position corresponding to tryptophan. This means that our estimate for this missense substitution is more accurate than that for the arginine position. Second, the cysteine missense substitution for tryptophan of 4 x 10^{-3} is roughly three times greater than that for arginine in L7/L12.

Missense errors in mutant bacteria

Some streptomycin-resistant (SmR) mutants with alterations in ribosomal protein S12 (*rpsL*) carry out translation more accurately than do their wild-type counterparts, while ribosomal ambiguity (Ram) mutants with alterations in protein S4 (*rpsD*) are less accurate (Gorini, 1971). We have therefore studied the effects of these mutations on the cysteine-arginine and cysteine-tryptophan missense substitutions to assess the contribution of the ribosome to these errors.

As shown in Table III, we have repeated the missense frequency measurements described above for strain 017 with its SmR derivative UK 235. The data show that the SmR strain has a cysteine-arginine missense frequency in L7/L12 of 4 x 10^{-4}, which is one third that of the wild-type strain. The effect of the S12 alteration is more pronounced in the case of the cysteine-tryptophan substitution in S6. Here, the missense frequency decreases by almost a factor of seven to 6 x 10^{-4}. These data suggest that the major site of both of these errors in the wild-type bacterium is the ribosome.

Table III. Errors in streptomycin-resistant strain (017-141)

Protein or peptide	Molar ratio Cys/Leu[a]	Experimental d.p.m. ratio $^{35}S:^{3}H$[b]	d.p.m. ratio normalized to 10 leucines[c]	Mol ^{35}S per mol polypeptide x 10^{3} [e]	Specific misincorporation x 10^{3} [f]
S4	1:20	691 140:68 770	20.1[d] Average		
L10	1:15	390 520:25 150	23.3[d] 21.7		
L7/L12	x:8	160:5500	0.023	1.07	1.07 − 0.67 = 0.4
L7/L12p1	x:2	52:717	0.014	0.67	
L7/L12p2	x:6	2:5110	Undetectable		
S2	1:21	519 730:29 910	36.5[d] Average		
L11	1:7	210 590:3760	39.2[d] 38		
S6	x:5	495:4270	0.058	1.5	
S6p1	x:4	264:4980	0.021	0.56	1.5 − (0.56 + 0.33) = 0.6
S6p2	x:1	72:570	0.12	0.33	

As Table II but with strain UK235 (*rpsL*).

Table IV. Errors in wild-type strain (UE 144)

Protein or peptide	Molar ratio Cys/Leu[a]	Experimental d.p.m. ratio $^{35}S:^{3}H$[b]	d.p.m. ratio normalized to 10 leucines[c]	Mol ^{35}S per mol polypeptide x 10^{3} [e]	Specific misincorporation x 10^{3} [f]
S8	1:9	121 100:5256	20.74[d] Average		
S4	1:20	505 825:50 710	19.95[d] 20		
L10	1:15	279 860:21 230	19.77[d]		
L7/L12	x:8	405:4547	0.070	3.6	
L7/L12p1	x:2	82:640	0.026	1.3	3.6 − (1.3 + 1.1) = 1.2
L7/L12p2	x:6	62:1640	0.022	1.1	
S6	x:5	493:1774	0.14	6.9	
S6p1	x:4	204:1947	0.14	2.1	6.9 − (2.1 + 1.7) = 3.1
S6p2	x:1	68:202	0.03	1.7	

As Table II but with strain UE144 (*rpsL*$^{+}$, *rpsD*$^{+}$).

Table V. Errors in Ram strain (UE 145)

Protein or peptide	Molar ratio Cys/Leu[a]	Experimental d.p.m. ratio $^{35}S:^{3}H$[b]	d.p.m. ratio normalized to 10 leucines[c]	Mol ^{35}S per mol polypeptide x 10^{3} [e]	Specific misincorporation x 10^{3} [f]
L10	1:15	57 100:5500	15.6[d] Average		
S2	1:21	25 400:3680	14.5[d] 15		
L7/L12	x:8	290:3520	0.066	4.4	
L7/L12p1	x:2	114:940	0.024	1.6	4.4 − (1.6 + 1.4) = 1.4
L7/L12p2	x:6	17:490	0.02	1.4	
S6	x:5	290:814	0.17	11	
S6p1	x:4	27:800	0.12	0.8	11 − (0.8 + (0.3) = 9.9
S6p2	x:1	11:232	0.05	0.3	

As Table II but with strain UE145 (*rpsD*).

To study the effect of the Ram mutation on the missense frequencies we have used a second wild-type strain, UE144, and its Ram derivative UE145. The data in Table IV show that UE144 is characterized by cysteine missense substitutions for arginine in L7/L12 and for tryptophan in S6 that are very similar to those for strain 017 (Table II). In contrast, the Ram derivative UE145 has a 3-fold higher cysteine substitution frequency for tryptophan in S6 (9.9×10^{-3}), but a virtually identical cysteine substitution rate for arginine in L7/L12 (Table V). Thus, the effect of the Ram phenotype on these

Table VI. Summary of error frequencies

Strain	L7/L12 (Cys/Arg) x 10^3	S6 (Cys/Trp) x 10^3
Wild-type (017)	1.3	4.0
Streptomycin-resistant (UK 235)	0.4	0.6
Wild-type (UE 144)	1.2	3.1
Ram (UE 145)	1.4	9.9

The data from Tables II – V are summarized here for convenience.

two errors is selective. The data for the cysteine missense incorporation of the different strains is summarized in Table VI.

