MICROBIOLOGICAL REVIEWS, Sept. 1989, p. 273–298
0146-0749/89/030273-26$02.00/0

Vol. 53, No. 3

Errors and Alternatives in Reading the Universal Genetic Code

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INTRODUCTION

The fidelity of biological information flow is critical. Deoxyribonucleic acid (DNA) replication, with its attendant proofreading and repair processes, has an error level in the range of 10^{-8} to 10^{-11} (71, 183). Different kinds of mutations occur at different frequencies, and sequences are known in which errors occur preferentially and at a higher frequency (see, e.g., references 15, 80, and 268). However, in an average *Escherichia coli* cell the great majority of genes will have been copied error free. For such a cell to function correctly, each of the subsequent steps in information flow from DNA to protein must also occur with reasonable fidelity. However, it is not obvious what constitutes reasonable fidelity. From the relatively few measurements of the accuracy of transcription, it seems that this process is much less accurate than DNA replication, if only because of the apparent lack of repair pathways. The in vivo error level for transcription may be on the order of 10^{-4} (258), and such errors may also be context dependent (259).

The universal genetic code was elucidated in the late 1960s primarily by in vitro biochemical means (see reference 324). It is substantiated by an enormous and growing body of data generated by comparing DNA and protein sequences. Therefore, given a DNA sequence, it seems simple to predict the sequence of its protein product. Indeed, reputable journals contain articles that routinely discuss "codons" such as TAT or ATG, as if ribosomes in cells read DNA directly. There can be dramatic pitfalls in this practice. Intron splicing creates rather large differences between transcripts and messenger ribonucleic acids (mRNAs). More subtle RNA editing processes, in which there are specific insertions or deletions of one or a few bases internally in a transcript (14, 273), or even a specific base substitution (47, 63, 247), can also occur. Knowledge of these different RNA-processing and editing mechanisms adds an element of caution when translating DNA directly to protein (and the mechanisms themselves introduce new possibilities for error). Note that in each case the problem can be overcome if the sequence of the mRNA is known. Although this is also true of protein made from mRNA containing transcriptional or processing errors, these few error-containing mRNAs will almost certainly be impossible to detect. In addition, several organisms and organelles that use genetic codes slightly different from the universal code have now been discovered. In each of these codes there are one or a few codons which have different assignments. These alternative codes have recently been reviewed by Fox (85). Alternative codes are translated correctly only when the translational apparatus has appropriate and corresponding predictable changes. As is the case for RNA-processing or transcriptional errors, knowledge of the mRNA sequence and the appropriate codon table is required for predicting protein sequence.

However, even disallowing posttranslational modifications, proteins whose primary sequence cannot be predicted from the most simple reading of the RNA code will be synthesized in the cell. Some of these will arise because of errors in the translation process. Errors are of interest because they allow insight into the normal functioning of the translational machinery and its evolution. However, not all differences between the predicted sequence of a protein and its actual sequence are the result of errors (see below). In some cases it is clear that certain codons or mRNA sequences can have alternative readings and that organisms have taken advantage of this flexibility in reading the genetic code.

The primary concerns of this review are the types of errors

and alternative readings that occur in cells using the universal code, the frequencies with which they occur, and some of the factors that influence these frequencies. There have been many excellent reviews of translational fidelity, such as those of Buckingham and Grosjean (36), Kurland and Gallant (163), and Yarus and Thompson (328). Several other recent reviews deal with certain aspects of the topics covered here and will be referred to at appropriate locations in the text.

MISSENSE ERRORS

The substitution of one amino acid for another is the archetypal error in protein synthesis. Coding ambiguity was noticed almost as soon as in vitro polypeptide-synthesizing systems were developed (33), and the use of simple synthetic mRNAs effectively excludes the study of other errors. Such substitutions are called missense errors, as an analogy with missense mutations. Missense errors are the result either of an erroneously charged transfer RNA (tRNA) being used or of an anticodon-codon mismatch on the ribosome. The former is known as misacylation or mischarging, and I shall refer to the latter below as misreading. Originally, missense errors were studied almost exclusively by using in vitro systems (see reference 324), and much important work on accuracy in protein synthesis must still be done in vitro, particularly in dissecting the various kinetic parameters involved, testing models, and examining errors that occur at a very low frequency. However, the purpose of this review is to disclose what is known about errors that occur in vivo. Therefore, in vitro data are discussed only when they shed light on particular events that occur in living cells.

Amino acid substitution during protein biosynthesis is difficult to detect because the aberrant protein has essentially the same size and amino acid composition as the native protein. Such errors can be most readily detected if the substitution involves an amino acid that does not normally occur in a peptide or protein or if the misincorporation changes the normal electrophoretic or chromatographic behavior of a protein. The assays used are often specific; i.e., only a very few of all possible errors can be detected. After detection of misincorporation, it is necessary to determine whether misacylation or misreading was involved (such determinations often also exclude errors in transcription). For *E. coli* this determination is greatly aided by the availability of restrictive *rpsL* mutations. These mutations lead to alteration in ribosomal protein S12 and result not only in streptomycin resistance, but also in a reduction of a variety of translational errors both in vivo and in vitro (reviewed in reference 103). Therefore, if the amount of misincorporation is decreased in such *rpsL* mutants, it is strong evidence that misreading events on the ribosome are involved. In addition, errors can be increased by aminoglycoside antibiotics, such as streptomycin and neomycin, and mutants with a Ram phenotype (ribosomal ambiguity), which also antagonize the *rpsL* restriction (reviewed in reference 103). In some cases the decision is based on assumptions about the likelihood of an error. Early in vitro work showed that the most likely misreading errors involved a single-base-pair mismatch between the codon and anticodon and that this relationship was maintained when the error-inducing antibiotic streptomycin was used (64). Misacylation presumably involves amino acids that are structurally related (reviewed in reference 82), but whose codons may not be. Nonetheless, the fact that closely related amino acids tend to have closely related codons can make such interpretation difficult.

Misacylation

The aminoacyl-tRNA synthetases recognize their substrates with a high degree of accuracy, and several have one or more proofreading steps (reviewed in reference 82). However, careful analysis indicates that some errors involving closely related amino acids may occur in the frequency range of 4×10^{-4} to 5×10^{-5} (179, 220), and some substitutions at this level should be detectable. The mechanism by which the synthetases recognize their tRNAs is currently being intensively studied (reviewed in reference 325). As part of these studies, a number of synthetic tRNAs with a low accuracy of charging have been created and are able to support the incorporation of more than one amino acid at a given codon in vivo (194).

The classic work on in vivo missense errors is that of Loftfield (184) on the misincorporation of the branched-chain amino acids into certain peptides of chicken ovalbumin. Although considerable uncertainties existed, it was concluded that valine could substitute for isoleucine at a frequency of about 3×10^{-4}. Similar studies with rabbit hemoglobin also showed misincorporation of valine at a similar frequency, i.e., 2×10^{-4} to 6×10^{-4} (185). Although at the time it was presumed that the errors involved first-position misreading of the isoleucine codons (AUU, AUC, or AUA) as valine (GUN), it has been shown by sequence analysis that at least at one site the valine was incorporated at a threonine codon (S. F. Coons, L. F. Smith, and R. B. Loftfield, Fed. Proc. **38**:328, 1979). Since the valine and threonine (ACN) codons are dissimilar, it seems likely that this error was misacylation.

A common motif in mistranslation is the occurrence of errors in cells whose metabolism is unbalanced. An example of mischarging in unbalanced cells involves the glutaminyl-tRNA synthetase of *E. coli*. This enzyme misacylates several mutant tRNAs and in vitro can use an amber-suppressing derivative of $tRNA^{Tyr}$ encoded by *supF*. Cells that are made to overproduce wild-type glutaminyl-tRNA synthetase do misacylate the *supF* tRNA, and this misacylation can be prevented by simultaneously overproducing $tRNA^{Gln}$ (288). There is no evidence of appreciable glutamine-for-tyrosine substitution in cells with wild-type tRNA, but cells containing a less accurate mutant synthetase do show a reduced growth rate (132).

The gram-positive bacteria, and at least some cyanobacteria, archaebacteria, and plant and animal mitochondria, apparently have no glutaminyl-tRNA synthetase and generate $Gln\text{-}tRNA^{Gln}$ by first charging $tRNA^{Gln}$ with glutamate and then using an amidotransferase (168, 269, 319, 320). The intermediate, $Glu\text{-}tRNA^{Gln}$, could present severe problems for the accuracy of protein synthesis if it is released by the synthetase and is thus available for protein synthesis, but there are no reports of an abnormally high level of glutamate for glutamine substitution in any of the systems. As discussed by Schön et al. (269), misincorporation by the free intermediate could be prevented by discrimination by elongation factors (EFs) or on the ribosome.

The study of the incorporation of amino acid analogs is far too extensive to be reviewed here. However, there is one recent report that illuminates the unexpected metabolic flexibility of unbalanced or stressed cells. When *E. coli* is used to produce very high levels of bovine somatotropin, a polypeptide particularly rich in leucine, the drain of leucine pathway intermediates apparently leads to the biosynthesis of norleucine (22). Norleucine is a methionine analog, and

these cells incorporate it randomly into protein in place of methionine at levels up to 14%.

Misreading

There are also reasonable amounts of information on specific codon-misreading errors that occur in vivo, and in many cases the search for these errors has been guided by inference from the in vitro systems. Only a few examples from *E. coli* have been rigorously demonstrated to be misreading errors, but there are several others for which there is strong evidence, and some of these are from eucaryotes.

The first measurements in *E. coli* involved the misincorporation of [^{35}S]cysteine into flagellin (74). This amino acid is not encoded by the *hag* gene (165). Edelmann and Gallant (74) found it to be present at 6×10^{-4} mol/mol of flagellin and, in addition, found that this misincorporation was increased approximately 10-fold when streptomycin and neomycin were used. To attempt to determine the actual nature of the substitution, they took advantage of previous work on the accuracy of translation in *relA* mutants of *E. coli*. These mutants are termed relaxed, because of their altered regulation of stable RNA synthesis during amino acid starvation (reviewed in reference 39). During amino acid starvation, relaxed strains synthesize defective, full-length protein, presumably as a result of missense errors (107). It could be predicted that if the cell was starved for the amino acid cognate for the codon being misread, the incorporation of cysteine would be increased. Since arginine limitation increased the incorporation fivefold, they reasoned that the substitution was a first-position misreading error of the arginine codons CGU and CGC as the cysteine codons UGU and UGC (74). Since there are 11 such codons in *hag* (10 CGUs and 1 CGC), this would give an average misreading frequency of 5×10^{-5} per codon in unstarved cells.

Since cysteine is used infrequently in protein, a number of other experimenters have used similar techniques to test for misincorporation (see, e.g., references 5, 170, and 251). In each case demonstrable misincorporation was found. Although the amount of misincorporation could usually be increased or decreased by using antibiotics and/or mutants in a predictable fashion, the amount of change was usually much smaller than that observed by Edelmann and Gallant (74). There are considerable technical difficulties with these labeling experiments, but it is possible that several different types of missense errors were being observed in addition to a misreading of arginine codons. These include misreading of the codons for tryptophan (UGG [see below]), and phenylalanine (UUU and UUC [170]), as well as possible misacylation.

