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Relationship between Guanosine Tetraphosphate and Accuracy of Translation in *Salmonella typhimurium*†

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ABSTRACT: In bacteria a high level of mistranslation is observed in amino acid starved *rel*⁻, but not *rel*⁺, strains, and mistranslation can be studied qualitatively by means of "stuttering" experiments in two-dimensional protein gels. It has been suggested that the low level of mistranslation that occurs in *rel*⁺ strains is assured by guanosine 5′-diphosphate 3′-diphosphate (ppGpp), a nucleotide whose intracellular concentration greatly increases in *rel*⁺ cells under amino acid starvation. In the present study the relationship between level of ppGpp and mistranslation was analyzed by performing stuttering experiments in amino acid starved bacteria that contained either high or low levels of ppGpp. Three strains of *Salmonella typhimurium* were used in these experiments: a *relA*⁺ *hisT*⁺ strain (TA997), a *relA*⁺ *hisT* strain (TA1001), and a *relA hisT* strain (PD2). These strains were first characterized with respect to macromolecular syntheses and ppGpp levels under exponential growth and under amino acid starvation. Both *rel*⁺ strains exhibited stringent control over RNA synthesis. ppGpp accumulated to high levels when TA997 was starved for either of three amino acids. Starvation of TA1001 for histidine did not cause accumulation of ppGpp, whereas starvation for lysine and arginine produced high levels of ppGpp. Extracts from the three strains, obtained either under exponential growth or under amino acid starvation, were then subjected to two-dimensional electrophoretic analysis: mistranslation was observed whenever ppGpp was absent. In particular, starvation of TA1001 for histidine resulted in high mistranslation frequencies, while under lysine and arginine starvation mistranslation was undetectable, regardless of whether the cells were *rel*⁺ or *rel*⁻. The results reported in this study provide strong evidence that ppGpp is indeed the molecule responsible in vivo for maintaining the frequency of mistranslation at a low level in *rel*⁺ bacteria.

Starvation for amino acids in eukaryotes causes incorporation of illegitimate amino acids at the starved-for codons according to a predictable scenario, whereas amino acid starvation of *rel*⁺ bacteria produces only a small increase in mistranslation (Hall & Gallant, 1972; Pollard, 1984). The *relA* gene of *Escherichia coli* and its homologue in *Salmonella typhimurium* preside over a variety of physiological events during amino acid starvation of these bacteria, including protection against translation errors. When mistranslation is studied in *rel*⁻ strains, however, the error frequencies that are produced are higher by several orders of magnitude (Hall & Gallant, 1972; Gallant & Foley, 1979; Cashel & Rudd, 1987). Thus, amino acid starved *relA* bacteria have been very useful in the study of in vivo translational errors.

The events that take place in *rel*⁺ bacteria under amino acid starvation have been collectively termed the stringent response (Gallant, 1979; Cashel & Rudd, 1987). A central element of the response is the production of guanosine 5′-triphosphate 3′-diphosphate (pppGpp) and of guanosine 5′-diphosphate 3′-diphosphate (ppGpp). There is good evidence that the two nucleotides are produced as a result of the interaction between a codon present in an RNA message and a codon-specific uncharged tRNA on ribosomes possessing an active *relA* product (Block & Haseltine, 1974). High levels of these nucleotides are normally produced by amino acid starved *rel*⁺, but not *rel*⁻, bacteria, and it is thought that ppGpp is an effector molecule that mediates many of the other metabolic events that take place during the stringent response (Cashel & Rudd, 1987).

The evidence for a causal role of ppGpp is circumstantial, since it is based on the correlation between production of the

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nucleotide and appearance of the other elements of the stringent response; it is also controversial, since it has been shown in several organisms that ppGpp need not be present for at least part of the response to occur (Spadaro et al., 1981; Belitsky & Kari, 1982; Acosta & Lueking, 1987).