Discussion

Direct measurements of the missense events in translation require that it be possible to distinguish such events from simple contamination of the test protein by other peptides. Although our procedures yield proteins as well as peptides that appear to be electrophoretically pure, and duplicate measurements have been reproducible (compare Tables II and IV), we have no definitive control that completely eliminates the influence of contaminants on our error estimates. Nevertheless, the response of the error frequencies to changes in the ribosome phenotype strongly suggests that contamination is not seriously perturbing our estimates.

Thus, the reduction of the cysteine-tryptophan substitution frequency in the protein S6 by a factor of seven in bacteria having streptomycin-resistant ribosomes suggests that >85% of this error in the wild-type bacterium occurs on the ribosome. The corresponding calculation for the cysteine-arginine substitution in L7/L12 yields a value of 75% or more for the error due to wild-type ribosomes.

Such results, as well as those obtained with the Ram mutants, are difficult to interpret in terms of contamination of our test proteins. Furthermore, these observations suggest that the errors attributable to RNA polymerase and the aminoacyl-tRNA synthetases are in both cases much smaller than those of the ribosome.

That the Ram ribosome selectively enhances the cysteine-tryptophan ambiguity in S6 but has an insignificant effect on the cysteine-arginine ambiguity in L7/L12 is reminiscent of the effects of Ram ribosomes on the read-through of nonsense codons. Thus, different Ram mutants exhibit different patterns of nonsense suppression that are sensitive to context and codon, but they invariably suppress UGA codons better than the other termination signals (Gorini, 1971; Andersson *et al.*, 1982). It has been suggested that this codon preference reflects the ease with which Ram ribosomes accept a third position mismatch between the UGA codon and Trp-tRNA. Similarly, the cysteine substitution for tryptophan in S6 must involve a third position mismatch between Cys-tRNA and the UGG codon. In contrast, the cysteine substitution for arginine in L7/L12 must involve a first position mismatch with the CGU codon. Hence, the present results are superficially consistent with the notion that Ram ribosomes preferentially enhance third position ambiguity during translation. However, we have observed in other experiments, that three different Ram ribosomes, one of which was from the Ram mutant studied here, enhance *in vitro* the error with both $tRNA_2^{Leu}$ and $tRNA_4^{Leu}$, i.e., a first position and third position mismatch, respectively (Andersson and Kurland, 1983). Therefore, we must look elsewhere for our explanation of the differential effects of the Ram ribosome in the different errors observed in the present study. Indeed, it is possible that these differential effects are caused by the messenger context or some peculiarity of the tRNA species involved.

Estimates of the RNA polymerase missense frequency *in vivo* place this error in the neighbourhood of 1.4×10^{-4} or greater per nucleotide (Rosenberger and Foskett, 1981). Assuming that there are no other errors introduced by the synthetases and ribosomes, such a polymerase error would lead to an average missense frequency in proteins close to 3×10^{-4} per codon. This latter level of error is close to what we observe in the translation products of SmR ribosomes (Table III).

Our relatively high figures invite comparison with the previous estimates for the cysteine/arginine substitution rate in flagellin which we have reproduced (unpublished data) and which correspond to values anywhere between 2×10^{-5} and 6×10^{-4} depending on the assumptions of the calculation (Edelmann and Gallant, 1977). Since we have no idea about how much, if any, of the cysteine incorporation into flagellin occurs at sites corresponding to a CGU codon, comparison with the present data for the error at this codon is in fact difficult. In addition, the lower range of the flagellin estimate is not commensurate with the error rate estimated for transcription (Rosenberger and Foskett, 1981). Furthermore, we have no estimates of the degree to which post-translational editing can affect the different proteins. To this may be added our uncertainty about how effectively erroneous proteins will be exported to and assembled into the flagella. In contrast, we do know that ribosomes seem rather tolerant of errors in their structural components (Dabbs, 1979; Olsson and Isaksson, 1980; Stöffler *et al.*, 1981).

Likewise, the more recent global estimate for the missense frequency in translation of 2×10^{-4} (Ellis and Gallant, 1982) turns out, upon closer examination, to be somewhat less than global, since it is based on indirect calculations for three proteins of unknown sequence. In contrast, the experimental studies of MS2 coat protein coded for by the viral RNA (Parker *et al.*, 1980) or a copy-DNA (Parker, personal communication) have identified a range of error frequencies including at least one missense error with a frequency $>10^{-3}$ *in vivo*. We are inclined to believe that the widely disparate estimates of missense frequencies reported so far are a faithful reflection of the non-uniform distributions of the errors of translation. Indeed, the range of variation observed so far for the missense frequencies is still well within the range of nonsense suppression frequencies determined by the context alone (Fluck *et al.*, 1977; Bossi and Roth, 1980; Engelberg-Kulka *et al.*, 1981; Andersson *et al.*, 1982).