A very similar set of experiments was done by Bouadloun et al. (28) with the ribosomal proteins S6 and L7/L12. However, peptide analysis of the labeled proteins allowed the site of the misincorporation to be identified and quantitated. The arginine codon CGU in L7/L12 was misread at a frequency of 10^{-3}, and the tryptophan codon in S6 was misread at a frequency of 3×10^{-3} to 4×10^{-3}. The misincorporation at the UGG codon was affected in a predictable way by ribosomal mutations. However, the misincorporation at the CGU was reduced only threefold by a restrictive *rpsL* allele and was not changed significantly by a *rpsD* mutant with a Ram phenotype. Also, misreading of the UGG was reduced 30-fold in a *miaA* mutant, which has an undermodified $tRNA^{Cys}$, but misreading of the CGU was unchanged (29). Mischarging of $tRNA^{Arg}$ cannot explain the incorporation at CGU, because such a high level of error at this commonly used codon should lead to demonstrable charge heterogeneity of most *E. coli* proteins, and this is not seen (see Fig. 1 in reference 228). The same argument can be used to establish that 10^{-3} cannot be the misreading frequency of all such codons, and this is further confirmed by the results of Edelmann and Gallant (74) discussed above. Therefore, it seems likely that this is a particularly error-prone context and that codons in such a context may be less susceptible to restriction and enhancement. The exact nature of the context is unclear but may involve both 5′ sequences and the 3′ codon GGC (169), although other codons in a similar context are mistranslated at expected, albeit high, frequencies and are restricted by *rpsL* mutations (139, 248). Of course, it is conceivable that aberrant flagellin is not incorporated into flagella at a high efficiency and therefore the measurements of Edelmann and Gallant represent a minimum, but there is no evidence of this.

On the basis of in vitro measurements, Woese (324) predicted that most misreading errors occur at the third position of the codon. This position is, of course, the most degenerate, so that most such misreading will not lead to an amino acid substitution (167). Following this prediction, and with the knowledge that *relA* mutants have increased levels of mistranslation, O'Farrell (219) sought to specifically increase the number of misreading errors that could be analyzed as charge heterogeneity of the mistranslated protein on polyacrylamide gels. He found that starvation for histidine (encoded by CAU and CAC) in *relA* mutants caused high levels of missense errors, apparently because of third-position misreading by glutaminyl-tRNA (CAA and CAG). He also found some misreading during arginine starvation, which might have been first-position errors (i.e., cysteine incorporation), since the arginine codons subject to potential third-position error, AGA and AGG, have subsequently been found to be rarely used in most *E. coli* genes (192). We confirmed the finding with histidine starvation and expanded it to asparagine starvation (232), in which the asparagine codons, AAU and AAC, are apparently misread as lysine, AAA and AAG. In addition, we demonstrated an identical pattern of starvation-induced charge heterogeneity in cultured mammalian cells (see below).

Investigators in my laboratory have continued to study these errors, and there is now considerable information on the substitution of lysine for asparagine. By sequence analysis of lysine-labeled, mistranslated MS2 coat protein synthesized in unstarved cells, we have demonstrated that this error occurs at a frequency of approximately 2×10^{-3} at AAU codons and 4×10^{-4} at AAC codons (231). The errors are strongly reduced in *rpsL* mutants (229), increase approximately 10-fold when otherwise wild-type cells are starved, and increase approximately 100-fold when *relA* mutants are starved (139, 230). Indeed, during starvation, the frequency of misreading of AAU codons can increase to above 0.5 in relaxed strains. This error can also be increased by polyamine deprivation (195). Asparagine starvation increases the errors proportionally at the two codons (139), as does treatment with streptomycin (140). We have found that context effects do exist, but are only about 2-fold, whereas an interchange of the codons AAU and AAC leads to an approximately 10-fold change in the error level (248). Data from MS2 and Qβ coat proteins indicate that the average error rate at an AAU codon in *E. coli* may be as high as 5×10^{-3} (149, 231, 248). What we presume to be the opposite error, substitution of asparagine for lysine, occurs only in lysine-starved cells and then only at a frequency of 8×10^{-3}

(228). If this also represents a 100-fold increase, it must occur at a frequency of 10^{-4} or below in normal cells.

An elegant activity assay has shown that the glycine codon GGC has been misread as serine (297). An AGC codon specifying an essential serine residue in β-lactamase was changed to GGA and to GGC, both of which are glycine codons. Both mutant *bla* genes produced equivalent amounts of protein, but activity could be found only in cells that contained the GGC-containing allele. The activity was increased in *rpsD* mutants with a Ram phenotype, but was unaffected by an *rpsL* allele. The effect of *rpsD* and the fact that no error at GGA was detected provide convincing evidence that misreading of the GGC codon is involved, probably by the $tRNA^{Ser}$, which normally reads AGU and AGC. The misreading frequency observed was approximately 10^{-3}. A frequency of at least 10^{-4} could have been detected, which thus is a maximum estimate of both misacylation and misreading of GGA. This is also the clearest example of a misreading error not affected by a restrictive *rpsL* allele.

There are two examples in which misincorporation is known to occur but the nature of the substitution is unknown. Histidine incorporation into MS2 coat protein, whose gene contains no histidine codons, has been measured in vivo at a level of about 1.4 mmol/mol of coat (195). This level is increased with streptomycin addition and decreased in *rpsL* strains. Although it is clearly a misreading error, there are 35 positions in the gene where a single-base mismatch could occur (195). A similar level of histidine misincorporation was seen in the coat protein of Qβ, but this was unaffected by a Ram mutation (149). Tryptophan misincorporation was also detected in Qβ coat protein (149).

Many of the examples of misreading discussed above have been most intensively studied in stressed cells, in which the error frequency was abnormally high. However, with one exception, the basal-level frequency of these errors in normally growing cells was also measured. Examples in which the error has been detected only in stressed cells are discussed here. In some cases it seems very likely that these errors occur at such low levels in normal cells that current methodology would fail to detect them, whereas in others the nature of the substitution makes the assay difficult.

As mentioned above, histidine starvation induces missense errors that appear to be glutamine substitutions. These errors are reduced in *rpsL* mutants and in *hisT* strains (227). The *hisT* strains are missing pseudouridine in the anticodon loop of a number of tRNAs (reviewed in reference 20), including $tRNA^{His}$ and both $tRNA^{Gln}$ isoacceptors, strongly supporting a misreading event. On the basis of the amount of heterogeneity observed in different proteins, we believe that the histidine codon CAU is also misread more frequently than CAC (231) and should be detectable in unstarved cells. However, deamidation artifacts in simple preparation can mimic the expected charge change, and as yet there are no sequencing data to support this hypothesis.

The codons AAC and CAC are preferred over their partners in highly expressed genes, although in each case only a single tRNA is involved (130). We postulated that part of the selective pressure that established this preference was the avoidance of mistranslation (231). The phenylalanine codon UUC is also preferred over UUU in *E. coli*. Although leucine-for-phenylalanine substitutions do not yield protein with charge changes, they can be analyzed by sequencing protein made during phenylalanine starvation of *relA* mutants if high error levels are obtained. Unexpectedly, in our original experiments with the *argI* gene, we found misreading of a UUC encoding residue 8 but not of the UUU at residue 3 (233). Recently we have shown that misincorporation at residue 8 is observed whether the codon is UUC or UUU, and we cannot detect misreading of either codon at position 3 (J. Precup, A. K. Ulrich, O. Roopnarine, and J. Parker, unpublished results). We have no further information on the nature of this strong context effect, nor have we been able to measure the basal level of error at either codon. We believe that phenylalanine starvation in the MS2 coat protein induces a frameshift (233), which is known to happen in other genes (see below), but if so, this frameshifting event seems insensitive to a restrictive *rpsL* mutation. Laughrea et al. report unpublished experiments showing that cysteine can be incorporated at phenylalanine codons during phenylalanine limitation, confirming their inference that such errors also occur in unstarved cells (170).

An example of how cells stressed by protein overproduction can make errors is given above. A similar example has been found in *E. coli* producing insulinlike growth factor 1. High-level expression of a synthetic gene encoding insulinlike growth factor 1, but with codon usage optimized for *Saccharomyces cerevisiae*, yielded lysine incorporation of 5 to 12% at each arginine residue that could be measured: residues 21, 36, and 37 (271). Each of these was encoded by the normally rarely used AGA. This error can be inferred to be misreading from the fact that the tRNA corresponding to this codon is minor, and hence there should be an excess of synthetase. However, the lysyl-tRNA synthetase can mischarge some synthetic tRNAs (194), and so the possibility of misacylation cannot be eliminated. The error is codon specific in that it is not seen if the gene is optimized for *E. coli* and contains only CGU arginine codons. However, if in this latter case one of the CGU codons (position 36) is changed to AGA, no misreading is observed. This could be some type of context effect and/or be related to lower demand on the tRNA.

We have sequenced [^{35}S]methionine-labeled protein to detect basal-level misreading of the rarely used isoleucine codon AUA (L. Li and J. Parker, unpublished results). We have been unable to detect any misincorporation above background levels, indicating that in this case the error frequency was below 2×10^{-4}. We can detect an error at a frequency of about 3×10^{-2} at both AUA and the commonly used AUU codons during starvation (Li and Parker, unpublished). The low level of third-position errors by Met-tRNA seems to be related to the N-acetylation of the cytosine at the wobble position of its anticodon (282). I have also failed to find detectable misreading of the glutamine codons, CAA and CAG, and the serine codons, AGU and AGC, even during starvation (J. Parker, unpublished results).

The *E. coli* data described above are summarized in Table 1. In the few confirmed examples, misreading errors seem to occur most frequently at the first or third position of the codon and can involve both pyrimidines and purines. Second-position errors may also occur. Some errors clearly occur at or above a frequency of 10^{-3} in normally growing cells. It is possible that some of these high levels, such as the misreading of CGU, are context dependent, but this seems not to be the case with AAU. Many errors occur at a level closer to 10^{-4}, and misreading at many codons has so far been undetectable. It seems most likely that the average misreading error frequency may be well below 10^{-3} per codon. Nonetheless, some misreading occurs at frequencies well above this, and some particular tRNAs may be error prone, perhaps those for cysteine, lysine, and glutamine. The data are simply too limited to make generalizations

TABLE 1. Codon misreading in vivo in *E. coli*

Codon and error type	Amino acid		Error	Frequency of error	Comments	References
	Normal	Substituted				
Basal-level errors						
AAC	Asn	Lys	Third-position C as purine	4×10^{-4}		231
AAU	Asn	Lys	Third-position U as purine	2×10^{-3}		231
CGU	Arg	Cys	First-position C as U	1×10^{-3}	One codon in *rplL*	28, 29
CGU/C	Arg	Cys	First-position C as U	5×10^{-5}	Average at 11 codons in *hag*	74
GGC	Gly	Ser	First-position G as A	1×10^{-3}		297
UGG	Trp	Cys	Third-position G as pyrimidine	3×10^{-3}		28, 29
UUU/C	Phe	Cys	Second-position U as G			170
Errors in stressed cells[a]						
AAA/G	Lys	Asn	Third-position pyrimidine as purine	8×10^{-3}	Substitution inferred	228
AGA	Arg	Lys	Second-position G as A	0.05–0.12	Substitution confirmed but misacylation not eliminated	271
AUA	Ile	Met	Third-position A as G	3×10^{-2}	Li and Parker, unpublished	
AUU	Ile	Met	Third-position U as G	3×10^{-2}	Li and Parker, unpublished	
CAC/U	His	Gln	Third-position pyrimidine as purine	>0.1	Substitution inferred	219, 232
UUU	Phe	Leu	Third-position U as purine	0.0–0.6	Very context dependent, misread position inferred (Precup, Ulrich, and Parker, unpublished)	233
UUC	Phe	Leu	Third-position C as purine	0.0–0.6	Very context dependent, misread position inferred (Precup, Ulrich, and Parker, unpublished)	233

[a] These errors are detected in stressed cells only. Stresses include limitation for an amino acid and, in the case of AGA, high-level production of a particular protein.

about whether overall patterns of codon usage reflect misreading.

