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"Two out of Three" Codon Reading Leading to Mistranslation in vivo

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Summary. Strains of *Escherichia coli* were starved for asparagine or lysine in order to increase the in vivo level of mistranslation. In a *relA* strain, asparagine starvation increased the error frequency in elongation factor Tu to 0.12 mistake per asparagine codon, while with lysine starvation in the same strain the error frequency per lysine codon was 0.008. The pattern of isoelectric point changes in the altered protein produced is consistent with third position misreading in the AAN codon group. This high level of mistranslation is not seen in streptomycin resistant (*rpsL*) strains or in most *relA*$^+$ strains.

Introduction

Recent in vitro studies on the reading of the genetic code have indicated that in a codon group (a set of four codons differing only at the third position) which codes for a single amino acid (codon family, Lagerkvist, 1978), the third position of the codon has even less specificity than predicted by the wobble hypothesis (Crick, 1966; Mitra et al., 1977; Goldman et al., 1979). This kind of codon reading has been termed "two-out-of-three" reading (Lagerkvist, 1978). However, in the case of the leucine codon family CUX, the third position possesses at least some specificity in codon-antocodon interaction (Goldman et al., 1979).

There are several codon groups where more than one amino acid is encoded (i.e., UUU, UUC, Phe: UUG, UUA, Leu). Here "two-out-of-three" reading would lead to mistranslation (i.e. amino acid substitution) and obviously could not be tolerated to any great extent in vivo in normally growing cells. Apparently even in an in vitro system, third position specificity is much greater in these codon groups (Goldman et al., 1979; Elias et al., 1979).

Mistranslation resulting from third position misreading was originally thought to be the predominant mistake made in translating the genetic code (e.g. Woese, 1967). However, the degeneracy of the code at this position made definitive experiments on third position misreading very difficult. Work with streptomycin, which increases ribosome ambiguity, showed that mistranslation could also result from first and second position misreading, and most subsequent work on mistranslation concentrated on such errors (see review by Gorini, 1974). Recently, however, it has been shown that severe amino acid restriction appears to lead to a great increase in the in vivo level of mistranslation (Parker et al., 1978; O'Farrell, 1978). Furthermore, this mistranslation seems to be a result of third position misreading. This mistranslated (altered) protein can be easily observed and quantitated on the two-dimensional polyacrylamide gel system of O'Farrell (1975) if the incorrectly inserted amino acid differs in charge from the normal amino acid. This type of mistranslation has been observed on this gel system by restriction for amino acids which share codon groups with amino acids of differing charge (CAU, CAC-His, CAA, CAG-Gln; AAU, AAC-Asn, AAA, AAG-Lys; AGU, AGC-Ser, AGA, AGG-Arg).

We have chosen to study this phenomenon primarily with asparagine (Asn) starvation in *Escherichia coli*. Asn starvation leads to isolectric point heterogeneity of a given protein with the altered protein being increasingly basic (Parker et al., 1978). This is what would be predicted if lysine (or any other basic amino acid) were inserted in place of Asn. Histidine (His) or arginine (Arg) limitation leads to increasingly acidic altered protein also as predicted by third position misreading. This latter type of protein charge heterogeneity can also be achieved by a variety of

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post-translational modifications, both in vivo and in vitro (e.g. Robinson, 1974; Stark et al., 1960), which lessens the utility of using His or Arg starvation.

In this paper we present results that strengthen the hypothesis that this phenomenon is the result of "two-out-of-three" misreading of the code. Further we show that asparagine starvation, coupled with two-dimensional gels, can be used as a sensitive assay for ribosomal mutations involved with changes in the level of ribosomal ambiguity.

Materials and Methods

Bacterial Strains. All strains of *Escherichia coli* constructed for this study are given in Table 1. The *asnA31, asnB32* mutations are those in the original asparagine auxotroph isolated by E. Reich (Cedar and Schwartz, 1969), kindly supplied to us by B. Bachmann. The *asnS* mutation is from strain HO202, kindly supplied to us by M. Nomura (Ohsawa and Maruo, 1976; Yamamoto et al., 1977). Strains were constructed by standard genetic techniques.

Media. The minimal medium used in this study is the MOPS buffered medium of Neidhardt et al (1975). The medium was supplemented with 0.4% (wt/vol) glucose and 5 μg/ml thiamine. Amino acid requirements were supplied by the addition of 50 μg/ml of the appropriate amino acid except in the case of Asn which was added to 200 μg/ml.