Materials and methods

Bacterial strains

All bacterial strains used in this investigation were derived from *E. coli* K12 and are listed in Table I.

Enzymes and chemicals

Protease from the submaxillary gland of mouse ('Endoproteinase Arg-C') was from Boehringer-Mannheim; *S. aureus* protease V8 was purchased from Miles Laboratories, 2-(2-nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine (BNPS-skatole) was from Pierce Chemical Company. [^{35}S]Sulphate (30–50 Ci/mg) as well as NCS Tissue Solubilizer were purchased from Amersham International. [^{3}H]Leucine (~500 Ci/nmol) was either from Amersham International or from New England Nuclear.

Growth and labelling of bacteria

Bacteria were grown in M9 minimal medium (Miller, 1972), containing 0.2% glucose and modified as follows: the concentration of sulphate was 12 μM, all the amino acids were present at the concentrations described for the MOPS medium (Neidhardt *et al.*, 1977), except for methionine which was present at a concentration of 60 mg/l (i.e., in large excess), and for leucine (35 mg/l). Cysteine was completely omitted. Adenine and cytosine were present at 27 and 22 mg/l, respectively. 10 ml of an overnight culture in this medium were added to 1 l of fresh medium in a 3 l flask on a rotary shaking water bath at 37°C. When the cell density had reached an absorbance of 0.2 at 420 nm, 50 mCi of [^{35}S]sulphate was added along with 2 mCi of [^{3}H]leucine. The cells were grown further to an A_{420} of 1.0–1.2, chilled quickly, harvested and washed twice by standard procedures, and stored frozen at −20°C.

We measured the radioactivity present in the total culture and in the supernatant after centrifugation of the bacteria. We found that up to 80% of the isotopes were taken up by the cells.

Ribosome purification

The cells were sonicated, and an S30 extract was prepared. Ribosomes were pelleted by centrifugation in a Beckman 70 Ti rotor at 55 000 r.p.m. for 70 min, washed once in 1 M NH_4Cl, and then either pelleted again through a 20% sucrose cushion containing 1 M NH_4Cl or applied on a Sephacryl S300 column following the procedure of Jelenc (1980). In some experiments, subunits were prepared by centrifugation through a 5–30% sucrose gradient as described by Sköld (1981).

Protein purification

The ribosomal proteins were extracted with 66% acetic acid in the presence of 100 mM magnesium (Hardy *et al.*. 1969). The protein-containing supernatant thus obtained was dialysed overnight at 4°C against 10 mM Bis-Tris, 8 M urea and 1% β-mercaptoethanol, pH 4.2 (Madjar *et al.*, 1979). The proteins were then separated on the two-dimensional gel system I of Madjar *et al.* (1979) with the slight modifications of Sköld (1981). Stained spots of the relevant proteins were cut out of the gels, mashed in 66% acetic acid, extracted overnight and purified from the Coomassie Blue, all according to the procedure of Bernabeu *et al.* (1978, 1980), dialysed against water for 2 h, and finally lyophilized.

Directed cleavage

Arginine-specific. Ribosomal protein L7/L12 was redissolved in 200 μl of 100 mM glycine buffer, pH 8.2. 10 units of protease from the submaxillary gland of mouse dissolved in water was added, and the digestion was allowed to proceed overnight at 37°C in an Eppendorf tube (Schenkein *et al.*, 1977). Sample buffer was then added, and the sample was applied directly onto a peptide separation gel (see below).

Tryptophan-specific. BNPS-skatole was used for the cleavage of ribosomal protein S6. We followed the procedure of Fontana (1972), and the reaction was allowed to occur in the dark for 28–36 h, under gentle shaking at 37°C. Excess reagent was then removed by at least five extractions with ether, and the acetic acid phase was diluted to 10% with water and lyophilized. The dry residue was redissolved in sample buffer (see below) and applied onto a peptide separation gel.

Hydrolysis with S. aureus protease. The proteolytic degradation was carried out using an enzyme/protein ratio of 1:100 under the conditions of Leijonmarck *et al.* (1981). The digestion time was, however, extended to 20 h in order to obtain one main peptide covering residues 58–82 (M.Leijonmarck, personal communication).

Peptide separation

The polyacrylamide SDS and urea gel electrophoresis system of Swank and Munkres (1971) was used as modified by Liljas *et al.* (1978). Reference proteins and uncleaved proteins were run next to the peptides for quantitation.

Quantitation

After staining and destaining the gels, the different bands representing proteins and peptides were carefully cut out of the gel, mashed separately in scintillation vials, and 1 ml of NCS Tissue Solubilizer was added to each vial. After a 3 h incubation at 58°C of the tightly sealed vials, 34 μl of acetic acid was added to neutralize the sample as well as to reduce chemoluminescence, and the vials were allowed to stand overnight in the dark at 4°C. Scintillation fluid was then added (5 g PPO/l toluene), and the radioactivity was measured in a Beckman LS 7500 scintillation counter programmed to make quench corrections. Here, a series of quenched standard samples were used to generate quench curves from which the individual experimental samples were corrected by the programmed counter.

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