The studies on specific misreading events in eucaryotes have been done by using amino acid starvation of cultured cells. The sign of the charge change in aberrant protein synthesized during starvation has indicated that the third-position misreadings of glutamine for histidine and lysine for asparagine substitutions occur exactly as in bacteria (232). Although it has not been possible to measure these errors directly in unstarved cells, one can assume that the frequency of errors is linearly related to the amount of time a ribosome pauses at the hungry codon and hence can calculate that the basal-level error frequency during actin synthesis in these cells is 0.3×10^{-4} to 3.5×10^{-4} at histidine codons and 0.7×10^{-4} at asparagine codons (110, 246). The exact level of error at histidine codons depends on the cell type, with simian virus 40-transformed lines having a higher frequency than that of their untransformed counterparts (246). These few measurements of in vivo misreading in eucaryotes could be used to argue that misreading levels are about the same as in procaryotes or that they are as much as 10-fold lower, but either argument seems premature. More information from a wider range of organisms, both procaryotic and eucaryotic, is needed.

Missense Alternatives

Although some misreading errors occur at frequencies approaching 1%, we do not yet know of any naturally occurring system in which this level of misreading during elongation is essential. However, many examples are known in which it is essential that a sense codon in the universal code be translated with an alternative meaning—initiation at codons other than AUG. It is well known that in both procaryotes and eucaryotes, some cistrons are normally initiated at codons other than AUG and that the initiation is with methionine. The factors involved in initiation are being intensively investigated, and this area has been recently and excellently reviewed (97, 98, 154, 155). I shall not repeat that information here. However, what is important for this review is that initiation in both procaryotes and eucaryotes is strongly context dependent and, in procaryotes, involves complementarity between a 16S ribosomal RNA (rRNA) sequence and a site on the mRNA; the Shine-Dalgarno sequence (275). The alternative start codons used in *E. coli* genes include, in order of preference, GUG, UUG, and AUU; all are single-base changes from AUG, as expected. In eucaryotes the codon ACG is used to initiate individual genes in both adeno-associated and Sendai viruses (11, 58). Other degenerate derivatives of AUG can function in *E. coli* (reviewed in references 98 and 154) and in *Saccharomyces cerevisiae* (338). In many cases the naturally occurring genes which use alternative codons are more efficiently initiated when an AUG is substituted, indicating that the use of an alternative codon is an effective means of setting the synthesis level.

In both procaryotes and eucaryotes, multiple initiation sites can be used to generate different proteins from the same mRNA. These overlapping reading frames are most often found in viruses (reviewed in reference 217; see also references 11 and 58). However, in addition to alternatives, initiations could involve obvious errors. In procaryotes, ribosomes can reinitiate internally in a cistron after being terminated at a nonsense mutation (see, e.g., reference 83). It seems possible that erroneous internal de novo initiation also occurs, giving rise to nonfunctional proteins missing the normal amino terminus (187), but such errors would be very difficult to detect and to differentiate from breakdown products. The strong context requirements for initiation may keep such errors at a very low level (72).

TO STOP OR NOT TO STOP

Leaky Stop Codons

In the universal code the codons UAA (ochre), UAG (amber), and UGA (opal) are used as signals to terminate peptide synthesis and are thus referred to as termination, stop, or nonsense codons (true nonsense codons, which are not recognized as coding for an amino acid or for termination, may occur in certain mitochondrial genomes [171]). The precise mechanism of termination is unknown, but it appears (reviewed in reference 56) that in bacteria, when these codons are in the ribosomal A site (or very near it [291]), they are specifically recognized by a release factor protein, which causes the hydrolysis of the peptide from the peptidyl-tRNA in the ribosomal P site. In *E. coli* several ribosomal proteins as well as rRNA seem to be involved (reviewed in reference 56; see also reference 209). The genes *prfA* and *prfB* from *E. coli*, encoding release factors 1 and 2 (RF1 and RF2), respectively, have been sequenced (57, 146), and, as discussed below, a novel type of translational regulation seems to control the expression of *prfB*. RF1 recognizes UAG and UAA, whereas RF2 recognizes UGA and UAA. There is a third RF, RF3, which seems to aid termination in a non-codon-specific fashion, but very little is known about this protein. In eucaryotes the mechanism of termination differs slightly and there seems to be a single release factor (reviewed in reference 40).

Mutations that generate these codons internally in a cistron are referred to as nonsense mutations. The nonsense codons (32) were found to be suppressed by mutant tRNAs. The study of nonsense suppressor tRNAs and suppression has proved extremely profitable for studying tRNA structure and function, tRNA-aminoacyl tRNA synthetase interactions, anticodon-codon interactions, mRNA contexts, and termination itself (reviewed in reference 75). In addition, a number of mutations in genes other than those encoding tRNA have been characterized that increase or decrease the level of suppression. In *E. coli* these include the restrictive *rpsL* mutations and mutations in genes encoding a number of other ribosomal proteins (see below), as well as mutations in the structural genes for RF1 (265, 266), RF2 (146), the genes for EF-Tu (126, 127, 306), 16S rRNA (209), and genes controlling some of the modifications of tRNA (reviewed in reference 20). In *S. cerevisiae*, in addition to mutations in tRNA and ribosomal proteins, two recessive omnipotent nonsense suppressors which suppress UAA, UAG, and UGA mutations (reviewed in reference 287) are known to occur. These map to genes encoding essential proteins, at least one of which is closely related to the EFs (164, 321), but whose normal roles in protein synthesis are unknown (118). Although it is conceivable that these factors are unique to *S. cerevisiae*, it is equally likely that there are translational factors in most organisms that are as yet poorly characterized.

For the most part I will confine myself to situations in which a native tRNA, not a mutant tRNA, reads the stop codon as a sense codon. If this happens at a nonsense mutation it can be called natural suppression; if it happens at a stop codon in any location it can be referred to as readthrough. There are other mechanisms of stop codon avoidance, such as frameshifting (see below), but in these cases the stop codon is not read, and so calling these processes readthrough seems inappropriate. Stop codon avoidance of both types in eucaryotes has recently been reviewed (301).

Some information on readthrough of stop codons comes from studies on nonsense suppression in cells that are suppressor-free, i.e., cells whose tRNA, and presumably the rest of their translational apparatus, is wild type. Natural suppression appears most often to be the result of translational leakiness of the particular stop codon, but in some cases transcriptional errors can also be involved (258, 259). The UGA codon has often been described as particularly leaky. Roth (260) found that in *Salmonella typhimurium*, leakiness of UGA codons occurred at a frequency of at least 10^{-2} to 10^{-3} and correlated this with the relative scarcity of identifiable UGA mutants in various genes in enteric bacteria. Similar evidence of UGA readthrough at or above a frequency of 10^{-2} in *E. coli* is available (see, e.g., references 266, 267, and 284), although UGA codons with a readthrough frequency as low as 10^{-4} have also been observed (204). Correlated with high-level readthrough are the facts that wild-type $tRNA^{Trp}$ can read UGA codons in vitro (120) and that a protein from a coliphage is known to contain tryptophan at the readthrough site (310). Thus, there seems to be convincing evidence that UGA can be read as tryptophan at an appreciable frequency. The possibility of UGA being read as cysteine (41) seems now to be largely ignored, even though cysteine can be incorporated at UGG (see above).

Similar experiments have been used to examine the natural leakiness of both UAG and UAA in the enteric bacteria. Although it is less clear which amino acid is being inserted, in vitro experiments with the error-inducing antibiotic streptomycin suggest that it may be glutamine (65, 241). In most cases UAG seems to be naturally suppressed with a frequency of 7×10^{-3} to 1.1×10^{-4} (26, 92, 266), but several examples of readthrough frequencies of over 10^{-2} were also seen (26, 204). The readthrough of UAA seems to occur at frequencies of 9×10^{-4} to less than 1×10^{-5} (266). The available data show that in *E. coli*, readthrough of UGA occurs at a higher frequency than that of UAG and that both occur at a significantly higher frequency than that of UAA; furthermore, this difference does not depend simply on the context (284). A review of stop codons actually used in the genes of *E. coli*, *Bacillus subtilis*, and *S. cerevisiae* (readthrough in eucaryotes is discussed below) reveals that UAA is preferentially used in all three organisms and UAG is rarely used (272). UGA is also used at a considerable frequency, except in highly expressed genes. The use of UAA, at least in *E. coli* and *B. subtilis*, correlates well not only with its lack of leakiness but also with the fact that either RF can use it (171). The reason(s) for the preference of UGA over UAG is less clear. However, the fact that most UGA mutations seem to be more leaky than UAG does not mean that this will be so in all contexts. Suppressor tRNAs seem not to be as effective at stop codons at the end of cistrons, at least in eucaryotes (19). Suppression of nonsense mutation is quite context dependent (see references 26, 27, and 204 and references therein). The exact nature of the important element(s) in the context is not completely known, but they often include a base or bases 3′ to the codon and probably involve RF context preferences as well as that for the suppressing tRNA (191). It has been pointed out that most leaky UGA codons are followed by an A (77), a context that is often correlated with efficient suppression of UGA nonsense mutations (204).

Suppression of nonsense mutations in *S. cerevisiae* has also been intensively studied, and a variety of suppressor mutants are available (reviewed in reference 274). Presumed natural suppression has been seen at a level of about 10^{-3} for some nonsense mutations (188). Most interestingly, a native

glutamine tRNA whose anticodon is UUG can suppress UAA mutations to a limited degree when overexpressed (249), and another, whose anticodon is CUG, can read UAG when the tRNA is overexpressed (177). Both of these essential tRNAs thus exhibit a low level of first-position wobble. Since they function as suppressors only in high copy, and then inefficiently, it is unlikely that there is a high level of erroneous readthrough of these stop codons in *S. cerevisiae*.

Geller and Rich (94) detected a natural readthrough product of the UGA terminator of β-hemoglobin in rabbit reticulocytes, both in vitro and in vivo (the latter at a very low level). A tryptophan tRNA was implicated. Nonsense mutations have been discovered in higher eucaryotes, but suppression of these mutations is quite difficult (reviewed in reference 113). Since programmed readthrough of stop codons by native tRNA seems to occur commonly in the higher eucaryotes (see below), it seems quite likely that there is considerable compartmentalization of tRNA function in these organisms (113).

As pointed out by Hatfield (113) and discussed below, some native tRNAs that read stop codons seem specific for them, whereas those discussed above read sense codons primarily. Extreme examples of the former would occur in cells or organelles with a genome in which one or more of these codons is read exclusively as a sense codon, i.e., one with a nonuniversal genetic code. Interestingly, the use of one or more of these codons as sense codons is one of the most common motifs in alternative codes, with the UGA codon becoming a tryptophan codon or the UAA and UAG codons becoming glutamine codons (reviewed in reference 85). This is almost certainly related to the fact that in genomes using the universal code, natural suppression of these codons may well involve these amino acids. The strong preferences of some organisms for stop codons used in normal termination may free these codons for normal use as sense codons. Natural populations of *E. coli* often contain organisms with efficient UAG suppressor tRNAs (190, 254), so one can argue that in these strains the rarely used stop codon UAG may become a sense codon. The takeover of stop codons has recently been proposed as a fundamentally important step in the evolution of the universal code (172).