Growth and Starvation Conditions. Cultures were grown aerobically in Erlenmeyer flasks with rotary shaking. Growth was monitored by absorption at 420 nm.

Strains JK100 and JK120 (both *asnA, asnB, lysA*) were starved for amino acids by filtering 10–20 ml of a culture growing logarithimically at 37° C ($1–2 \times 10^8$ cells/ml) onto a 4.7 cm dia membrane filter (0.22 μm pore size). The cells were washed twice with 10 ml of prewarmed medium lacking amino acids. The filter was then removed, the cells were washed off the filter and distributed to new flasks. Control (unstarved) cells were then supplemented. Starved cells remained unsupplemented. The entire filtration, washing, and resuspension process was accomplished in less than 3 min. No measurable lag in the growth of the control culture was observed.

Strains with the temperature-sensitive *asnS* mutation were starved by shifting a logarithimically growing culture from 30° C to 42° C.

Labelling with Radioactive Amino Acids. Total protein was labelled by transferring 2 ml of culture to a prewarmed tube containing 10 μCi [^{35}S]-L-methionine. Five minutes later, nonradioactive methionine is added to 500 μg/ml. After a two minute chase, the sample is chilled to 2° C. Extracts were made as previously described (Pedersen et al., 1976).

The rate of protein synthesis was determined by incorporation of ^{14}C labelled L-Pro or L-Leu. Thirty to sixty minutes before measurements of protein synthesis were to begin, L-proline or L-leucine was added to the logarithimically growing culture to a final concentration of 10 μg/ml. At the appropriate time 1 ml of culture was added to a prewarmed tube containing 0.5 μCi of the appropriate ^{14}C labelled amino acid. After 5 min incubation, 1 ml of 10% trichloroacetic acid containing 1 mg/ml of that amino acid was added. The samples were placed at 100° C for 20 min, filtered and counted in a scintillation counter.

Two Dimensional Gels. Two-dimensional separation of proteins on polyacrylamide gels was carried out essentially as described by O'Farrell (1975). The pH range in the isoelectric focussing dimension was 5–7 and the electrofocussing was carried out for 7,000 V h. The second dimension was a sodium dodecyl sulfate/10% polyacrylamide slab. Gels were stained with Coomassie brilliant blue (Pedersen et al., 1975), dried under vacuum and autoradiograms made. Densitometer scans of the autoradiograms were performed with a Transidyne RFT Scanning Densitometer # 2950 (Ann Arbor, Michigan, U.S.A.)

Table 1. Bacterial strains constructed for this study

Strain	Genotype
JK100	*asnA31*[a], *asnB32, spoT, fuc, relA1, lysA*
JK120	same as above except *fuc*$^+$ *relA*$^+$
JK163	*asnS, leu-40, lacZ608, nalA*
JK164	same as JK163 except *relA1*
JK177	same as JK164 except *rpsL125*

[a] Allele numbers used are those assigned by B. Bachmann, Curator, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn., U.S.A.

Results

Protein Synthesis during Asparagine Starvation

E. coli can be conveniently starved for asparagine (Asn) in two ways: 1) removal of exogenous Asn from an *asnA asnB* double mutant, and 2) raising the incubation temperature of a temperature-sensitive *asnS* mutant growing in the absence of exogenous Asn from 30° C to 42° C. Both types of starvation lead to the synthesis of protein which shows the same type of charge heterogeneity.

Figure 1 shows autoradiograms of gels of total protein from strain JK164 (*relA asnS*) pulse labelled before (Fig. 1A) and between 55 and 60 min after (Fig. 1B) a shift from 30° C to 42° C. In each panel the upper arrow points to elongation factor G (EF-G) and the bottom arrow to elongation factor Tu (EF-Tu). The authentic (unmodified) protein species can be located in the extract from the starved cells by a protein stain of the gel. Almost all the protein present is made before starvation and is therefore unlabelled in such an extract. In the case of the proteins labelled during Asn starvation it can be seen that a trail of discrete protein spots follows the authentic protein, and that they are increasingly more basic. Ribosomal proteins, most of which do not run in the pH range used in the first dimension of these gels, show a similar pattern (results not shown). This is the pattern one would expect if Lys was substituted for Asn. Each spot of altered protein in a given trail would be composed of those protein species with the

