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Translation of the sequence AGG-AGG yields 50% ribosomal frameshift

(accuracy/minor tRNA/rare codon/Shine–Dalgarno complementarity)

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ABSTRACT **We have inserted the sequence 5′-AAG-GAGGU-3′, which is complementary to the 3′ terminus of *Escherichia coli* 16S rRNA, in a reading frame and analyzed its effect on the accuracy and overall rate of translation *in vivo*. Translation over the sequence yields a 50% ribosomal frameshift if the reading phase is A-AGG-AGG-U. The other two possible frames do not give shifts. The introduction of a UAA stop codon before (UAA-AGG-AGG-U) but not after (A-AGG-AGG-UAA) the AGG codons abolishes the frameshift. The change in the reading phase occurs exclusively to the +1 direction. Efficient frameshifting is also induced by the sequence A-AGA-AGA-U. The arginine codons AGG and AGA are read by minor tRNA. Suppression of frameshifting takes place when a gene for minor $tRNA^{Arg}$ is introduced on a multicopy plasmid. We suggest that frameshifting during translation of the A-AGG-AGG-U sequence is due to the erroneous decoding of the tandem AGG codons and arises by depletion of $tRNA^{Arg}$. The complementarity of tandem AGG codons to the 3′ terminus of 16S rRNA is a coincidence and apparently not related to the shift. Replacing the AGG-AGG sequence by the optimal arginine codons CGU-CGU does not increase the overall rate of translation.**

The error accompanying the translation of mRNA into protein is on the order of 5×10^{-4} per codon (1). This degree of accuracy does not simply reflect the specificity of codon–anticodon interaction but is obtained through fidelity-amplification processes that involve a substantial mass of the ribosome (2–4). One such error-suppressing activity, for which evidence has been presented, is a GTP-consuming proofreading step at the level of aminoacyl-tRNA binding (5, 6). The active role of the ribosome in setting the translational accuracy is confirmed by the existence of mutations in ribosomal protein genes that profoundly affect the error rate. For instance, mutations in ribosomal protein S12 that confer resistance to streptomycin result in a greatly increased fidelity (ref. 7 and references therein).

A second potential source of translation errors is an unbalanced supply of aminoacyl-tRNA. Since the accuracy of selection is basically proportional to the ratio of correct to incorrect substrates available, it follows that limitations in one or more amino acids could drastically increase the error at those codons for which aminoacyl-tRNA is in short supply. Such an increase has indeed been found *in vitro* and in *relA* mutants, but in wild-type bacteria this error is suppressed by the induction of guanosine 3′-diphosphate 5′-diphosphate (ppGpp) (8–10). ppGpp is produced on the ribosome by the *relA* gene product when, due to aminoacyl-tRNA shortage, uncharged tRNA binds to the ribosomal aminoacyl-tRNA (A) site.

In spite of this degree of sophistication, the error-control mechanism is, by accident or by design, not at all foolproof. We describe here an example of the breakdown of this elaborate system in wild-type *Escherichia coli* cells. The presence of two consecutive AGG codons in a reading frame raises the ambiguity level by 3 orders of magnitude. Our results identify $tRNA^{Arg}$ shortage as the cause of frameshifting. Bacterial mechanisms to prevent such errors do not appear to exist.

This is not the first time that an abnormally high error rate has been found *in vivo* at a single position. Recent studies (reviewed in ref. 11) have shown that some messengers contain the information for the desired protein in two different reading frames and that, accordingly, specific phase changes must, and in fact do, occur. Notably, during translation of the *E. coli* mRNA encoding peptide chain-release factor 2 (RF2), shifts of up to 50% have been reported (12). Apparently, nature itself has found ways to bypass the rules of triplet decoding. We will shortly discuss differences and similarities between shifts at naturally occurring sequences and the one we describe here.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. In all experiments the *E. coli* K-12 strain M5219 was used (13). This strain harbors a defective, nonexcisable λ prophage carrying a mutant *c*I gene (*c*Its857) and the gene for the transcription antitermination factor N. Cultures were grown in L broth, which contained (per liter) 10 g of tryptone, 5 g of yeast extract (Difco), 8 g of NaCl, and 5 ml of 1 M Tris (pH 7.3). Cells were grown at 28°C to an OD_{650} of 0.25 and then induced at 42°C for 30 min.