Programmed Readthrough

Although termination at stop codons at the end of a cistron may be less prone to error than at internal nonsense mutations, there are, as in the case of β-hemoglobin, several instances in which readthrough is known to occur. In some cases the readthrough is no doubt simply by error, but there are several examples in which the readthrough protein serves some necessary function. Therefore, I shall consider all of these to be examples of programmed alternatives.

The RNA bacteriophage Qβ was found to synthesize a protein that results from the insertion of tryptophan at the UGA codon terminating the coat gene (310). Readthrough of the UGA occurs about 6.5% of the time (149). This level can be increased by using suppressor-containing strains, but only by 3.5-fold (309), as would be expected if the readthrough was already abnormally high. Although this protein is assembled into the viral capsid at a lower level than it is synthesized (309), in vitro reconstitution experiments indicate that its presence in the virion is required for infectivity (122). Some restrictive *rpsL* mutations (see above) reduce the level of the readthrough protein (309) and prohibit the productive infection of this phage (78, 79), as would be predicted if readthrough was essential. Several other bacteriophages, e.g., lambda and the filamentous single-stranded phages (f1, fd, and M13), are also restricted by certain *rpsL* mutants (79, 329), but in these cases the requirement for any type of ribosomal error has not yet been otherwise established, and other aspects of the pleotropic *rpsL* mutations could also be involved. There is similar evidence that readthrough of the UGA at the end of the tryptophan operon attenuator is required for normal attenuation (153), but it has not been rigorously demonstrated that the UGA codon is involved, and other explanations are possible (300). It is interesting that the use of UGA and UAG is common in the attenuators of *E. coli* (Parker, unpublished observations).

Most of the remainder of the examples of required stop codon readthrough also involve RNA viruses, but in this case both plant and animal viruses are included. In many cases this seems to be a way not only to maximize genome coding capacity, but perhaps also to overcome the inefficient use of polycistronic mRNA in their hosts (see below).

One of the best-characterized examples of readthrough is in tobacco mosaic virus (TMV). The most 5′ open reading frame encodes a 126-kilodalton protein and terminates at a UAG (95). Reading this termination codon would allow the synthesis of a 183-kilodalton protein, which is seen both in vivo and in vitro (see, e.g., references 235 and 239). The apparent level of readthrough in vivo is 10% or above and may vary during the infection cycle (235). The level of this protein can be increased in vitro by using suppressor tRNAs from *S. cerevisiae* or native Tyr-tRNAs from a variety of organisms (18, 239). The Tyr-tRNAs from tobacco which can read this UAG are the major isoacceptors (12). It has been shown that Tyr-tRNAs with the 3′ anticodon position modified from G to Q cannot read UAG (12, 18). This modification is not found in most of the tRNA in tobacco but is found in some other plants. The modification is both tissue specific and developmentally regulated (see reference 13 and references therein). It should be noted that the UAG codon in TMV is bounded on either side by the glutamine codon CAA, a sequence which can cause +6 frameshifting in *E. coli* (314) (see below). However, although the 183-kilodalton protein has not been sequenced, circumstantial evidence strongly supports the hypothesis that this UAG is read as Tyr at a frequency of about 10%.

There is as yet no proof that the 183-kilodalton protein is required for infection. However, the readthrough portion of this protein is homologous to proteins independently encoded by a segment of the genomes of other plant RNA viruses and to a protein from the animal alphaviruses Sindbis virus and Middleberg virus (111). These alphavirus proteins are encoded as part of a polyprotein but are then processed (186). In both these animal viruses this protein is synthesized by the readthrough of a UGA codon (283). These proteins contain homology to the RNA replicase of poliovirus and therefore are almost certainly involved in RNA replication (143). The protein from Sindbis virus is found in very small amounts (although this increases in temperature-stressed cells) and apparently is required in "exceedingly small quantities" so that readthrough of the UGA need not be particularly efficient (186).

A wide variety of plant RNA viruses seem to produce readthrough proteins (106, 109, 124, 128, 334). In most cases the amount of readthrough can be increased in vitro by suppressor tRNAs, as expected. Only a few of the expected products have been identified in vivo. As in TMV, several of these are almost certainly involved in RNA replication (111,

143), but some may not be essential. For instance, the readthrough products of the small RNA of soil-borne wheat mosaic virus can be easily detected in vivo, but mutants with large alterations in these readthrough proteins seem viable (124). In the plant viruses so far examined, only tobacco rattle virus has a leaky UGA (108); in all the other sequenced viruses the stop codons involved are UAG. In addition to TMV, both turnip yellow mosaic virus (106) and beet yellow vein virus (30) have the sequence CAA UAG CAA.

There are also animal viruses that encode readthrough proteins. In addition to the alphaviruses discussed above, some retroviruses encode these proteins. Several of the proteins encoded by retroviruses are synthesized from genomic RNA as a part of a polyprotein initiated with the 5' *gag* gene (reviewed in reference 305). Although the use of alternative translational events is the norm with these viruses, stop codon readthrough is much less common than frameshifting (see below). However, both murine leukemia virus and feline leukemia virus synthesize the polyprotein by using readthrough of an in-frame UAG which terminates the *gag* gene (242, 332, 333), possibly at a frequency of 4 to 10% (138). In both cases the substituted amino acid is glutamine (332, 333). The UAG codons are apparently read by a minor glutamine tRNA, whose anticodon is UmUG (Um is 2'-*O*-methyluridine) and whose level increases following infection (157).

In all of these examples from bacteria, plants, and animals, there are no required readthrough events that have been shown to use UAA. Although this should instill a certain confidence that this codon very efficiently causes termination, it seems unlikely that such a simple translational event does not occur. There is in fact one possible example of UAA readthrough being important. The coliphage T7 only abortively infects *E. coli* cells that contain the F plasmid, because of the plasmid-borne *pif* genes (see reference 156 and references therein). This inhibition of T7 replication is overcome by certain *rpsL* alleles that restrict suppression (42, 156). The inhibition is increased with UAA suppressors, and is related to the termination of translation of the T7 RNA polymerase whose stop codon is UAA (156). Interpretation is made difficult by the unknown role of the *pif* genes and by the fact that the relief of inhibition brought about by *rpsL* mutations is overcome by mutations in the host RNA polymerase (43). Nonetheless, it seems likely that in wild-type *E. coli* K-12 the readthrough of some UAA codon prevents productive infection by T7 (156).

Readthrough proteins almost certainly can have important regulatory functions and are used by several viruses to make proteins that seem to be essential. The use of readthrough could simply ensure that a protein is made in low abundance or targeted to a particular location (the amino-terminal portion of the protein serving as a leader sequence as well as an independent role [45]).

UGA as an Alternative Sense Codon in the Universal Code

Although in many contexts UGA seems quite leaky and some required readthrough events may occur in vivo at levels of as high as 10%, termination is by far the most likely response when a ribosome encounters a stop codon. Recently, though, an example of readthrough of UGA codons has been described which appears to occur at a very high efficiency, close to 100%. Furthermore, this readthrough occurs in organisms that use the universal code and in which UGA is known to be able to function efficiently as a stop codon.

A few redox-type enzymes from bacteria and higher eucaryotes have been found that contain an essential selenium component, which in most cases is present as a selenocysteine residue in the polypeptide chain (reviewed in reference 281). Recently, the genes and/or mRNA encoding two such enzymes have been sequenced. For glutathione reductase from mice (44) and humans (285) and for formate dehydrogenase H from *E. coli* (336), it has been shown that the codon corresponding to the selenocysteine residue is UGA. It has been convincingly demonstrated that in the bacterial system the selenocysteine is cotranslationally incorporated and is not a product of posttranslational modification (335, 336), and there is evidence that the same may be true in mammalian systems (116) (see below).

The UGA codon is also used in mammalian and *E. coli* mRNAs as a stop codon; therefore, its use as a selenocysteine codon is not simply an example of an alternative code. In addition, since selenocysteine is found in very few proteins, at least in *E. coli* (54, 236), it would seem that its incorporation is also not via a general readthrough of UGA codons, with these being particularly efficient examples. Rather, selenocysteine incorporation represents a programmed, alternative reading of UGA codons.

The mechanism by which selenocysteine is incorporated has not yet been fully elucidated, but Böck and colleagues have made important progress by using the formate dehydrogenase system of *E. coli*. Both formate dehydrogenases of *E. coli* contain selenocysteine residues. Although the sequence of the gene encoding the formate dehydrogenase N has not yet been reported, mutant analysis would indicate that the mechanism of incorporation of selenocysteine into this protein is the same as that for formate dehydrogenase H. Mutations in the genes *selA*, *selB*, and *selC* block incorporation of selenocysteine into protein (173). (The incorporation of selenium into a few tRNAs as a base modification [48] is apparently unaffected by these mutations.) The *selC* gene encodes a tRNA with an anticodon complementary to UGA. This tRNA is aminoacylated with L-serine, which seems to be subsequently converted to selenocysteine (174). The mutant form of this gene has a base change in a universally conserved sequence. Natural UGA-reading tRNAs that are aminoacylated with L-serine (which is subsequently phosphorylated) have also been identified in eucaryotic cells (114). Furthermore, it has been established that the carbon backbone of the selenocysteine in rat liver is from serine (286). Therefore, these tRNAs, which are found in a variety of species (114, 115, 222), could well be analogous to the *selC* product. All these tRNAs are large and have many interesting sequence differences from normal elongator tRNAs. It is possible that some of these tRNAs are also involved in other UGA readthrough, but this has not been demonstrated in vivo. The products of the *selA* and *selB* genes are proteins whose function is not known. Mutants of these can at least charge the tRNA with L-serine (174), but the UGA codon cannot be read, and truncated proteins are formed. Truncated proteins are also formed in bacteria deprived of selenium. Therefore, having a chargeable, UGA-reading tRNA in the cell is not sufficient to read the UGA codon in this context. The same may hold true for mammals, since it has been shown that selenium-deprived cells have greatly diminished amounts of glutathione peroxidase (289). This finding also supports the view that selenium is cotranslationally incorporated, although the possibility that phosphoserine is converted to selenocysteine posttranslationally in mammals has not been formally excluded (206).

Since the UGA codon itself cannot be a sufficient signal,

this is another case in which the context of the codon must be critical. As yet, the nature of this context is not known, although it was noticed that in *E. coli* the UGA codon could be involved in a small region of secondary structure (336). When the selenocysteine-encoding UGA in *E. coli* is replaced by the cysteine codon UGU or UGC, selenocysteine incorporation is still observed at 10% of the normal level, whereas no incorporation is seen with the serine codon UCA (335). This shows that in this context the cysteine codons have a basal-level, third-position missense error frequency of 10%. Once again, labeling experiments would indicate that in other contexts this particular misreading event occurs at a very low frequency. Another interesting observation is that replacing the UGA with any sense codon increases the yield of the full-length product (335). Although this was observed in an overproducing system, it could indicate that ribosome pausing is involved in the normal system.