The low level of mistranslation present in amino acid starved *rel*$^+$ bacteria is one of the components of the stringent response for which a causal involvement of ppGpp has been hypothesized (Cashel & Rudd, 1987). The evidence for the involvement lies in the good correlation between level of ppGpp and mistranslation, not only in amino acid starved *rel*$^+$ bacterial cells but also in amino acid starved *rel*$^-$ cells that have been caused to synthesize ppGpp by different ppGpp-producing pathways (Fiil et al., 1977; Friesen et al., 1978; Gallant & Foley, 1979). There is also compelling in vitro evidence for such a hypothesis (Dix et al., 1983; Dix & Thompson, 1986). If the hypothesis is true, then the *rel*$^+$ organsisms that fail to increase their intracellular concentration of ppGpp during amino acid starvation should furnish high mistranslation frequencies under such starvation conditions.

One of the *rel*$^+$ bacteria that does not raise its ppGpp level but still exhibits many of the other elements of the stringent response under amino acid starvation is a strain of *S. typhimurium* containing a mutation in the *hisT* gene (Spadaro et al., 1981). Mutations in this gene cause a pseudouridine deficiency close to the anticodon (positions 38–40) in all those tRNA chains whose unmodified molecule has a uridine residue in such positions (Cortese et al., 1974; Turnbough et al., 1979). One possibility is that the inability of amino acid starved *rel*$^+$ *hisT* bacteria to produce ppGpp is due to the fact that the uncharged tRNAs that interact with their cognate codons on the *rel*$^+$ ribosomes are not properly modified. In fact, a *hisT* mutant fails to raise its ppGpp level under starvation for histidine, and histidyl-tRNA is subject to pseudouridine modification in the anticodon region; starvation for threonine and arginine do result in ppGpp accumulation, and most cognate tRNA species are not subject to modification (Spadaro et al., 1981).

In the present study the occurrence of mistranslation as evidenced by "stuttering" (O'Farrell, 1978; Parker et al., 1978; Pollard, 1984) was determined in a *hisT* mutant of *S. typhimurium* under exponential growth conditions and under starvation for amino acids that either do or do not produce high levels of ppGpp.

Materials and Methods

Bacterial Strains and Growth Conditions. The three strains of *S. typhimurium* used throughout experiments were previously described (Spadaro et al., 1981): strain TA997 (*relA*$^+$, *hisT*$^+$, *aroC5*, *purF145*, *hisD2655*), strain TA1001 (*relA*$^+$, *hisT1514*, *aroC*$^+$, *purF145*$^+$, *hisD2655*), and strain PD2 (*relA*::Tn*10*, *hisT1514*, *aroC*$^+$, *purF145*$^+$, *hisD2655*). Strain TA1001 was obtained by cotransducing the markers *aroC*$^+$, *hisT1514*, and *purF*$^+$ into strain TA997, and strain PD2 was constructed by transducing *relA*::Tn*10* into strain TA1001 (Spadaro et al., 1981).

Bacteria were grown at 37 °C in a minimal medium (Kjeldgaard, 1961) containing 100 mM Tris-HCl at pH 7.4, 27 mM KCl, 37 mM NH_4Cl, 2.7 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM Na_2SO_4, 0.007 mM $FeCl_3$, 0.5 mM KH_2PO_4, 0.15 mM $CaCl_2$, 10 mM glucose, and 0.015 mM thiamine. Histidine was added at a final concentration of 50 μg/mL.

Starvation experiments were performed either by transferring exponentially growing cells to a fresh medium lacking histidine as already reported (Donini et al., 1978) or by treating cells with the amino acid analogues lysine hydroxamate or arginine hydroxamate (400 μg/mL each) obtained from Sigma (St. Louis, MO), which produce starvation for lysyl-tRNA or arginyl-tRNA, respectively. Amino acid hydroxamates are indeed inhibitors of their corresponding aminoacyl-tRNA synthetases and therefore produce starvation for the aminoacyl-tRNA, not the amino acid itself (Tosa & Pizer, 1971b; Bali et al., 1984). The inhibition of bacterial growth by hydroxamates can be rapidly reversed by the addition of the normal amino acids to the culture medium (Tosa & Pizer, 1971a). For simplicity the effects of hydroxamates on protein synthesis will be hereafter referred to as lysine and arginine starvation.