Plasmids. In clones 1, 2, and 3 the plasmids were derivatives of p13.3 (14). Clones 4–21 were derived from pPLc236 (13). In all clones the MS2 cDNA present was the *Eco*RI fragment containing the sequence 103–1628 (MS2 numbers) (15).

Recombinant DNA Procedures. Standard cloning procedures were used as described (16). Restriction enzymes, bacteriophage T4 DNA ligase, and *E. coli* DNA polymerase I (Klenow) were obtained from Pharmacia. Unphosphorylated, annealed oligonucleotides were inserted into linearized vector DNA at a 250-fold molar excess. The nucleotide sequence of cloned inserts was determined in the phage M13 system developed by Messing and Vieira (17). Only in one instance (clone 7) was the sequence different than projected.

Radiolabeling of Proteins. Cells were grown in M9CA medium (16). After induction at 42°C, 30 μCi (11.1×10^5 becquerels) of [^{3}H]valine (New England Nuclear) was added and growth continued for 30 min at 42°C. The pellet of 1 ml of cells was dissolved in 25 μl of 2× Laemmli sample buffer (18) and analyzed as indicated in the text.

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Abbreviations: ppGpp, guanosine 3′-diphosphate 5′-diphosphate; A site, aminoacyl-tRNA site; RF2, peptide chain-release factor 2.

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RESULTS

The Sequence A-AGG-AGG-U Induces a +1 Frameshift. During our efforts to express the rat interferon α_1 gene in *E. coli* by translational reinitiation, we constructed the clone depicted in Fig. 1. Our strategy was that the efficiently translated coat-protein gene of RNA phage MS2 will direct a large proportion of the cell's ribosomes to the stop codon present 5 bases downstream of the start codon of the interferon gene. There is a variable probability that terminating ribosomes will reinitiate at a nearby start codon (refs. 19–22 and references therein). In an attempt to boost the reinitiation efficiency, an 8-base-long Shine–Dalgarno region (AAGGAGGU) was placed before the interferon start. Immunoblots of cell extracts of this clone showed that beside mature interferon, a coat–interferon fusion protein was produced that could only have arisen by a +1 frameshift. Fig. 2 shows an immunoblot of this clone (no. 1) developed with antibodies against the MS2 coat protein. The upper indicated band corresponds to the fusion product, and the lower band corresponds to the coat-protein fragment that is produced by termination just behind the interferon start.

To roughly identify the region where the shift had occurred, we placed a UAA stop codon before the SD region (clone 2); as shown in Fig. 2, this change abolished the frameshift product. In clone 3, the guanine residues in the SD region were replaced by pyrimidines (AAGGAGGU → AACUAUCU). This change also prevented appearance of the frameshift product. We consider these results as good indications that the frameshift originates in the AAGGAGGU region.

Since the construct shown in Fig. 1 is not convenient to assay large numbers of sequences for their capacity to change the ribosomal reading frame, we set up a simpler system (outlined in Fig. 3) in which the same coat gene fragment is present but the test sequence can be inserted as an *Eco*RI–*Bam*HI fragment. Translation beginning at the coat gene can proceed through the test sequence until the first in-phase stop is encountered 117 nucleotides inside the vector. This leads to the synthesis of a carboxyl-extended coat protein of 147 amino acids. The positioning of either a +1- or a −1-frame stop downstream of the SD region will reveal shifts in reading frame by the appearance of a protein of 106 amino acids. Both proteins can be unambiguously identified on immunoblots.