The programmed alternative reading of a codon and close relatives of this codon is highly reminiscent of the mechanism of initiation of protein synthesis: a mechanism which in vivo requires specific protein factors, a specialized tRNA, and the appropriate codon context. It would certainly seem possible that not only is there an essential codon context for these selenocysteine-encoding UGAs, but also some of the protein-encoding genes identified in *E. coli* are specific EFs for this process. There is speculation that selenocysteine is an early member of the genetic code whose place was almost entirely supplanted by cysteine in an increasingly aerobic biosphere (174). Whatever its origins, its continued use in protein synthesis stands as the clearest demonstration of the unexpected flexibility that can be used in translating the code.

Pausing and Premature Termination

Writings on translational errors and alternatives seem, possibly by design, to contain words or phrases with potentially ambiguous meanings. One of these is premature termination. A ribosome that is stalled at a codon because it is blocked in some way could appear in many types of analysis to give a premature termination product, but actually contains a nascent peptide. Also, a ribosome that frameshifts and then terminates at a nearby but previously out-of-frame stop codon prematurely terminates in the sense that the protein is shorter than expected. The first of these is clearly not termination but ribosome stalling, whereas the other is a secondary consequence of the location and direction of a frameshift. I shall define premature termination as the release of the growing peptide chain at a codon other than a stop codon. This could be accomplished in at least two quite different ways. The growing peptide chain which is still attached to the tRNA could drop off the ribosome and then be subsequently released from the tRNA by peptidyl-tRNA hydrolase. Alternatively, a release factor could function at a sense codon to catalyze termination and release. Experimentally, these two possibilities are difficult to distinguish, and both are difficult to distinguish from frameshifts and ribosome pauses.

Certainly, ribosomes appear to prematurely terminate in vitro (see, e.g., references 93 and 101), but the important question is whether they do so in vivo. Having a peptidyl-tRNA drop off the ribosome at a measurable frequency seems inefficient in that the total cost of making the protein is lost to the cell. However, there is evidence that this may happen in vivo at frequencies between 4×10^{-3} and 1×10^{-4} per elongation event, the range of frequencies being related to the particular tRNA being studied (197, 199) and the particular genetic background of the strain being used (240). All these measurements must of necessity be made with a strain having a temperature-sensitive peptidyl-tRNA hydrolase (6). The growth of this strain is quickly blocked under restrictive conditions, apparently because most tRNA species rapidly become blocked by peptide chains (199). Evidence that the peptides have been released from the ribosome (and that the mutation, *pth*, does not simply affect a termination factor) depends on in vitro assays of protein synthesis and the decreased level of polysomes in vivo (201), coupled to the increase in peptidyl-tRNA. The fact that mutations that alter tRNA modification can affect the peptidyl-tRNA level in the *pth* mutant also suggests that premature termination occurs in vivo (240). Menninger (198) has proposed that this release is coupled to anticodon-codon misreading errors and is a mechanism of reducing error-containing completed protein (see below).

Other evidence concerning premature termination involves the presence of decreased polysome levels in amino acid-starved wild-type cells (53, 257) and the apparent build-up of small polypeptides in amino acid-starved *relA* mutants (see, e.g., references 189 and 219). These data seem prima facie incompatible in that the short peptides are found in *relA* cells, in which higher levels of polysomes are found, and not in wild-type cells, in which polysome breakdown seems to occur at a higher rate (219). A variety of explanations can reconcile the data; e.g., the ribosomes in the *relA* polysomes may be inactive (53), or the small proteins in the wild-type cells may be more rapidly degraded (see below) or too small to be visualized. Data on polysome stability are open to a considerable variety of interpretations, but apparently contradictory reports in the literature can be reconciled if one takes into account the abundance of the particular amino acid in protein (189, 257). A similar relationship holds for the small proteins; i.e., starvation for an abundant amino acid leads to a considerable increase in small proteins (219), but this would also be the case if the ribosomes were simply paused or if there was a specific increase in frameshifting (see below).

When specific proteins are examined, there is less evidence of premature termination. Hall and Gallant (107) found that *relA* mutants starved for arginine synthesize one-third the amount of β-galactosidase monomers (as judged by molecular weight) synthesized by the wild-type strain, although missense errors greatly diminish the activity. However, the actual reduction in the level of protein can be explained exclusively at the level of transcription (84). There is good evidence that in normally growing cells a significant fraction of ribosomes that initiate *lacZ* do not reach the normal termination codon (see below) (187). However, there are no data relating to the mechanism by which the shortened peptides are generated, and this mechanism could therefore be frameshifting. There is no evidence that during tryptophan starvation in *E. coli* cells infected with MS2, ribosomes stalled at or queued behind the hungry tryptophan codons release their peptides, but, once again, protein degradation is a possibility (152).

Ribosomes in unstarved cells also stall or pause in vivo. *E. coli* ribosomes stalled at the rarely used arginine codon AGA found in the *tolC* mRNA do not seem to terminate under normal conditions, but may do so when a tRNA that reads the codon is overproduced (205). Similarly, eucaryotic ribosomes stalled at particular CAA codons in alfalfa mosaic virus RNA 1 do not terminate, although these data were obtained in vitro (180). There is a considerable amount of

information indicating that the movement of the ribosome down most mRNAs is variable, involving more or less time at individual codons (238, 303). This may be related both to tRNA abundance and to intrinsic differences in codons (see, e.g., references 25, 292, and 303). Therefore, it seems that pausing per se may be a poor signal to envision for premature termination.

It seems logical to assume that premature termination occurs at some frequency in vivo, and the data from Menninger and co-workers would indicate that the frequency is in the same range as that of missense errors. Clearly, if such events are frequent, they will have an important impact on the efficiency of protein synthesis (37, 197, 199–201). Completely unambiguous in vivo data are very difficult to obtain, but it seems clear that further work should be done.

FRAMESHIFTS

The reading frame for an mRNA is established at initiation. For a typical mRNA it is critical that this reading frame be maintained until the stop codon is reached and therefore that each translocation event be 3 bases. The simplest frameshift errors would be either 2-base or 4-base translocations. A 2-base translocation appears to be a backward or 5′ slip by the ribosome and will be referred to here as a −1 frameshift. A 4-base frameshift will likewise be known as a +1 frameshift. (Another system of nomenclature for translational frameshifting based on insertion and deletion [frameshift] mutations is possible [312]. However, this system is not widely used and can seem counterintuitive when discussing translation.) Ribosomes that make +1 or −1 frameshifts on a typical mRNA will then begin synthesizing a polypeptide having little homology with the normal product. The actual activity and size of the product, and its stability (reviewed in reference 203), will depend on the precise location and sign of the frameshift. Typically, however, the protein will be inactive and, because stop codons are abundant in the alternative frames, shorter than the native protein. This can make it difficult to detect translational frameshifts in vivo.

One method of studying ribosomal frameshifts involves searching for phenotypic, translational suppression of a known or specifically constructed insertion or deletion (frameshift) mutation. In this review I discuss frameshift events that seem to involve native tRNAs. There are also a large number of studies in which mutant suppressor tRNAs were used, and these have led to considerable insight into tRNA-mRNA interactions (reviewed in references 208 and 261; see also reference 60). The assays used are quite sensitive, e.g., plaque-forming ability or enzyme activity, but are usually limited in that they can detect only particular events; i.e., the suppression of a single-base deletion will occur only if the ribosome also makes a −1 frameshift in this region of the mRNA. Recently this problem has been circumvented by using appropriately designed gene constructs. In addition, gene constructs have been used to fuse easily assayed target genes to wild-type sequences that are believed to cause frameshifting. Frameshifting at native sequences can sometimes be detected by searching for proteins of appropriate size or immunologic specificity, or both. Although in vitro protein synthesis systems can have abnormally high error frequencies, they are often appropriate for assaying frameshift errors thought to occur at reasonably high frequencies and can also be used effectively to complement in vivo experiments.

When the possibility of a ribosomal frameshift is being considered, it is essential that transcriptional or RNA editing and processing events be excluded. For procaryotes, this can often be done by mutational analysis, but it is more difficult for eucaryotes. It is sometimes possible to examine presumed mRNA directly or rely on in vitro coupled transcription-translation systems, in which the transcriptional component is from a heterologous system (137). Other translational events, such as reinitiation, can also complicate interpretation. Despite these rather formidable obstacles, a considerable amount of information is now available on ribosomal frameshifting. It is also now clear that a variety of genes require a frameshifted ribosome for proper expression. I shall deal with these as "programmed" frameshifts, but there is not always a clear distinction between these and errors.

Frameshift Errors

A collection of *E. coli lacZ* frameshift mutations, mapping throughout the gene, were tested for low levels of enzyme production, and all were found to be phenotypically leaky (7). Since this leakiness, or suppression, is reduced by *rpsL* mutations, it is presumably caused by ribosomes that make compensating frameshifts on the mRNA at or near the mutated sequence. The efficiency of suppression varied about 100-fold, to a high of 0.06% of wild-type enzyme activity, but *lacZ* mutants which would have had even higher levels of leakiness were not examined (212). Leakiness could not be accounted for by reinitiation and was observed for both insertions and deletions. These studies clearly show that ribosomes can shift reading frames at reasonable levels and that these shifts are not confined to a particular region of the mRNA. The levels of frameshifting observed were similar to the frameshifting frequencies, 3×10^{-3} to 5×10^{-5}, found for a series of mutant tRNAs at a particular site in *lacZ* (59).

The antibiotic streptomycin increased the suppression, as did introduction of a Ram mutation, both apparently only increasing the frequency of errors and not stimulating new types of errors (7). Gallant and colleagues have increased ribosomal frameshifting in a more specific way. They found that by using amino acid starvation, the level of phenotypic frameshift suppression could be increased for both *lacZ* and T4 rII mutations (89, 311, 316). The rationale for such experiments was the prediction that a ribosome pausing at a hungry codon could shift into a new reading frame. If this hungry codon is near the mutant site and if the frameshift is compensating, phenotypic suppression will occur. As discussed above, starvation for particular amino acids has also been shown to increase the frequency of missense errors. As in the case of missense errors, starvation for only certain amino acids, e.g., isoleucine, lysine, phenylalanine, proline, tryptophan, or tyrosine, was found to increase frameshifts. These increases were also sensitive to the *rpsL* allele and the *relA* allele, as would be expected. Apparently, frameshifting occurs only at certain "shifty" codons, and as a corollary, only certain shifty tRNAs are involved. In addition, there are strong context effects that determine both the level and direction of the shift. These specificities are all obviously in addition to the requirement that only a limited number of translational events could phenotypically suppress a given mutation.

Two quite different mechanisms can be postulated by which starvation would lead to a frameshift. First, noncognate tRNA could read the hungry codon, and the nonstandard pairing would lead to an inaccurate translocation, error coupling (159; reviewed in reference 163). A specific mech-

anism of this type was postulated by Weiss (313) to give a unitary account for the data available at that time. Alternatively, one can envision a mechanism whereby a cognate tRNA reads a "codon" overlapping the hungry codon. These two possibilities can be easily distinguished only by sequencing the protein product of the frameshift event. The latter mechanism is clearly involved in the only starvation-induced frameshift error for which the product has been sequenced (312). In this case, the hungry lysine codon AAG overlaps with the serine codon AGC, and serine is found to be inserted at the frameshift. In this case Ser-tRNA$_3$ reads a cognate but out-of-frame codon.

An unexpected finding was that amino acid starvation of *relA* mutants could suppress nonsense mutations, also in a starvation-specific and nonsense allele-specific fashion (88). The simplest model to explain this involves a double frameshift. It is presumed that the ribosome pauses at a hungry codon upstream from the mutation, makes a frameshift, and then downstream from the mutation makes a frameshift in the opposite direction. In this model only the first frameshift is induced, whereas the second occurs spontaneously and is a frameshift back to the normal reading frame.