Macromolecular Synthesis. The synthesis of protein and RNA was measured by the incorporation of, respectively, [^{14}C]proline (0.04 μCi and 10 μg/mL; specific activity 250 mCi/mmol, CEA, Saclay, France) and [^{3}H]uracil (0.15 μCi and 5 μg/mL; specific activity 1 mCi/mmol). Samples of 400 μL each were withdrawn at different times from the culture medium and treated individually with 500 μL of cold 10% trichloroacetic acid. The resulting precipitates were collected on Millipore filters, and the radioactivity was counted in a scintillation fluid by using the appropriate double-label setting in a Tri-Carb Packard spectrometer. The labeling periods used were sufficiently long to ensure that the RNA under observation was essentially ribosomal RNA.

Analysis of Guanosine Polyphosphates. The method described by Gallant et al. (1970) was used. Briefly, exponentially growing bacteria were labeled with [^{32}P]orthophosphate (200 μCi/mL) for approximately one doubling of cell population to allow equilibrium of the nucleotide pools with the added radioactive precursor. In histidine starvation experiments, cells were collected on Millipore filters and resuspended in an histidine-deprived medium containing radioactive orthophosphate at the same specific activity as before. In lysine or arginine starvation experiments, the relevant hydroxamate was added directly into the prelabeled culture medium. In each case, aliquots (1 mL) were withdrawn and treated with an equal volume of 2 M formic acid for 30 min at 0 °C. The suspension was then subjected to low-speed centrifugation, and 10 μL of formic extract was analyzed by ascending chromatography on poly(ethylenimine)–cellulose plates. The nucleotides pppGpp and ppGpp were revealed by autoradiography using X-O-Mat films from Eastman Kodak Co. (Rochester, NY). It must be noted that under our analytical conditions some guanosine polyphosphates other than pppGpp and ppGpp could not be detected. This concerns, for instance, ppGp and pGpp, which are separated only by two-dimensional chromatography (Bochner & Ames, 1982; Lee et al., 1983).

Preparation of Cellular Extracts. In view of protein analysis by two-dimensional electrophoresis, extracts were prepared from normally growing or starved bacteria labeled for 45 min with [^{35}S]methionine (5 μCi/mL; specific activity 1400 Ci/mmol, Amersham Centre, Little Chalfont, England). Cells were suspended in a buffer containing 10 mM Tris-HCl at pH 7.4, 5 mM $MgCl_2$, and 50 μg/mL pancreatic ribonuclease and disrupted by repeated ultrasonic treatment. The sonicated extract was incubated for 15 min at 4 °C in the presence of 50 μg/mL pancreatic deoxyribonuclease. After centrifugation for 25 min at 30000*g*, the supernatant fraction was collected, and proteins were treated with 0.12 volume of a 3% sodium dodecyl sulfate/10% 2-mercaptoethanol mixture, then precipitated overnight with 5 volumes of 95% acetone at −20 °C, and finally centrifuged and dried under vacuum.

Two-Dimensional Analysis of Proteins. ^{35}S-Labeled proteins were analyzed by the O'Farrell (1975) gel technique.

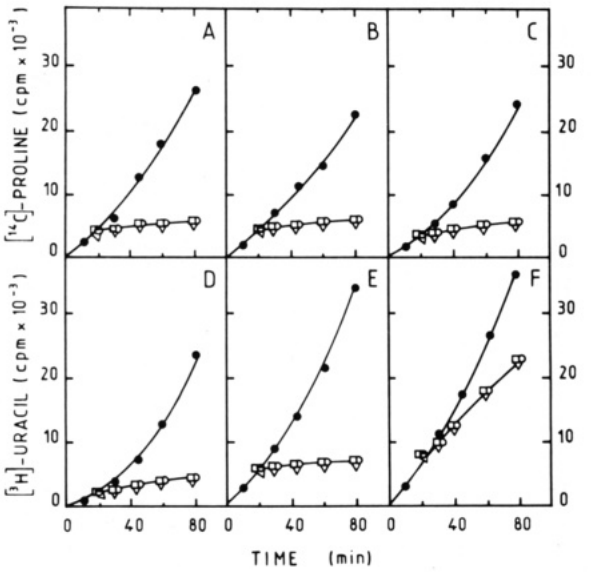

FIGURE 1: Protein and RNA synthesis in *S. typhimurium*. The synthesis of protein (A–C) and RNA (D–F) was measured by the incorporation of [^{14}C]proline and [^{3}H]uracil, respectively, in the acid-insoluble material of cells as described under Materials and Methods. Experiments were performed during either exponential growth (●) or starvation for histidine (○), lysine (□), or arginine (▽). (A, D) Strain TA997; (B, E) strain TA1001; (C, F) strain PD2.