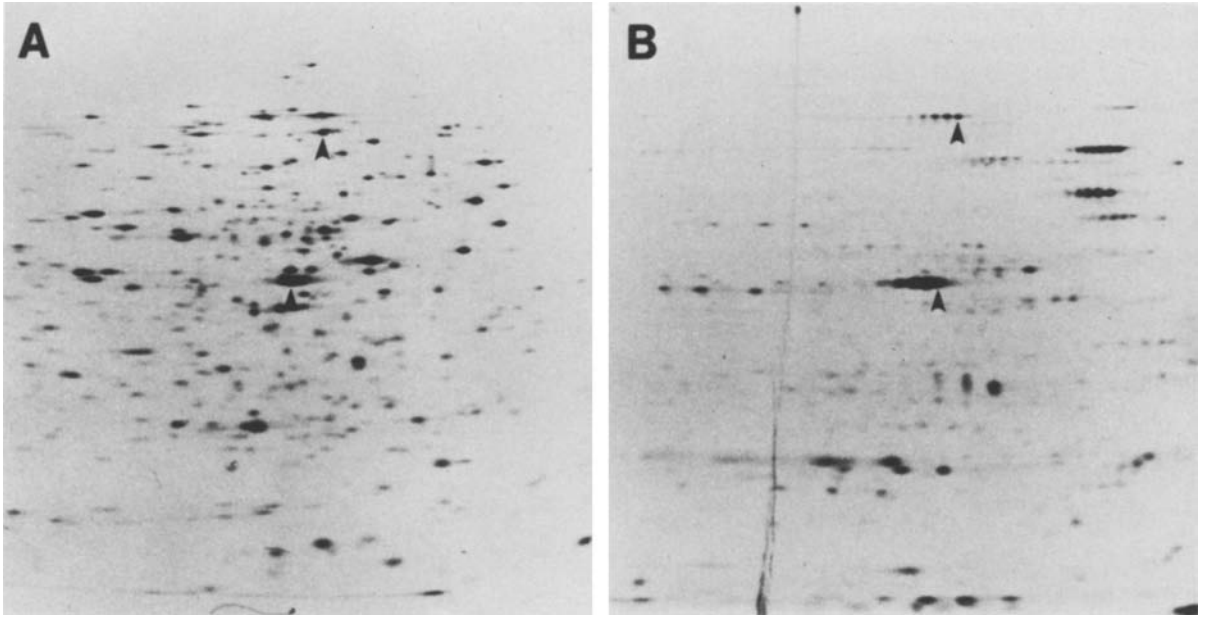

Fig. 1A and B. Autoradiograms of two-dimensional gels of [^{35}S] methionine-labelled proteins made before and after asparagine starvation. Strain JK164 was labelled before and 55–60 min after Asn starvation was induced by a shift form 30° C to 42° C. Growth, labelling and extraction conditions are described in Materials and Methods. Two-dimensional gels were run as described in Materials and Methods. The gels are oriented so that the pH range of 5–7 runs from right to left. **A** Proteins labelled before the shift. **B** Proteins labelled 55–60 min after the shift. In each part, the upper arrow points to authentic EF-G, and the lower arrow to authentic EF-Tu