Genetic manipulation with synthetic DNA has allowed the construction of genes designed specifically to study frameshifting. In the mRNA from such constructs, the ribosome enters a frameshift window in the normal frame, but to produce an active protein it must leave in a different frame. The 3′ end of the window is a stop codon, followed by a reporter sequence, usually *lacZ*, fused in the desired reading frame. To ensure that the ribosome shifts within the window, and not before it, the 5′ end of the window is either the initiation codon or a stop codon in the same frame as the reporter gene. The activity of the enzyme can be measured to quantitate frameshifting, and the frameshifted protein itself can be purified and sequenced. Weiss et al. (314) present data from a series of such constructs. For shifting at stop codons, one mechanism appears to be that the ribosome slides with the P-site tRNA having good codon-anticodon pairing in both the original and the shifted frame. This type of shifting is enhanced by runs of repetitive nucleotides 5′ to the stop codon. Both −1 and −2 frameshifts were observed at frequencies as high as 2% with Gly-tRNA, and Glu-tRNA also gave −2 frameshifts. In addition, forward +2, +5, and +6 frameshiftings were observed at frequencies of 0.4 to 1.0%. These forward shifts did not involve strings of bases, and it is difficult to envision a base-pair-dependent sliding mechanism for the P-site tRNA. Since the shifts are between homologous codons that can be read by a single tRNA (e.g., AAC to AAU), and since only one such amino acid is incorporated, the authors (314) characterized these shifts as "hops." It must be noted that the +6 shift involved hopping over a stop codon while maintaining frame and without inserting an amino acid in the position of the stop codon. Therefore, it is clear that mechanisms of stop codon avoidance involving translating the stop codon must be substantiated by protein sequence data, particularly if the codons on either side of the stop codon can be read by the same tRNA. Weiss et al. (314) point out that several plant RNA viruses have such sequences surrounding leaky stop codons; however, there is strong evidence that at least some of these stop codons are read (see above).

The only constructs reported in the above study were those that gave frameshifting frequencies above 0.1%, and most frequencies were considerably above this. These shifty sequences are apparently found "to occur frequently and unexpectedly during construction of specific sequences" (314). A similar construct (312) also has readily measurable activity. Such sequences cannot be common in actual mRNAs, however, otherwise large proteins could not be synthesized. Recently, a gene construct with an extremely high level of frameshifting was reported (280).

It has been observed that translation of downstream genes in polycistronic mRNAs can be coupled to that of upstream genes; i.e., ribosomes terminating at the upstream stop codon will preferentially reinitiate at appropriate nearby sites (see, e.g., references 223 and 279). Coupling of this type can be used to increase production from cloned genes (see, e.g., reference 270). In an attempt to increase the production of rat interferon in *E. coli*, Spanjaard and Van Duin (280) constructed a bicistronic operon under the control of a strong promoter in which the upstream gene (for MS2 coat protein) overlapped slightly with the rat interferon gene, whose reading frame was +1 compared with that of the coat protein gene. They then placed a strong Shine-Dalgarno sequence, AAGGAGGU, upstream from the interferon start codon. Upstream genes containing such initiation signals are not unusual in polycistronic mRNA; it is also not uncommon for genes to be in different frames. However, in this case the sequence did not lead to increased production of interferon, but, rather, induced the production of a coat-interferon fusion at a 50% frequency. By using a variety of constructions, it was demonstrated that the frameshift was the result of the codons AGG-AGG being in frame, not of its being able to act as a Shine-Dalgarno sequence even for control of the direction of the frameshift. The result was the same if AGA codons were substituted for AGG. Both AGG and AGA codons are read by minor tRNA and are rarely used in *E. coli* (130). When these codons are not in frame, or when a tRNA that could translate them is overproduced, the high level of frameshifting is abolished. Similarly, if there is only one such codon, there is no frameshifting.

Spanjaard and Van Duin (280) postulated that when ribosomes read the first rare codon on the abundant mRNA, there is a depletion of the tRNA such that very little is available to read the second codon. The stringent response (see below) is not induced, because the tRNA is sequestered in the P site. The ribosome pauses at the empty A site and then shifts to another reading frame, in this case exclusively to the +1 frame. Since the protein was not sequenced, it is not clear precisely what mechanism is involved. However, the pause and frameshift event do not diminish the number of ribosomes reading this sequence compared with a similar construct that does not induce frameshifting. Therefore, the limiting step in reading the mRNA must occur prior to these, most probably at initiation (280).

Several reports of ribosomal pausing in *E. coli* are discussed above. It seems clear that ribosomes do pause at certain codons and that tandem rare codons can increase this pausing (304). In most cases reported in the literature, frameshifting at pause sites would not have been noticed, because of the absence of a frameshift assay; however, it is possible that in some others, frameshifting would have been detectable (255), and it is therefore not clear that frameshifting occurs in all cases of tandem AGG codons.

The difficulty with any type of data on frameshift mutation suppression is that they measure the frequency with which a ribosome returns to the correct reading frame, rather than the frequency with which it leaves it. Also, this frequency might be increased because the alternate reading frames in an mRNA have different "codon" usage. Trifonov (298) has recently proposed a reading frame scanning model, in which the 16S rRNA monitors the mRNA by base pairing to a

preferred codon repeat consensus GNN-GNN. He found that this consensus was universal in the correct frame (thus, there is a tendency for proteins to use amino acids whose codons begin with G). Such a model would predict that frameshifting back to the normal reading frame should happen at a high frequency. Therefore, it becomes critical to have measurements of frameshifting out of the normal frame.

A series of in vitro experiments to measure actual errors in a typical mRNA have been performed, and at least one of these errors occurs in vivo (66). In vitro protein synthesis using the genome of the RNA phage MS2 yields a reasonable amount of minor products that have proved to be the results of specific frameshifts. The amount of these products can be altered by changing the ratios of specific tRNAs in the reaction mixture, so it is clear that normal tRNAs are involved (8). Bruce et al. (35) and Dayhuff et al. (66) have conclusively demonstrated that the alanine codon GCA can be read by a serine isoacceptor that normally reads AGC, leading to a −1 frameshift. Interestingly, the base preceding the GC does not have to be an A, nor must it be capable of base pairing with the tRNA. This seems a clear example of a tRNA recognizing a 2-base codon. Only certain tRNAs are capable of this type of shift, and tRNA mutagenesis experiments indicate that frameshifting depends primarily on the anticodon (35). Frameshifting can occur at any of the GCA codons so far examined and, to a lesser extent, at GCU and GCC. It also seems that Thr-$tRNA_3$ can read the proline codons CCG and CCA (but not CCU and CCC) by a similar mechanism (66).

Manley has found that over 30% of the β-galactosidase monomers synthesized in vivo that could be precipitated by antibody are truncated, apparently because of translational events, not protein degradation (187). These events could be erroneous in-frame terminations, or they could be frameshifts leading to termination at an out-of-frame stop codon. The products identified were all greater than 40% of the normal monomer size; presumably, smaller products were either degraded or not recognized by antibody. Therefore, the amount of truncated products found give a minimum estimate of the number of events. If all the truncated protein was caused by frameshifting, the average probability of a ribosome frameshifting at a typical *lacZ* codon could be as high as 5×10^{-4}. This number, although somewhat high, correlates rather well with the frameshift suppression frequencies discussed above. Only nine size classes of truncated proteins were found. This could argue for only a few very specific events of higher frequency, but it must be remembered that any +1 frameshift within a +1 frameshift window will yield essentially the same size product as will a −1 shift in a −1 window. Although there are over 40 frameshift windows in the appropriate portion of the *lacZ* mRNA, there are only about 10 of sufficient length for a reasonable amount of identically sized products. A constant 5×10^{-4} frequency of errors might be tolerable in *E. coli*, although synthesis of large proteins would be inefficient. However, it is possible that some eucaryotic proteins are translated from mRNAs containing well over 10,000 codons (182), and it is difficult to imagine that such a high probability of frameshifting would be tolerable here. It could be that codon usage and codon context in such mRNAs lead to high accuracy. Certainly, there is convincing evidence that only some codons and tRNAs are particularly error prone. Still, there is no direct evidence that the truncated proteins were the result of frameshifting, and there is no evidence that frameshift leakiness accurately reflects average events. It seems likely that such measurements would all represent maximum estimates, and therefore translocation at most codons in most contexts may have an error frequency of 10^{-5} or less.

Programmed Shifts

Although frameshift frequencies of 0.1 to 2.0% cannot be typical of the average ribosome at the average codon, it is clear that frameshifts can occur at this frequency and that this frequency can be increased in vivo (312). Therefore, it should not be difficult to believe that there are certain genes that use such frameshifting as an element in their expression.

Most of the known examples of programmed frameshifts occur in viral genes. As many as four frameshift proteins may be synthesized from bacteriophage T7 (73). In two cases, −1 frameshifts occur near sequences where it would be possible for Phe-tRNA to slip from UUC to UUU in the sequence U UUC AAA. Similar sequences have also been found near leaky frameshift mutations in the *trpE* gene of *Salmonella typhimurium* (9) and a yeast mitochondrial gene (87). However, the mutations in the yeast mitochondrial gene are leaky only in naturally occurring mutants with altered mitochondrial 15S rRNA (317, 318). Although some of the T7 frameshift proteins are synthesized in vivo, it is unclear whether they play any role in the developmental cycle of the phage.

The retroviruses contain several examples of essential proteins whose synthesis requires a frameshift event. As mentioned above, in retroviral genomes the *pol* gene product (encoding reverse transcriptase) is synthesized as a fusion protein with the product of the 5′ *gag* gene (and any intervening genes), with the polyprotein then being processed. Although there is a single mRNA, there is always considerably more *gag* protein synthesized than *gag-pol* fusion protein. In each case, this fusion protein is the result of a certain number of ribosomes reading through, or frameshifting around, the stop codon(s) of the preceding gene(s). Retroviruses that make use of readthrough are discussed above. For some of the viruses using a frameshift mechanism, the *pol* gene itself overlaps with the *gag* reading frame of the mRNA and can be read by a ribosome making a single −1 shift in the overlap region. In the other cases, there is an intervening gene, *pro*, which overlaps with both *gag* and *pol*. In these instances, the *pro* gene also requires a −1 shift for translation, and another −1 shift must be made between the *pro* and *pol* genes (reviewed in reference 305). These frameshifts occur in vivo and in vitro at frequencies ranging from 5 to 25%, so that in each case there is a fixed ratio between the *gag* and the *gag-pol* proteins (134–136, 207, 224).