Separation in the first dimension was achieved by isoelectric focusing to equilibrium (10000 V·h) in pH 5–7 ampholine in a 4% acrylamide gel containing 9.5 M urea. Electrophoresis in the second dimension was performed in 12.5% acrylamide and 1% sodium dodecyl sulfate (600 V·h). The amount of protein loaded on each gel (25–30 μg) was determined by the Bradford (1976) technique. For autoradiography the gel was incubated for 30 min at room temperature in 7.5% acetic acid/30% methanol, dried under vacuum, and exposed for 36–48 h by using direct-exposure DEF-5 films from Kodak.

RESULTS

In the three different strains TA997, TA1001, and PD2 of *S. typhimurium* used in this work, protein synthesis was analyzed both quantitatively and qualitatively in connection with the accumulation of pppGpp and ppGpp within the cells. This analysis was performed during either normal growth or amino acid starvation of bacteria. The three amino acids (histidine, lysine, and arginine) that cells were starved for separately were chosen for two main reasons: (i) for each of them, all the relevant isoacceptor tRNA species are either totally pseudouridylated in their anticodon region under normal conditions ($tRNA^{His}$ and $tRNA^{Lys}$) or not modified at all ($tRNA^{Arg}$), and (ii) their substitution by an erroneous amino acid due to codon–anticodon misrecognition was expected, in each case, to result in a modification of the charge of the synthesized protein detectable by isoelectric focusing analysis (O'Farrell, 1978; Parker et al., 1978; Parker & Friesen, 1980).

Quantitative Analysis of Protein Synthesis. To measure protein synthesis, exponentially growing cells were first labeled for 20 min in the presence of [^{14}C]proline and then either maintained in a complete culture medium or deprived either of histidine, of lysine, or of arginine. The amount of radioactive acid-insoluble materials was thereafter determined as a function of time. RNA synthesis was measured in parallel by labeling the cells simultaneously with [^{3}H]uracil. The results presented in Figure 1 show that, in each case, starvation for any amino acid results in the same strong inhibition of the rate of protein synthesis to only about 5% of the control value (diagrams A–C). In the wild-type strain TA997 and the *hisT* mutant TA1001, which both harbor the *relA*$^{+}$ genotype, the rate of RNA synthesis during starvation is also reduced to about 5% of the control diagrams D and E); by contrast, it is maintained to a relatively high level (over 70% of the control) in strain PD2 carrying the *relA hisT* genotype (diagram F). These observations therefore indicate that, from a quantitative point of view, the *hisT* mutation does not seem to affect per se macromolecular synthesis. They confirm, moreover, the well-known differential behavior of stringent and relaxed cells with regard to RNA synthesis during starvation (Gallant, 1979).

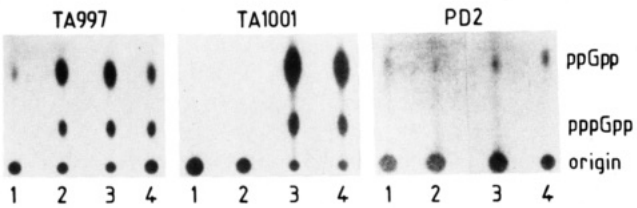

FIGURE 2: Synthesis of guanosine polyphosphates in *S. typhimurium*. Bacteria were labeled with [^{32}P]orthophosphate, and a formic extract was prepared and analyzed by ascending chromatography as described under Materials and Methods. The location of the nucleotides pppGpp and ppGpp was detected by autoradiography. For each strain, analysis was performed on extracts prepared from cells either in exponential phase of growth (track 1) or starved for histidine (track 2), lysine (track 3), or arginine (track 4).