Let us first consider clone 4, which carries the archetypal test sequence AAGGAGGU in combination with a +1-frame

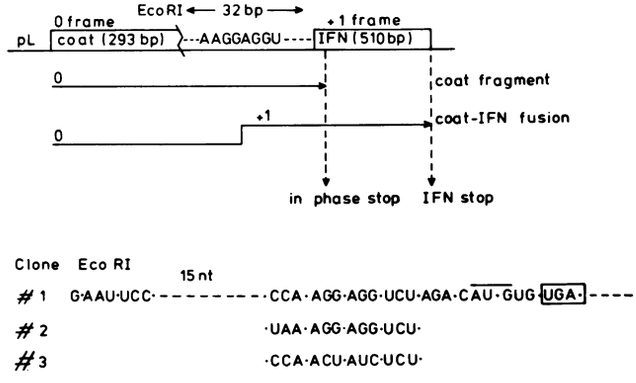

FIG. 1. Schematic presentation of clones 1–3. The coat gene of phage MS2 is fused out-of-frame to the leader of the α_1-interferon (IFN) gene of the rat. Translation originating at the coat gene will terminate at the boxed UGA codon, 5 nucleotides (nt) downstream of the overlined AUG start codon of the interferon gene. The complete construct is placed on a plasmid behind the thermoinducible P_L promoter of phage λ. Throughout this paper a +1 frame is defined as a 1-nt shift toward the 3′ direction in the mRNA. Dots indicate the reading frame in phase with the coat gene. bp, Base pairs.

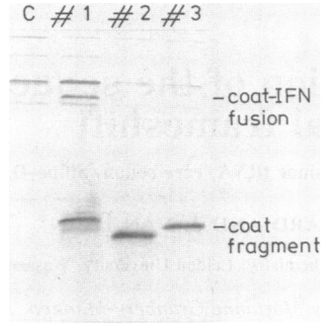

FIG. 2. Immunoblot analysis showing the presence of a coat–interferon fusion protein in clone 1. Stronger staining of the upper background bands in the control (C) and clone 1 lanes reflects higher cell extract loading. The relevant sequences of clones 1–3 are shown in Fig. 1. In lane C the coat gene fragment is cloned in the opposite orientation. An antiserum against MS2 coat protein was used and the procedure described by Towbin *et al.* (23) was followed to develop the blot.

stop codon (Fig. 3 and Table 1). Immunoblot analysis of this clone gave two bands (Fig. 4). The upper one was identified as the 147-residue coat protein, because it had the correct mobility and was absent in the control (lane C). The lower band needed identification as the product originating from out-of-phase termination at the overlined ochre (UAA) codon (Table 1). It was not a proteolytic degradation product of the 147-amino acid protein, because when we substituted the arginine codons AGG-AGG for their synonyms CGU-CGU the lower band disappeared (Fig. 4, clone 6). In addition, when the stop codon in the +1 frame was eliminated as in clones 5 and 7, the lower band disappeared (Table 1). The absence of the lower band in clone 5 (data not shown), in which the +1 stop is replaced by a −1 stop, shows that shifts do not occur to the −1 direction. Apparently, frameshifting takes place and is related to the AAGGAGGU sequence. The question is whether the translation error is due to the features of this sequence that are complementary to rRNA or due to the decoding of the two consecutive AGG codons for which very little tRNA is present (24). We shall refer to these two possibilities as the complementarity model and the decoding model, respectively.

Frameshifting Is Due to Decoding the AGG-AGG Sequence. We have mutated the first and/or the second adenine of the two AGG codons to uracil. This breaks up the demand for the minor tRNA but leaves complementarity to 16S rRNA largely intact (clones 8, 9, and 10). These substitutions fully abolish frameshifting, implicating the decoding of the AGG codons as

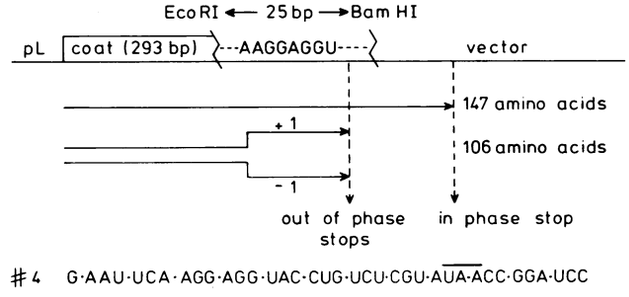

FIG. 3. Schematic presentation of our plasmid-borne frameshift assay system. The coat gene fragment of the MS2 phage directs translation as indicated by the straight arrowheaded line. Shifts that occur at the AAGGAGGU sequence will yield premature translation termination. The sequence presented below the diagram is the *Eco*RI–*Bam*HI fragment used to construct the archetypal clone, no. 4, carrying a stop codon (overlined) in the +1 frame. Dots indicate the reading frame in phase with the coat gene.