In the cases in which the mechanism of synthesis of the fusion proteins has been most thoroughly investigated, alternative explanations such as reinitiation and splicing seem to have been convincingly eliminated. Sites of frameshifting have been firmly established in a few cases by sequence analysis of the products (121, 134, 135). Sequence analysis of the genomes can establish the extent of the overlapping reading frames (frameshift windows) only, and these vary considerably in size. They are, of course, all bounded at their 3′ end by the stop codon of the upstream gene. A simple model would be that ribosomes pausing at these stop codons have a high propensity to frameshift, a situation analogous to frameshifting at hungry codons. Although in the best-studied cases the frameshifting does occur at or near the stop codon, the use of an in vitro assay system has demonstrated that the stop codons are not necessary for all

such shifts to occur at a high frequency. The coupled transcription-translation in vitro systems utilize cloned DNA transcribed by a bacteriophage RNA polymerase (134–137, 207). When a eucaryotic (but not procaryotic) extract was used for translation, frameshifting yielding the appropriate products is observed (137). Recently, a very systematic analysis of frameshifting at the *gag-pol* junction of Rous sarcoma virus was made by using this system (134). The reading frame in *gag* is A AAU UUA UAG, and the −1 shift for *pol* gives AAA UUU AUA G, the protein having the sequence Leu-Ile at the fusion point (134). Site-directed mutagenesis of this sequence indicates that any single base change of the sequence UUU greatly reduces frameshifting and that there is a moderate reduction if the sequence AAA is altered. However, changes in the stop codon have no effect. The heptameric sequence A AAU UUA, or a closely related sequence, appears in many retroviral genomes at suspected frameshift sites (134). Some of these are considerably upstream of the stop codon. However, some overlaps do not have this sequence (reviewed in reference 134). One which does not is mouse mammary tumor virus, whose *gag-pro* frameshift had previously been shown to occur at an A-rich sequence, most probably A AAA AAC (121). When this sequence is substituted for the heptamer, a high level of frameshifting is observed (however, simply inserting seven A's is much less effective). Similar sequences are found in all cases at suspected retroviral frameshifts (134). The results of the site-directed mutagenesis were explained by postulating that a ribosome with appropriate tRNAs in both the P and the A sites slips back 1 base; i.e., both tRNAs slip simultaneously (134). Good base pairing in the −1 frame for both tRNAs is required, but is not sufficient even in this context. Apparently Leu-, Phe-, and Asn-tRNAs are shifty in the A site, but Lys-tRNA is not. In addition, G GGG did not cause frameshifting, and therefore Gly-tRNA is apparently not shifty. Although these sequences caused high levels of frameshifting in this system, previous evidence indicated that when placed in novel mRNA contexts, they might not (136). It was noted that immediately downstream from some potential frameshift sites, the mRNA could form reasonably stable stem-loops, and it was postulated that these structures might also be important (136, 252). Using site-specific mutagenesis and either deleting or destabilizing the stem, Jacks et al. (134) showed that these sequences are critical, most probably by causing translational pausing. A very similar mechanism is likely to be involved in the −1 frameshift necessary to synthesize the polymerase of an avian RNA virus, which is not a retrovirus (34).

The yeast retrotransposons also synthesize the reverse transcriptase protein as part of a fusion protein with the upstream gene, apparently using a frameshift (51, 52, 323). Although the retroviruses all seem to use a −1 frameshift, the yeast retrotransposons use a +1 frameshift. Clare et al. (52) have recently demonstrated that a conserved 14-nucleotide sequence, CTT AGG CCA Gc/gA AC, in the overlap region between the *TYA* and *TYB* genes is sufficient to induce high-level frameshifting when placed into novel sites, except when it is immediately downstream from a start codon. Since these experiments were done in vivo, it is unclear what *trans*-acting elements may be involved, or at precisely which residues the frameshift occurs. However, RNA editing is not involved, and the mechanism of the frameshift is likely to be greatly different from that found in the retroviruses of the higher eucaryotes.

The previous examples of programmed frameshifting all seem to take advantage of a shifty sequence to provide a lower, but fixed, level of expression of a downstream, overlapping reading frame. Most of the systems are not yet well characterized, and other regulatory elements may play a role. Indeed, for the MS2 lysis protein, for which circumstantial evidence was very strong (144), it is now clear that frameshifting is not involved (16).

However, there is one example in which not only does programmed frameshifting occur, but also its frequency can be regulated: the *E. coli* gene encoding RF2. The gene (*prfB*) encoding RF2 consists of two separate overlapping reading frames (57). The first terminates in UGA at codon 26; the remainder of the 339-residue protein is encoded by the second reading frame, which requires a +1 frameshift. Apparently, a Leu-tRNA reading CUU slips to UUU, so that the sequence CUU UGA C is read as Leu-Asp (314) at frequencies as high as 50% (55). A number of elegant experiments involving site-directed mutagenesis of the appropriate sequence have established the mechanism of the frameshift. The most relevant portion of the sequence is AGG GGG UAU CUU UGA CUA. As could be expected, the string sequence CUU U was found to be crucial, and most tested replacements greatly reduced frameshifting. However, Gly-tRNA and Val-tRNA can apparently make the same type of shift at GGG U and GUU U, respectively, albeit at a somewhat reduced frequency (314). As could be expected, the stop codons UAA and UAG could be substituted for UGA. However, if the upstream sequence is unaltered, replacing the stop codon with a sense codon still leads to frameshifting levels of about 2%. However, for frameshifting with a sense codon, the sequence AGGGGG is crucial, as is the distance between it and the shift site. By mutating this upstream sequence and making compensating mutations in 16S rRNA, Weiss et al. (315) have demonstrated that this Shine-Dalgarno-like AGGGGG sequence interacts with the 16S rRNA. If this sequence is mutated in a cell with wild-type ribosomes, a stop signal is necessary for frameshifting at the RF2 site, and the frequency is greatly reduced. (These studies also indicate that the AGG is not required as a codon in this frameshift.) For maximal efficiency, this frameshift seems to require both the stop codon and ribosome-binding site, possibly to give an even more restrictive pause or to give direction to the shift, or both.

The UGA stop codon is necessary to form an in vivo regulatory circuit for the frameshift. This stop codon requires RF2 for efficient termination. Therefore, when RF2 levels are adequate, synthesis would be terminated here, but when RF2 levels are low, the ribosome would pause and subsequently shift into the +1 frame. Craigen and Caskey (55) confirmed one prediction of this model when they found that high levels of RF2 could reduce frameshifting in an in vitro system. Another prediction is that nonsense suppressor tRNAs should decrease frameshifting. This has been confirmed to occur in a synthetic *lac* fusion construct when the shifty stop is a UAG and an amber suppressor is used (61), although, inexplicably, it was not seen with the UGA codon and an opal suppressor.

When the RF2 frameshift site was used to create a fusion with *lacZ*, there was a 50% frameshift in presumably normal cells (55). Certainly, the very high level of frameshifting is striking, but so is the very low termination at the in-frame UGA. As discussed by Craigen and Caskey (55), there must be some mechanism which prevents termination, and there are several possibilities (61). It could be that the upstream Shine-Dalgarno sequence does not act simply to cause the ribosome to pause, or to give direction to the shift, but that its interaction with 16S rRNA prevents termination (315). It

has been postulated for some time that 16S rRNA-mRNA interactions may be important in termination (reviewed in reference 56) and that undermodified 16S rRNA has a slightly increased readthrough frequency (302). A mutant 16S rRNA has now been identified that suppresses UGA termination in some contexts, but the altered site is far from the 3′ end of the 16S molecule (209). It seems reasonable that the interactions apparently important in efficient initiation may preclude or reduce those involved in efficient termination.

The last, and most intriguing, programmed frameshift is a +53 frameshift which occurs when ribosomes translate the mRNA of the bacteriophage T4 gene 60 (125). Extensive control experiments would seem to indicate that translation is not from a minor mRNA species from which this sequence has been deleted. The potential codon at the 5′ end of the 50-nucleotide untranslated sequence is the stop codon UAG, and so this is yet another example of a frameshift involving a paused ribosome. This untranslated sequence also has extensive potential secondary structure, which could cause the translated codons to be aligned consecutively. A model could be developed that would allow the folded RNA to move through the ribosome without being unfolded, i.e., almost as if the untranslated RNA mimicked a tRNA. Fusions containing this sequence embedded within *lacZ* are translated with 70% efficiency. Fascinatingly, this unusual sequence is not found in either T2 or T6, which are very closely related to T4.

Programmed frameshifts have now been found in a very wide spectrum of organisms. Most seem to involve paused ribosomes (although how this is accomplished in the yeast retrotransposons is not clear), specific shifty sequences, and equally shifty tRNAs. The mechanisms seem to be diverse, though, and are likely to be adapted to the ribosomes of each organism. The very-high-level frameshifts seem to me to be the most striking examples of translational flexibility.

HOW CELLS CONTROL AND COPE WITH ERROR

The primary purpose of this review is to focus on errors and alternative readings which are known to occur in vivo. However, cells use a variety of mechanisms to control error frequency and to cope with errors that do occur. Some of these are discussed briefly here, particularly areas that point to future directions of research.

Codons and Context

Codon choice is highly biased in many organisms. In unicellular organisms this bias is often correlated with the level of the tRNA species that reads the codon and is most pronounced in highly expressed genes (reviewed in reference 131). Kurland (160) has postulated that using a limited number of codons, and hence a limited number of tRNAs, for highly expressed genes is a strategy for optimizing translational efficiency during high growth rate. The actual choice of codons may be made under a variety of selective pressures. It has long been argued that the third-position degeneracy of the code limits errors. We believe that third-position misreading of AAU and CAU still occurs at a high frequency and that the use of AAC and CAC in highly expressed genes may limit these errors (231). McPherson (196) suggests that codon usage and tRNA levels in *E. coli* are also balanced to reduce misreading by U · G mispairing at the second and first positions and to ensure that the most likely substitutions will be between related amino acids. The inherent stability of base pairing between codon and anticodon must be important, but, as best illustrated by the UGA-suppressing $tRNA^{Trp}$ (119), sequences far from the anticodon can also be involved. Base modifications in or near the anticodon play important roles in determining codon affinity and translational fidelity (reviewed in references 20 and 36). In many cases these modifications apparently affect only the relative ability to read synonymous codons; e.g., a mammalian $tRNA^{Lys}$ with threonyladenosine adjacent to the anticodon prefers AAG, whereas an undermodified form prefers AAA (278). However, situations in which modifications are important in controlling misreading, e.g., the N^4-acetylcytosine at the wobble position of $tRNA^{Met}$ which prevents misreading of AUA (282), have been discussed above, and others are discussed below. The fact that at least some cells can regulate the extent of particular modifications (reviewed in reference 20) opens the possibility that some alternative readings are also regulated. This may be involved in the readthrough of UAG in tobacco (13). Certainly, error hunts should be guided by due appreciation of the structure of the tRNAs involved.

One important regulator of translational flexibility is the context of the codon or codons involved. This context may involve simple sequence determinants or secondary structure of the mRNA. Programmed reading-frame shifts and the insertion of selenocysteine have very high codon and context specificity (see above). Considerable information is available about sites for high-level frameshifting from a variety of organisms. In most cases it seems clear that ribosome pausing is involved so as to halt the ribosomes at particularly shifty sequences, which often contain runs of bases. In the retroviruses this may involve stem-loops on the mRNA (134). In instances in which stop codons are used as part of the frameshift context, it is not clear how termination is avoided. The idea of the ribosome as a steamroller on RNA does not seem to be consistent with the identification of a +50 frameshift, which can be most easily explained by the retention of considerable secondary structure as mRNA moves from A to P sites (125). It seems unlikely that frameshifting across a wide phylogenetic spectrum can be explained by a unified mechanism, and at least some frameshifting sites from eucaryotes do not function in procaryotes and vice versa (8, 137). For selenocysteine incorporation at UGA, the importance of context is extremely clear, but currently the nature of the context is much less so. Several extensive studies on the importance of context for nonsense mutation suppression have been done. There is a tendency for stop codons followed by a purine to be read with the highest efficiency, but this is not always the case (26). Context may also play an important role in normal termination (19), although much less is known about this. Although programmed readthrough of stop codons has now been demonstrated in a large number of instances, almost no information is available on what sequences are important besides the stop codon. It has now been demonstrated by statistical analysis of DNA sequences that there are preferred sense codon contexts (277, 327), and at least some in vivo misreading errors seem to be strongly affected by context. This seems to be true of the CGU misreading as cysteine and of the leucine-for-phenylalanine substitutions (see above) and has also been demonstrated to occur in missense suppression by mutant tRNA (210, 211). It is possible that mRNA secondary structure plays a role in missense errors (276), maybe as a result of ribosomal pausing (328).