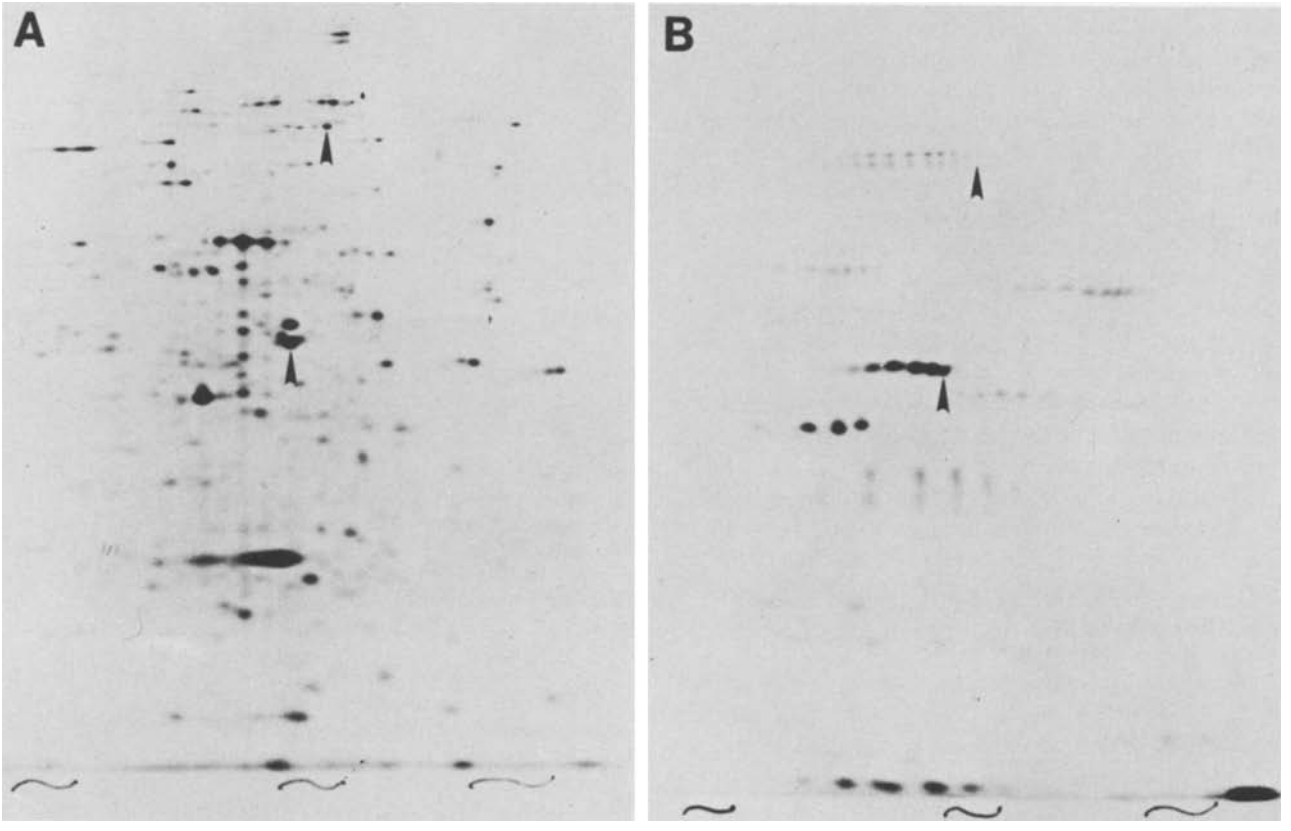

Fig. 2A and B. Autoradiograms of two-dimensional gels of [^{35}S] methionine-labelled proteins made after asparagine starvation in stringent and relaxed cells. JK120 (*relA*$^+$) and JK100 (*relA*) were starved for Asn by removal of exogenous asparagine from the growth medium as described in Materials and Methods. Both strains were labelled 15–20 min after starvation. **(A)** Jk120 **(B)** JK100. Gels are presented as described in the legend to Fig. 1

same number of Asn to Lys substitutions, i.e., the spot one charge more basic than the authentic spot contains protein with one such error and so on.

Although the addition of D, L-aspartyl-β-hydroxamate can be used to starve mammalian cell cultures for Asn (Uren et al., 1977), the same does not seem to be true in *E. coli*. D, L-aspartyl-β-hydroxamate does inhibit the growth of *E. coli*, but, in our hands, the growth inhibition observed is overcome by aspartic acid (Asp) and not Asn (data not shown). Further, unlike the case with the other two methods of Asn starvation, no protein charge heterogeneity can be seen in protein made during this starvation (data not shown).

Starvation in Stringent and Relaxed Cells

Figure 2 shows the pattern of protein synthesis after Asn starvation of the asparagine auxotrophs JK120 (*relA*$^+$, *spoT*), Fig. 2A, and JK100 (*relA*, *spoT*), Fig. 2B. As in Fig. 1 the arrows indicate authentic EF-G and EF-Tu. First, it can be seen that the *pattern* of protein synthesis is quite different in the two strains and is exactly the difference one would expect between relaxed and stringent cells (Reeh et al., 1976;Blumenthal et al., 1976). Second, the relaxed cells mistranslate much more than the stringent cells, although the stringent cells do mistranslate. It can be seen that the mistranslation in stringent cells is confined primarily to high molecular weight proteins, and that there are fewer altered protein species in a given trail than in the relaxed cells. This is as expected since the higher molecular weight proteins should, on the average, have more Asn codons, and therefore only in these proteins would a lower level of mistranslation be apparent. By comparing Fig. 2B with Fig. 1B, it can also be seen that JK100, which is an *asnA*, *asnB* strain, mistranslates more than JK164, which is *asnS*. This is almost certainly because after starvation, the residual rate of protein synthesis was 7.0% in JK100 and 16.5% in JK164 at the time the sample was taken. We have previously shown in cultured mammalian cells that there is a direct relationship between the severity of amino acid starvation and the amount of mistranslation (Parker et al., 1978). Thus, a similar relationship is apparent in *E. coli*.