Table 1. Relevant sequences of clones used in this study

Clone	Sequence	Frameshift peptide
4	UCA–AGG–AGG–UAC–CUG–UCU–CGU–AUA–ACC–GGA	+
5	AGG–AGG U–AAA–ACC	−
6	CGU–CGU U–AUA–ACC	−
7	AGG–AGG U–ACA–CCC	−
8	AGG–UGG U–AUA–ACC	−
9	UGG–AGG U–AUA–ACC	−
10	UGG–UGG U–AUA–ACC	−
4	UCA–AGG–AGG–UAC–CUG–UCU–CGU–AUA–ACC–GGA	+
11	UCC–AAG–GAG–GUA–CUG U–AUA–ACC	−
12	UCC–AAG–GAG–GUA–CUG U–AAA–ACC	−
13	UCU–CAA–GGA–GGU–ACU–CUG–UCU–CGU–AUA–ACC	−
14	UCU–CAA–GGA–GGU–ACU U–AAA–ACC	−
4	UCA–AGG–AGG–UAC–CUG–UCU–CGU–AUA–ACC–GGA	+
15	AGA–AGA U–AUA–ACC	+
16	AGA–AGA U–AAA–ACC	−
19	AGG–AGG–UAA U–AUA–ACC	+
20	UCA–CCU–CCU–GCA–AGC–UUC–CGU–AUA–ACC–GGA	−
21	CCU–CCU U–AAA–ACC	−

Sequences shown are those present between the *Eco*RI and *Bam*HI sites of the construct outlined in Fig. 3. The A → C change in clone 7 at nucleotide 25 is an error in the synthesized oligomer. Hyphens indicate the reading frame. Out-of-phase stops are overlined. The +1 shifts occurring in clones 5 and 7 yield a coat-like protein deriving its carboxyl-terminal region from a random reading frame in the vector. This product is unstable and produces smears in immunoblots.

the error source (Fig. 4). More subtle variations have been introduced in clones 11–14. Here, we have maintained full complementarity while changing the frame in which ribosomes pass over the AAGGAGGU sequence. In clones 11 and 12 this was achieved by the insertion of 1 base before the arginine codons and a compensatory deletion behind these. In clones 13 and 14 the third remaining reading frame was realized by the insertion of 2 bases before the shifty sequence while 1 base was added behind it to bring the reading phase into register again. If shifts are triggered by rRNA·mRNA complementarity, they will persist even when the complementary sequence slides 1 base backward or forward with respect to the translating ribosome. However, in none of these four constructs did we detect a +1 or a −1 frameshift protein (Fig. 4, Table 1). This result strongly favors the decoding model.

Next, we made a C → A substitution in our standard clone no. 4, creating a stop codon directly behind, and in-phase with, the AGG-AGG sequence (clone 19). In-frame translation will yield a protein of 102 amino acids, whereas shifts to the +1 frame will, as before, give a polypeptide of 106 amino acids. The decoding model predicts the persistence of the shift in this construct because the arginine codons will be translated before the ribosome reaches the termination signal. In the complementarity model, however, a shift is not expected since the ribosome will have terminated before the AGG-AGG sequence has had the opportunity to interact with the 3′ terminus of 16S rRNA. The fact that frameshifting persists in clone 19 (Fig. 4) supports the decoding model. One could still argue that the putative rRNA·mRNA interaction takes place ahead (i.e., downstream) of the ribosomal decoding site. In this unlikely, though not impossible, spatial arrangement of mRNA and rRNA, the frameshift in construct 19 could be accounted for by the complementarity model. However, when a nonsense codon was placed immediately in front of the purine sequence (UAA-AGG-AGG) as we did in clone 2, we found no shift (Fig. 2). This result is at variance with the special version of the complementarity model but in agreement with the decoding model.