Synthesis of Guanosine Polyphosphates. The metabolic properties of strains TA997, TA1001, and PD2 were further investigated by studying the effect of amino acid starvation on the intracellular concentration of pppGpp and ppGpp. In each case, exponentially growing cells were labeled with [^{32}P]orthophosphate and either maintained in a complete culture medium or starved separately for histidine, lysine, or arginine. An acidic extract was then prepared and subjected to ascending chromatography and autoradiography for analysis of guanosine polyphosphates (Gallant et al., 1970). The results presented in Figure 2 indicate, on the one hand, that both pppGpp and ppGpp accumulate in strain TA997 during starvation, whatever the missing amino acid. On the other hand, they show that in strain PD2 no significant increase of the nucleotide concentration is observed under the same conditions of deprivation. These data are consistent with the previously described capacity of starved stringent and relaxed bacteria to respectively accumulate, or not, guanosine polyphosphates (Gallant, 1979). The behavior of the stringent strain TA1001 is unusual, since in this case the accumulation of pppGpp and ppGpp depends on the nature of the missing amino acid. Thus, when this strain is deprived of lysine or arginine, the levels of the two nucleotides greatly increase within the cells; but when it is starved for histidine, the nucleotides do not accumulate, in agreement with the previous observation that stringency of macromolecular synthesis may occur without ppGpp accumulation (Spadaro et al., 1981). The different responses obtained under histidine or lysine starvation were unexpected, considering that the various isoacceptor tRNAs specific for these two amino acids share the common property of being normally pseudouridylated in their anticodon region (Turnbough et al., 1979). We took advantage of the peculiarity of strain TA1001, with respect to the absence of ppGpp under histidine starvation, to analyze the involvement of guanosine polyphosphates in the control of translational accuracy.

Qualitative Analysis of Protein Synthesis. The quality of proteins synthesized by strains TA997, TA1001, and PD2 was studied. Bacteria were first cultured in a complete medium for about two generations, then either maintained under