Mistranslation in stringent cells is not confined to Asn starvation. Figure 3 shows a small portion of autoradiograms of proteins synthesized in JK120 under 4 different conditions. The cells from a single culture of JK120 were filtered, resuspended, and then distributed to four different media for labelling: complete minimal (Fig. 3A), minus Asn (Fig. 3B), minus Lys (Fig. 3C) and complete plus Val (Fig. 3D).

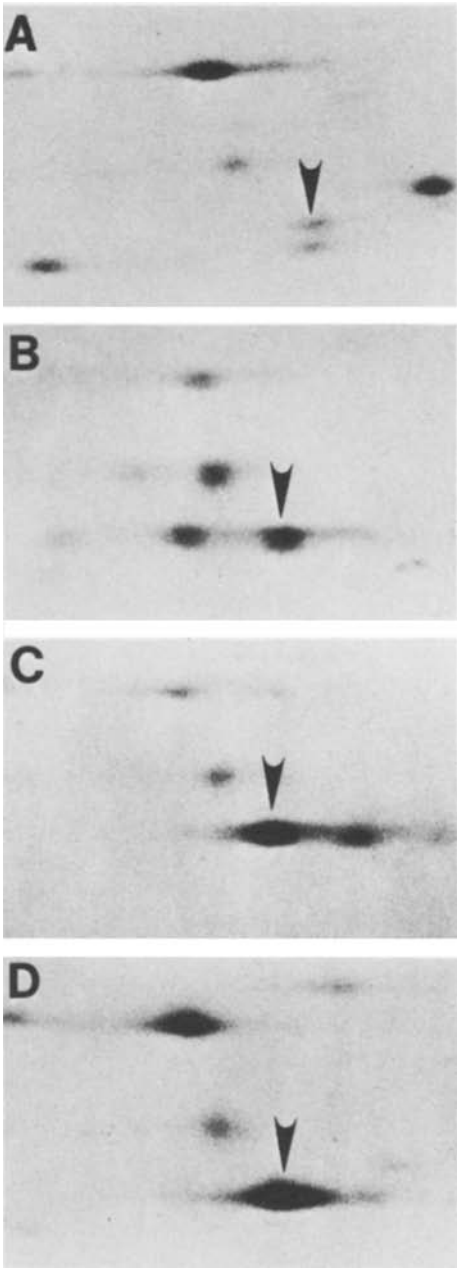

Fig. 3A–D. Portions of autoradiograms of proteins made during various amino acid starvations of a stringent strain. JK120 (*relA*$^+$) was labelled growing normally **(A)**, starved for Asn **(B)**, Lys **(C)** or Ile **(D)**. Starvation and labelling conditions are as given in Materials and Methods. Ile starvation was achieved by adding Val to 200 μg/ml. The protein marked with the arrow has an apparent molecular weight of 61,000 daltons. the protein above and to the left of this protein is threonyl-tRNA synthetase, protein G65 on the nomenclature system of Pedersen et al (1978). The gels are oriented with the more acidic proteins to the right

The protein marked by the arrow has an apparent molecular weight of 61,000. It can be seen that both Asn and Lys starvation cause mistranslation with the altered protein species being more basic in the case

of Asn starvation and more acidic in the case of Lys starvation. Val addition (Ile starvation) caused no mistranslation apparent in this gel system. These results are exactly as one would predict with two out of three misreading.

Figure 4 shows the region of autoradiograms around EF-Tu in an experiment similar to that described above, but using the relaxed strain JK100. The direction of charge change with Lys (Fig. 4A) and Asn (Fig. 4B) starvation is exactly the same as in Fig. 3. EF-Tu contains 7 Asn residues and 23 Lys residues (Richard Laursen, personal communication). Densitometer scans of the spots and calculation of misreading frequencies gives 0.12 error per Asn codon during Asn starvation, and 0.008 error per Lys codon during Lys starvation. This difference in frequency between Asn and Lys induced mistranslation was not confined to EF-Tu, but was true of all proteins.