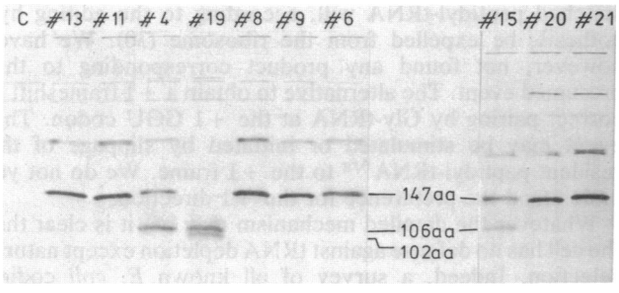

FIG. 4. Immunoblot analysis of several clones used in this study. The relevant nucleotide sequence of each clone is shown in Table 1. Control (C) is the same as in Fig. 2. The band visible in the middle of this blot is due to readthrough at the UGA stop present in the vector at the end of the zero reading frame. This band is absent in clone 19, where an in-frame ochre stop is present right after the AGG-AGG sequence. Ochre codons are known not to be leaky. Lengths of polypeptides are indicated in amino acids (aa).

Other Minor-tRNA Codons That Promote Frameshifting. The decoding model predicts that other minor-tRNA codons will likewise disturb triplet reading. Indeed, the translation of two successive AGA codons, decoded by minor $tRNA^{Arg}$, also triggers a high percentage of frameshifting (Fig. 4, clone 15). In contrast, the tandem minor-tRNA codons CCU-CCU do not trigger measurable frameshifting (Fig. 4, clones 20 and 21). Presumably, the corresponding tRNA is less minor than that for $tRNA^{Arg}$.

Frameshifting Is Abolished by Cloned $tRNA^{Arg}$ from Bacteriophage T4. Bacteriophage T4 carries several tRNA genes in its genome, one of which encodes $tRNA^{Arg}$. Its anticodon is UCU and the U in the wobble position is probably modified (25, 26). Overproduction of this tRNA in our constructs is expected to relieve the frameshift on tandem AGA codons (clone 15). Accordingly, we placed the tRNA gene, contained in a 640-bp *Eco*RI fragment (25), in the *Eco*RI site of the chloramphenicol acetyltransferase (*cat*) gene of plasmid pACYC184, which is compatible with the pBR322 derivatives used in this study. When the T4 $tRNA^{Arg}$ gene was cloned in the same orientation as the *cat* gene promoter the frameshift protein disappeared, but it persisted when the gene was cloned in the inverse orientation (Fig. 5, lanes 3 and 2,

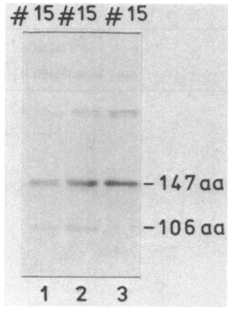

FIG. 5. Suppression of frameshift at double AGA codons by $tRNA^{Arg}$ (anticodon UCU) of bacteriophage T4 cloned in the compatible vector pACYC184 (16). Lanes: 1, unmodified vector pACYC184; 2, $tRNA^{Arg}$ gene cloned in the *cat* gene of pACYC184 against the orientation of the *cat* gene promoter; 3, $tRNA^{Arg}$ gene cloned in the *cat* gene in the same orientation as the *cat* gene promoter.

respectively). Similarly, frameshifting was not relieved by the empty vector pACYC184 (lane 1). In addition, a mutation introduced in the anticodon resulted in loss of frameshift suppression. These results confirm that the phase shift studied in this paper arises by tRNA depletion. We have also noted a substantial decrease in frameshifting when the mRNA concentration is kept low by partial induction of the P_L promoter.