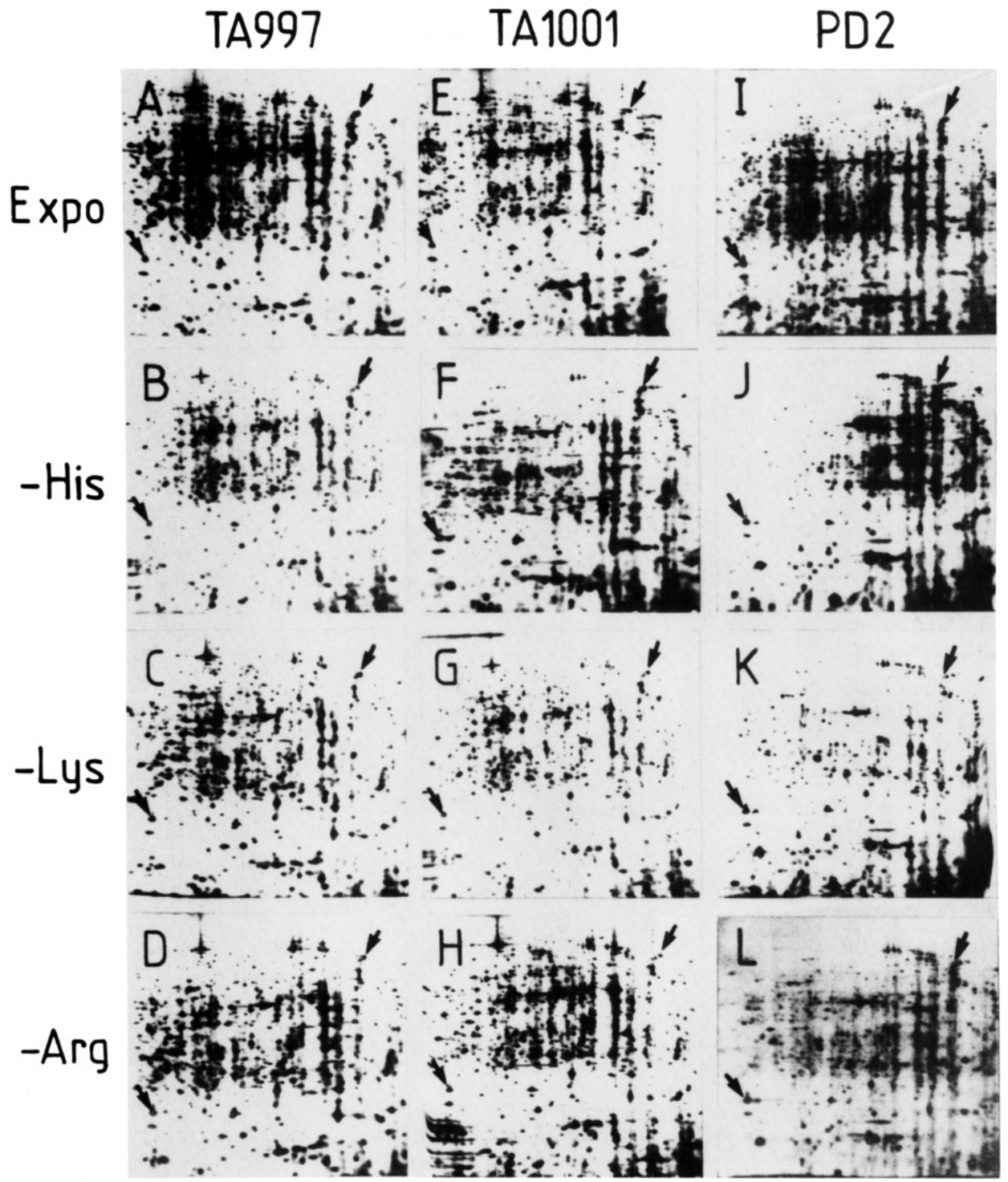

FIGURE 3: Qualitative analysis of proteins of *S. typhimurium*. Bacteria were labeled with radioactive methionine, and proteins were analyzed by two-dimensional electrophoresis followed by autoradiography. Experiments were performed with cells of strain TA997 (A–D), strain TA1001 (E–H), and strain PD2 (I–L), collected during either exponential phase of growth (Expo) or starvation for histidine (–His), lysine (–Lys), or arginine (–Arg). Two specific proteins, shown by arrows, were examined with particular attention to estimate the extent of erroneous translation. In each case the arrow points to the location of the qualitatively normal protein.

normal conditions of growth or starved for either histidine, lysine, or arginine, and labeled in all cases with [^{35}S]-methionine. Proteins were extracted and analyzed by two-dimensional gel electrophoresis according to the O'Farrell (1978) technique followed by autoradiography. In this procedure abnormal proteins are detected on the autoradiograms as a series of satellite spots with molecular weights similar to that of the authentic protein but separated from it in the isoelectric focusing dimension (O'Farrell, 1978; Parker et al., 1978), a phenomenon termed stuttering. The direction of displacement of the mistranslated forms generated by starvation for a given amino acid can be predicted from the genetic code by applying the rules derived from the patterns and hierarchy of errors previously reported (Woese, 1967; Yarus, 1979; Ellis & Gallant, 1982). Such prediction is presented in Table I for each of the three amino acids that bacteria were starved for.

The results corresponding to the various situations analyzed are presented in Figure 3. Depending of the type of strain and on the culture conditions, several proteins appeared or did not appear to undergo mistranslation, as exemplified by the two proteins indicated by arrows on the autoradiograms. No

Table I: Amino Acid Substitutions Predicted and Observed during Starvation[a]

cognate			noncognate			direction of charge shift	
codons read	amino acid	charge	codons read	amino acid	charge	predicted	observed
CAU/CAC	His	basic	CAA/CAG	Gln	neutral	acidic	acidic
AAA/AAG	Lys	basic	AAU/AAC	Asn	neutral	acidic	acidic
AGA/AGG	Arg	basic	AGU/AGC	Ser	neutral	acidic	acidic

[a] For each of the three basic amino acids (His, Lys, Arg) that strains of *S. typhimurium* were separately starved for, the nucleotide sequence of the codons read by the cognate tRNAs is indicated. Assuming the occurrence of misreading at the third codon position, the nature and the charge of the substituted amino acid are presented in each case, as well as the predicted direction of the subsequent charge shift. The rightmost column indicates the direction of the protein displacement actually observed on the autoradiograms of Figure 3.

stuttering occurred in strain TA997 whatever the missing amino acid or in strain TA1001 starved for lysine or arginine. By contrast, when strain TA1001 was deprived of histidine or when strain PD2 was deprived of any amino acid, a significant charge heterogeneity of proteins was detected. In these two cases, the leftmost (most basic) spot of each series corresponds to the protein made in unstarved cells, and the displacement of satellite spots is toward the acidic end of the gel. Such a shift is therefore due to the substitution of the missing amino acids by less basic amino acids, which experimentally confirms the predictions made in Table I. Concerning the relationship between ppGpp level and translational fidelity during starvation, it seems clear from the present data that, with no exception, the frequency of errors is greatly increased whenever the nucleotide does not accumulate within the cells, whether the cellular background be stringent or relaxed.