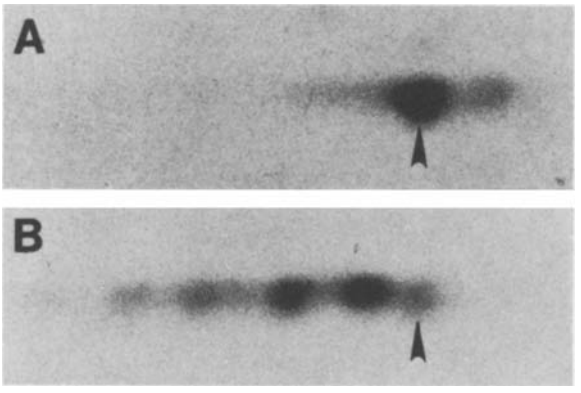

Fig. 4A and B. Autoradiograms of two-dimensional gels showing elongation factor Tu made during lysine and asparagine starvations of a relaxed strain. JK100 (*relA*) was labelled during starvation for Lys **(A)** and Asn **(B)**. Starvation and labelling conditions are as given in Materials and Methods. The gels are oriented with the more acidic proteins to the right. The arrow marks authentic EF-Tu

Starvation in Strains with Ribosomal Mutation

Certain ribosomal mutations are known which decrease translation fidelity and others which increase it. Therefore we decided to test the effects of these mutations on protein synthesis during Asn starvation.

One such experiment is shown in Fig. 5. Figure 5A is a portion of an autoradiogram showing protein synthesized in JK177 (*asnS relA rpsL125*), and in Fig. 5B, JK164 (*asnS relA rpsL*$^+$), both at 42° C. As can be seen, the introduction of the *rpsL* allele, leading to streptomycin resistance, abolished the mistranslation observable in this system. Preliminary results indicate that the introduction of *rpsE* mutations have no effect on mistranslation in *relA* strains, but increase mistranslation in the *relA*$^+$ strains (results not shown).

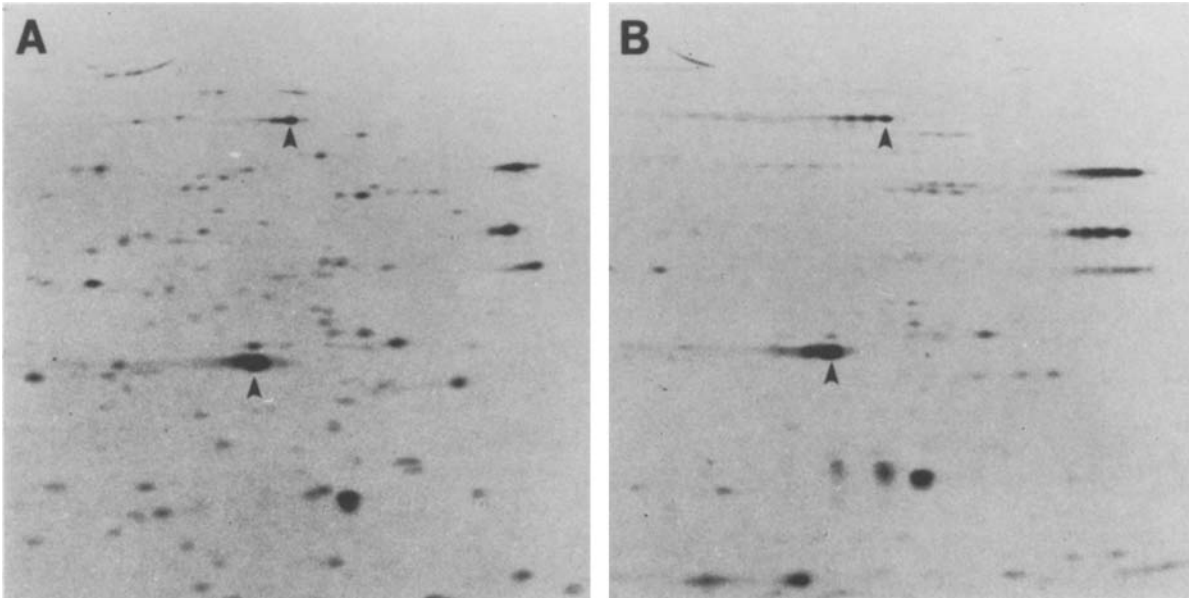

Fig. 5A and B. Autoradiograms of two-dimensional gels of proteins made during asparagine starvation of streptomycin resistant and sensitive strains. Strains JK177 (*rpsL*) and JK163 (*rpsL*$^+$) were labelled with [^{35}S]-methionine 45–50 min after Asn starvation was induced by a shift from 30° C to 42° C. Gels are oriented as described in the legend to Fig. 1, but only the upper two-thirds of the gels are shown. **A** JK177. **B** JK163

Discussion

Previously it has been shown that severe amino acid restriction (residual protein synthesis $<20\%$) leads to observable charge heterogeneity in the proteins still synthesized (Parker et al., 1978; O'Farrell, 1978). The pattern of heterogeneity observed is most easily explained by third position misreading of the genetic code. The altered protein is only synthesized during restriction for a few amino acids, and, more importantly, the charge change of the altered protein is also predictable by third position misreading.