Quantitation of Frameshifting. It is clear from the approximately equal staining intensities in the immunoblot shown in Fig. 4 (clone 4) that the probability to frameshift at double AGG codons is considerable. To quantitate this ratio, we labeled a culture with [^{3}H]valine and immunoprecipitated the cell extract with anti-coat antiserum. The pellets were electrophoresed in a $NaDodSO_4$/polyacrylamide gel, which was then autoradiographed (Fig. 6). According to the DNA sequence, the two coat-derived proteins contain the same number of valine residues. The bands in lane 2 (clone 4) were cut out and their relative radioactivities were determined as 47:53 (upper/lower), showing that about half of the ribosomes lose the right frame in passing the AGG-AGG element. It was also of interest to know whether the total incorporation of amino acids was affected by using AGG-AGG instead of CGU-CGU (CGU is the statistically favored arginine codon

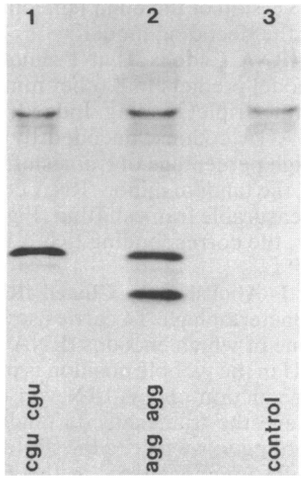

FIG. 6. Autoradiograph showing the correct and frameshift protein synthesized in clone 6 (lane 1), clone 4 (lane 2), and the control (lane 3). Labeling of cells is described in *Materials and Methods*. Radioactivity of gel slices was measured in a liquid scintillation counter. Values were corrected for differential gel loading.

and is recognized by a major tRNA species). Thus, we compared the total incorporation of valine in correct plus incorrect protein in clone 4 to that incorporated in the correct protein in clone 6 (Fig. 6, lanes 2 and 1, respectively). Surprisingly, total incorporation was the same. Apparently, the shortage of $tRNA^{Arg}$ that triggers frameshifting is not reflected in the number of amino acids polymerized per ribosome per time unit.

DISCUSSION

Mechanism of Frameshifting. The presence of two consecutive AGG codons in a reading frame leads to 50% +1 ribosomal frameshift. This error appears not to be related to the complementarity of the sequence AAGGAGGU to the 3′ terminus of 16S rRNA but originates during the decoding of the tandem codons, recognized by a minor $tRNA^{Arg}$. An error level of 50% at a single position is extreme for a bacterium equipped with the natural machinery to control such misfortune (i.e., our strain is *relA*$^+$). In addition, M5219 is streptomycin-resistant, a condition that further increases translational fidelity. Furthermore, the cells were not starved for arginine.

Varenne and Lazdunski (27) presented calculations showing that the depletion of a minor tRNA is greatly increased when the corresponding codons occur in tandem rather than scattered through the message. Our finding of suppression of frameshifting by overexpression of T4 $tRNA^{Arg}$ is in perfect agreement with the predictions of these calculations. We suggest, therefore, that when the ribosome has the first AGG codon and the corresponding peptidyl-$tRNA^{Arg}$ bound to its peptidyl-tRNA (P) site, there is a serious shortage of cognate Arg-$tRNA^{Arg}$ for the second AGG codon now exposed in the A site. In this situation we envisage that competition with noncognate tRNA becomes overwhelming and errors cannot be avoided.

One possible cause for the extremely high error rate is that, unlike in amino acid starvations, the cell cannot react by the synthesis of ppGpp because the circumstances in our experiment do not yield uncharged tRNA but result rather in shortage of one tRNA species. Previous *in vitro* experiments have indeed shown that when an unbalanced supply of normal tRNAs is forced by the addition of an excess of one tRNA species, a high level of frameshifting is induced (28).

In the absence of the sequence of the frameshift protein we can only speculate on a mechanism that causes ribosomes to slip on the AGG-AGG sequence. Basically, two possibilities may be considered. One is an aminoacyl-tRNA mismatch at the second in-phase AGG codon, which, once transpeptidation is completed, may lead to erroneous translocation (29). This possibility seems somewhat unlikely because mismatched peptidyl-tRNA will, according to the editing hypothesis, be expelled from the ribosome (30). We have, however, not found any product corresponding to this presumed event. The alternative to obtain a +1 frameshift is correct pairing by Gly-tRNA at the +1 GGU codon. This event may be stimulated or initiated by slippage of the resident peptidyl-$tRNA^{Arg}$ to the +1 frame. We do not yet understand the preference for the +1 direction.