Discussion

The introductory experiments reported in this study confirm the previous finding (Spadaro et al., 1981) that the *hisT S. typhimurium* strain TA1001 under histidine starvation retains its *rel*$^+$ phenotype with respect to the synthesis of stable RNA in spite of its inability to accumulate ppGpp (and pppGpp). Thus, several cases of lack of correlation between ppGpp accumulation and stable RNA synthesis now exist together with the earlier reported quantitative inconsistencies in the correlation (Khan & Yamazaki, 1974; Hansen et al., 1975; Donini et al., 1978; Pao & Dyess, 1981). These findings are consistent with the notion either that ppGpp is not involved at all in the control of stable RNA synthesis during the stringent response or that, if it is involved, there is more than one mechanism and the ppGpp mechanism is dispensable.

Contrary to expectation, starvation of the *hisT* mutant for lysine, whose cognate tRNAs normally contain pseudouridine in the anticodon region, resulted in accumulation of both polyphosphorylated nucleotides to a level similar to the one that is observed in the classic stringent response. Thus, the decision whether to accumulate or not to accumulate ppGpp is not determined in a simple and direct manner by the absence or the presence of pseudouridines in the anticodon region. The *S. typhimurium* and *E. coli* $tRNA^{His}$ molecules have identical sequences, and they both contain pseudouridines at positions 38 and 39. Only the $tRNA^{Lys}$ of *E. coli* carrying the UUU anticodon has been sequenced, and it contains a pseudouridine only at position 39. It may be that position 38 is the key position where the presence or the absence of a pseudouridine determines whether ppGpp is to be produced or not; perhaps the presence of a pseudouridine at this position, in those tRNAs that require it, causes the tRNA molecule to assume the three-dimensional conformation most appropriate for interaction with the *relA* product on the ribosome. Only further experimentation will decide this issue.

The main goal of this study was to determine the frequency of mistranslation at a low ppGpp level in the course of a stringent response by exploiting the features of the *hisT* mutation. The result obtained is that when the *hisT* mutant is starved for histidine, which does not elicit ppGpp accumulation, the mistranslation frequency is as high as that obtained with the quasi-isogenic *rel*$^-$ control strain. The high frequency of mistranslation is not due to the mere presence of the *hisT* gene, since when starvation of the mutant strain is carried out for two other amino acids that do cause ppGpp to accumulate, the level of mistranslation is as low as in the starved *hisT*$^+$ control. Thus in the same strain and in the course of a stringent response, a high frequency of errors occurs in protein synthesis when ppGpp is at its basal level, whereas the error frequency is low when the level of ppGpp is high.

This finding would appear to conflict with the one reported by Parker (1982) in a mistranslation experiment performed under histidine starvation in the only available *hisT* mutant of *E. coli*. In reality this particular mutant does not seem to be appropriate for this type of analysis, since under histidine starvation ppGpp is reduced, but only to a level that is about 50% of normal (P. Donini, unpublished results). In principle, errors can be introduced into proteins through amino acid substitutions caused by misreading at the first, second, or third codon position (Edelmann & Gallant, 1977; Thompson et al., 1981; Yarus & Thompson, 1983; Johnston & Parker, 1985). The errors that are observed by means of stuttering experiments such as the ones presented here are the ones that are provoked by misreading at the third position (Lagerkvist, 1978; Parker & Holtz, 1984; Pollard, 1984).

The results reported in this study provide strong evidence that ppGpp is indeed the molecule responsible in vivo for maintaining the frequency of mistranslation at a low level in *rel*$^+$ bacteria. However, it cannot be excluded that some other guanosine polyphosphates such as pGpp and ppGp (Pao & Gallant, 1979; Bochner & Ames, 1982) may participate as well in this regulatory process. Further experiments are therefore needed to test this possibility.

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