The work reported in this paper strengthens the view that the charge heterogeneity observed is the result of mistakes at the level of the ribosome. Streptomycin resistant mutants are known to have increased fidelity of protein synthesis (see review by Gorini, 1974). Starvation of streptomycin resistant, relaxed mutants does not lead to any apparent alteration of the protein still synthesized. This would indicate that it is not misacylation of tRNA which leads to the observed mistranslation but events on the ribosome affecting codon-anticodon interaction. Our unpublished work with *rpsE* mutants (cited above) supports this view.

Relaxed strains were also previously shown to mistranslate to a greater extent than stringent strains (e.g. Hall and Gallant, 1972). O'Farrell (1978) was unable to detect mistranslation in starved stringent cells. Our results differ from his only in degree; we see a low level of mistranslation in most stringent cells. Possibly this is because in our experiments with auxotrophs a residual rate of protein synthesis of about 7% was obtained, and O'Farrell (1978) had a rate of about 14%. In *asnS relA*$^+$ strains, the rate of protein synthesis after the temperature shift to 42° C seems to be maintained at above 15%, and we have not detected mistranslation in such strains unless we introduce other mutations such as *rpsE*.

We have reported (Parker et al., 1978) that some stringent strains mistranslate to a high degree, almost as high as that observed in relaxed strains. This work had been done primarily in strain CP78 (*relA*$^+$) (Fiil and Friesen, 1968) and its derivatives. In our hands this apparent high level of mistranslation in CP78 during His starvation is very reproducible. Possibly CP78 has an otherwise cryptic mutation which affects translation fidelity under these conditions. Experiments are now in progress to repeat the work using Asn starvation and to attempt to localize any cryptic mutation in this strain.

The difference in the frequency of mistranslation during starvation for the two amino acids which share the same codon group is not restricted to the Asn/Lys group. Histidine starvation leads to easily observable levels of mistranslation but glutamine starvation does not (Parker et al., 1978). Arginine starvation leads to measurable mistranslation in this system (O'Farrell, 1978) but serine starvation does not (J. Parker, unpublished results). It would seem, then, that under conditions of severe depletion of the correctly aminoacylated tRNA there are still constraints on third position codon-anticodon interaction. This is similar to what Goldman et al. (1979) and Elias et al. (1979) have found in in vitro protein synthesizing systems.

The error frequency per codon we have observed with this system is very high; at least 0.12 with Asn starvation (Fig. 4A) and 0.23 with His starvation (Parker et al., 1978). This frequency is approximately three orders of magnitude higher than that observed previously in normally growing cells (Loftfield, 1963; Loftfield and Vanderjagt, 1972; Edelmann and Gallant, 1977). These studies, however, were concerned with mistranslation in other codon groups. The method we employ to detect errors is relatively simple, but can not be used to detect the very low levels of mistranslation in normally growing cells. The error frequency in EF-Tu induced by Lys starvation was 0.008. Possibly error frequencies of slightly above 10^{-3} could be detected by this method, but this would still be almost ten times the normal frequency.

The indirect evidence supporting this phenomenon as mistranslation caused by third position misreading is very strong. Work is now in progress to do peptide analysis of mistranslated protein in order to have definitive proof.

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References

Blumenthal, R.M., Lemaux, P.G., Neidhardt, F.C., Dennis, P.P.: The effects of the *relA* gene on the synthesis of aminoacyl-tRNA synthetases and other transcription and translation proteins in *Escherichia coli* B. Mol. Gen. Genet. **149**, 291–296 (1976)

Cedar, H., Schwartz, J.H.: The asparagine synthetase of *Escherichia coli* I. Biosynthetic role of the enzyme, purification, and characterization of the reaction products. J. Biol. Chem. **244**, 4112–4121 (1969)

Crick, F.H.C.: Codon-antocodon pairing: The wobble hypothesis. J. Mol. Biol. **19**, 548–555 (1966)

Edelmann, P., Gallant, J.: Mistranslation in *E. coli*. Cell **10**, 131–137 (1977)