Whatever the detailed mechanism may be, it is clear that the cell has no defense against tRNA depletion except natural selection. Indeed, a survey of all known *E. coli* coding regions (GenBank, tape release 44) shows the existence of 10 AGGAGG sequences, none of which has the reading frame AGG-AGG.

tRNA Shortage Affects Fidelity but not Efficiency of Translation. An interesting aspect of our study is that the apparent shortage of a tRNA species does not decrease the rate of amino acids incorporated by the ribosome (Fig. 6). This shows that the ribosomal pause at the "hungry" AGG codon

is short compared to the time required for initiation of translation. In other words, even in the presence of the tandem AGG codons, ribosome loading at the highly expressed coat gene is the rate-limiting step in product formation. This seems to imply that an organism cannot meaningfully control its gene expression by the exploitation of minor-tRNA codons. Such a strategy may only decrease the number of good proteins at the price of synthesizing an increased amount of faulty product. In fact, selection against minor-tRNA codons in highly expressed genes may in part occur to avoid the generation of such nonsense proteins. Several researchers have indeed concluded from other sorts of evidence that the cell does not use minor-tRNA codons to restrict the rate of protein production at the level of elongation (31, 32).

Our results also relate to a report by Robinson *et al.* (33) that the insertion of a row of AGG codons in the *cat* gene decreases the efficiency of translation as measured by the yield of Cat enzyme. This result is consistent with our finding that the AGG codons reduce the amount of correct product. Presumably, the reduction in Cat enzyme synthesis must be attributed to the derailment of a substantial fraction of the ribosomes rather than to a rate-limiting ribosomal pause. We note, however, a report by Misra and Reeves (34) claiming the relief of a translational pause site at a single AGA codon in the *tolC* gene by cloned $tRNA^{Arg}$ from bacteriophage T4.

Natural Frameshifts. Translational frameshifts are no longer a laboratory curiosity. Many examples have been found where the information for a protein is encoded in two different reading frames. Accordingly, organisms must exploit and control reading shifts to synthesize the intact protein. Among the well-studied cases are the RF2 gene of *E. coli* and the polyproteins encoded in retroviral RNA and the Ty transposon of yeast (reviewed in ref. 11). The *E. coli* RF2 messenger contains an in-frame UGA stop codon preceded at 6 nucleotides by the sequence AGGGGG. The stop codon marks the position where the coding information for the release factor continues in the +1 frame. Ribosomes shift at the stop codon to this +1 frame when RF2 is in short supply (12). Weiss *et al.* (35, 36) have presented strong evidence that the AGGGGG sequence contributes to the shift by interacting with the 3′ terminus of 16S rRNA. The stop codon enhances the shift, possibly by creating a pause, which may provide the time for the Shine–Dalgarno interaction. Clearly, this mechanism differs basically from the one described here. Our shift occurs during the decoding of tandem rare codons due to tRNA limitation. In spite of these different mechanisms, there seems to be one common feature. In both examples the shift occurs when there is no or not enough substrate for the vacant ribosomal A site. In our study the vacancy results from $tRNA^{Arg}$ depletion, but during RF2 messenger translation it is the release factor that is in short supply. Apparently, substrate shortage for a vacant A site is a highly error-prone state for the ribosome.

The frameshifts that produce *gag*-related fusion proteins in Rous sarcoma virus and mouse mammary tumor virus RNA (37, 38) also arise at an in-frame stop codon. It is possible that here, too, the empty A site triggers rephasing. The secondary structure element found downstream of the stop codon that reportedly cooperates in the shift may function as a steric barrier that prevents the binding of the translation termination factor to the ribosomal A site facing the stop codon. As a result competition with noncognate tRNA may be drastically amplified.

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