Elias, P., Lustig, F., Axberg, T., Åkesson, B., Lagerkvist, U.: Reading of the lysine codons in the MS 2 coat protein cistron during protein synthesis *in vitro*. FEBS Lett. **98**, 145–151 (1979)

Fiil, N., Friesen, J.D.: Isolation of "relaxed" mutants of *Escherichia coli*. J. Bacteriol. **95**, 729–731 (1968)

Goldman, E., Holmes, W.M., Hatfield, G.W.: Specificity of codon recognition by *Escherichia coli* $tRNA^{Leu}$ isoaccepting species determined by protein synthesis *in vitro* directed by phage RNA. J. Mol. Biol. **129**, 567–585 (1979)

Gorini, L.: Streptomycin and misreading of the genetic code. In: Ribosomes (M. Nomura, A. Tissières, and P. Lengyel, eds.), pp. 791–803. New York: Cold Spring Harbor Laboratory 1974

Hall, B., Gallant, J.: Defective translation in RC^- cells. Nature New Biol. **237**, 131–135 (1972)

Lagerkvist, U.: "Two out of three": an alternative method for codon reading. Proc. Natl. Acad. Sci. U.S.A. **75**, 1759–1762 (1978)

Loftfield, R.B.: The frequency of errors in protein biosynthesis. Biochem. J. **89**, 82–92 (1963)

Loftfield, R.B., Vanderjagt, D.: The frequency of errors in protein biosynthesis. Biochem. J. **128**, 1353–1386 (1972)

Mitra, S.K., Lustig, F., Åkesson, B., Lagerkvist, U., Strid, L.: Codon-anticodon recognition in the valine codon family. J. Biol. Chem. **252**, 471–488 (1977)

Neidhardt, F.C., Bloch, P.L., Smith, D.F.: Culture medium for enterobacteria. J. Bacteriol. **119**, 747–763 (1974)

O'Farrell, P.H.: High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**, 4007–4021 (1975)

O'Farrell, P.H.: The suppression of defective translation by ppGpp and its role in the stringent response. Cell **15**, 545–557 (1978)

Ohsawa, H., Maruo, B.: Restoration by ribosomal protein S1 of the defective translation in a temperature-sensitive mutant of *Escherichia coli* K-12: characterization and genetic studies. J. Bacteriol. **127**, 1157–1166 (1978)

Parker, J., Pollard, J.W., Friesen, J.D., Stanners, C.P.: Stuttering: high level mistranslation in animal and bacterial cells. Proc. Natl. Acad. Sci. U.S.A. **75**, 1091–1095 (1978)

Pedersen, S., Bloch, P.L., Reeh, S., Neidhardt, F.C.: Patterns of protein synthesis in *E. coli:* a catalog of the amount of 140 individual proteins at different growth rates. Cell **14**, 179–180 (1978)

Pedersen, S., Reeh, S.V., Parker, J., Watson, R.J., Friesen, J.D., Fiil, N.P.: Analysis of the proteins synthesized in ultraviolet light-irradiated *Escherichia coli* following infection with the bacteriophages $\lambda drif^d$ 18 and $\lambda dfus$-3. Mol. Gen. Genet. **144**, 293–343 (1976)

Reeh, S., Pedersen, S., Friesen, J.D.: Biosynthetic regulation of individual proteins in $relA^+$ and *relA* strains of *Escherichia coli* during amino acid starvation. Mol. Gen. Genet. **149**, 279–289 (1976)

Robinson, A.B.: Evolution and the distribution of glutaminyl and asparaginyl residues in proteins. Proc. Natl. Acad. Sci. U.S.A. **71**, 885–888 (1974)

Stark, G.R., Stein, W.H., Moore, S.: Reaction of the cyanate present in aqueous urea with amino acids and proteins. J. Biol. Chem. **235**, 3177–3181 (1960)

Uren, J.R., Chang, P.K., Handschumacher, R.E.: Effects of asparagine synthetase inhibitors on asparaginase resistant tumors. Biochem. Pharmacol. **26**, 4408–4410 (1977)

Woese, Carl R.: The present status of the genetic code. Prog. Nucleic Acid Res. and Mol. Biol. **7**, 107–172 (1967)

Yamamoto, M., Nomura, M., Ohsawa, H., Maruo, B.: Identification of a temperature sensitive asparaginyl-transfer ribonucleic acid synthetase mutant of *Escherichia coli*. J. Bacteriol. **132**, 127–131 (1977)

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