Although it is important to continue work directed toward

determining the nature of the context in these processes, it is equally important to determine how the context itself is monitored. The increase in suppression seen when the nonsense codon is followed by a purine is not the result of a fourth base pair between that purine and the universal U at position 33 of the tRNA (10). Once again, base modifications probably play an important role. The *miaA* mutation results in the loss of a modification near the anticodon (see Table 2) and affects the context preferences of nonsense suppression (29). Such modifications could directly affect the strength of the association of the tRNA with the codon (reviewed in reference 20) or could alter tRNA-tRNA interactions on the ribosome (26, 278). In addition, it seems likely that at least in procaryotes, the small-subunit rRNA is involved in monitoring the context of initiation (reviewed in reference 98), elongation (298), and termination (209). Although the hypothesis that the GNN-GNN sequence of mRNA is monitored by 16S rRNA to prevent translocation errors has not been proven, it is clear that 16S rRNA-mRNA interaction can occur during elongation (315). Martin et al. have also shown that some part of the context effect in nonsense suppression is probably a result of the release factors (191).

Proofreading and Editing

The accuracy of DNA synthesis depends not only on the initial selectivity of the polymerase but also on subsequent proofreading and repair (reviewed in reference 183). For many synthetases, aminoacylation also includes one or more proofreading steps (reviewed in reference 82). Proofreading steps were also postulated to take place on the ribosome so that the initial codon-anticodon selection step could be repeated to ensure enhanced fidelity, although this would occur at the energy cost of extra GTP hydrolysis (123, 213). Evidence of proofreading by bacterial ribosomes was soon forthcoming (295) and has now been confirmed (see, e.g., references 264 to 294). It was also demonstrated that the most error-prone $tRNA^{Leu}$ isoacceptors are proofread with the lowest efficiency (264). Increased proofreading will increase accuracy but will also lead to decreased efficiency, not only in energy costs per peptide bond but also in the rate of elongation (reviewed in references 161 and 293).

Menninger postulated that when a missense error occurs during protein synthesis, there is an increased likelihood of premature termination, and that this would represent an editing scheme to abort the synthesis of aberrant protein (198). Kurland and Ehrenberg (162) argued that this cannot be a designed editing step, because the energy cost to the cell would be too high. They postulated that the possible relationship between missense errors and drop-off or frameshifting might have been a strong selective force for a low missense error frequency.

The actual relationship between these types of errors has been very difficult to demonstrate. The data of Menninger and co-workers show that physiological or genetic manipulations that are known to affect missense errors also affect the level of peptidyl-tRNA in the *pth* mutant (37, 38, 200). They have also isolated a mutation that allows continued growth in the presence of a defective peptidyl-tRNA hydrolase, but at the cost of increased levels of aberrant protein (1). Amino acid starvation increases both missense and frameshift errors, but in the only case in which a starvation-induced frameshift protein was sequenced, no missense substitution was found (312). Our analysis of lysine-for-asparagine substitutions indicates that this error occurs at a high frequency at AAU codons wherever they are found in the mRNA, and we have seen no evidence of increased frameshifting or drop-off (248). With leucine-for-phenylalanine substitutions, the situation may be different. We can find no substitution at the third codon of *argI* mRNA (233), whether this codon is UUU or UUC (Precup et al., unpublished). This could be explained in a number of ways, but there is no evidence against premature termination or frameshifting. Support for a coupling between missense and other errors also comes from an in vitro system containing polyuridylic acid, for which was shown that noncognate tRNAs dissociate more readily than $tRNA^{Phe}$ (93). Fascinatingly, this occurs preferentially early in peptide synthesis. If the ribosome editor functions only at early steps in protein synthesis (or if any type of error coupling preferentially occurs here), this would not only overcome objections to the overall cost to the cell but would also explain the difficulty of finding evidence for this process, except in *pth* mutants, in which turnover of the small peptides would be blocked (see below). However, the data of Menninger (197) would indicate that the majority of peptidyl-tRNA released has more than seven amino acid residues, and so the process cannot be confined to the first few peptide bonds. It is also clear that very high levels of certain missense errors can be measured at early residues of the full-length protein.

Mutations Affecting Translational Fidelity

Considering the range of errors and alternatives discussed above, it should appear likely that there are a very large number of mutations that affect fidelity but still allow the continued growth of the organism. Some of these are discussed above. Table 2 summarizes the well-characterized mutations that are known in *E. coli* and *S. typhimurium*. I have omitted the many known mutant frameshift-, missense-, and nonsense-suppressing tRNAs, since these are well analyzed in other reviews (75, 208, 261). Mutations that seem to affect nonsense readthrough only in cells containing suppressor tRNAs are also not included. However, of the several mutations that affect readthrough, only for *rpsL* is there in vivo evidence indicating that readthrough of an in situ stop codon, not just a nonsense mutation, is affected (78), and this is corroborated by in vitro data (329). It must be emphasized that mutations that alter readthrough can do so by a variety of mechanisms, since competition between elongation and termination is involved. Any change in the balance of this competition will alter the frequency of readthrough, even if the primary effect in normal cells is on elongation. However, Murgola et al. (209) make a strong argument that the mutation leading to an altered 16S rRNA affects termination specifically. Some of the information on whether a mutation affects missense errors comes from in vitro assays, although there is no reason to believe that this differs from the in vivo situations.

Many of the mutations in the genes encoding ribosomal proteins have been known for some time and have been extensively and recently reviewed (31, 75). Mutations in genes encoding S12, S17, and L6 confer resistance to the error-enhancing antibiotics streptomycin, neamin, and gentamicin, respectively, and some of the mutants show increased fidelity; i.e., they have a restrictive phenotype (reviewed in reference 102; see also references 23, 158, and 296). Some mutations resulting in altered proteins S4, S5, and L7/L12 have decreased accuracy, i.e., a Ram phenotype (102, 151, 221, 243). The S4, S5, S12, and L7/L12 mutants have been most extensively analyzed. The various restrictive S12 mutations increase proofreading proportionally to

TABLE 2. Mutations affecting error levels in enteric bacteria

Gene	Component affected[a]	Activity of mutant	References
glnS	Glutaminyl-tRNA synthetase	Increased misacylation	132
hisT	ψ in anticodon loop and stem of several tRNAs	Decreased missense	225, 227
ksgA	Synthesis of $m^6{}_2A$ at 1517 and 1518 of 16S rRNA	Increased frameshift and readthrough	302
miaA	Synthesis of ms^2i^6A, base 37 of some tRNAs	Decrease of some missense and readthrough, possible increase of other missense	29, 241
prfA (*uar*)	RF1	Increased readthrough of UAG and UAA (probably allelic to *ups* and *sueB*)	62, 218, 265, 266
prfB (*supK*)	RF2	Increased readthrough of UGA	146, 250
relA	Stringent factor (synthesis of ppGpp)	Increased frameshift, missense, and readthrough during amino acid starvation	89, 107, 219
rplF	Ribosomal protein L6	Decreased missense and readthrough in the presence of gentamicin	158
rplK (*relC*)	Ribosomal protein L11 (synthesis of ppGpp)	Increased errors during amino acid limitation	232, 234
rplL	Ribosomal protein L7/L12	Increased missense and readthrough	150, 151
rpsD (*ramA*)	Ribosomal protein S4	Increased frameshift, missense, and readthrough	7, 102, 221
rpsE (*spcA*)	Ribosomal protein S5	Increased missense and readthrough	2, 243
rpsL (*strA*)	Ribosomal protein S12	Decreased frameshift, missense, and readthrough	7, 102, 103
rpsQ (*neaA*)	Ribosomal protein S17	Decreased missense and readthrough	23, 296
rrsB	C to U at position 1538 of 16S rRNA	Context-specific readthrough of UGA	209
trmD	m^1G at position 37 of some tRNAs	Increased frameshifting by $tRNA^{Pro}$	20[b]
tufAB	EF-Tu	Increased frameshift, missense, and readthrough	126, 127, 290, 306

[a] The following abbreviations are used for the modified bases: $m^6{}_2A$, 6-dimethyladenosine; ms^2i^6A, *N*-6(2-isopentenyl)-2-methylthioadenosine (or its 4-hydroxyisopentenyl derivative in *Salmonella* spp.); m^1G, 1-methylguanosine; ψ, pseudouridine.

[b] Unpublished observation of P. M. Wikström, A. S. Byström, and G. R. Björk cited in reference 20.

their ability to restrict (263), but the increased proofreading is accompanied by decreased growth rates (4, 263). (It is important to note that not all S12 mutants have increased accuracy; thus, an increase in accuracy is not a necessary requirement for streptomycin resistance.) The Ram mutants in S4, S5, and L7/L12 all have decreased proofreading (2, 3, 150). In some cases there is a pleasing relationship between the efficiency of a particular error and the increased or decreased proofreading of the strain (326), and the particularly error-prone tRNAs are not as efficiently proofread (264). However, once again, there are certainly considerable complications. Some nonsense suppression does not respond as expected to decreased proofreading (81), and there is very strong evidence that the same is also true of some misreading errors (28, 297). Since errors depend on both codon and context, it seems logical to assume that the same is true of error-correcting mechanisms. The mutation altering ribosomal protein L11 affects accuracy by altering the stringent response (232, 234), as do mutations in *relA* (see below).

Base modification in tRNAs are clearly involved in fine tuning anticodon-mRNA interactions (reviewed in references 20 and 36). The 2′-*O*-methyluridine wobble base modification is probably important for UAG readthrough in some retroviruses, and Q-base modification is important for regulating readthrough of UAG in TMV (see above). However, the *tgt* mutant of *E. coli* lacks a Q base in its tRNA, and although this seems to affect codon choice by Q^- tRNAs, there is no demonstrable effect on fidelity (216). However, a number of mutations do affect fidelity. The lack of a modified G next to the anticodon of $tRNA^{Pro}$ in *trmD* mutants seems to increase spontaneous frameshifting (P. M. Wikström, A. S. Byström, and G. R. Björk, unpublished observation reported in reference 20). The absence of certain pseudouridine modifications in *hisT* mutants seems to decrease misreading in vivo (227) while decreasing the elongation rate (225).

The case with *miaA* promises to be very interesting. Mutations in this gene prevent the formation of the highly modified A found next to the anticodon in most tRNAs from *E. coli* and *S. typhimurium* that read the codons UNN. Diaz et al. (69) report that at least one such tRNA is more efficiently proofread. This supports the finding that nonsense readthrough and cysteine (UGU/C)-for-tryptophan (UGG) errors are reduced in *miaA* strains (29, 241). However, the first-position error, cysteine for arginine, was unaffected, and Wilson and Roe (322) have recently reported that undermodified $tRNA^{Phe}$ (UUU/C) has increased errors at CUU codons in vitro, also a first-position error. Increased proofreading could have been negated by relative tRNA levels (322). However, these results correlate very well with the hypothesis of Ninio (215) on the evolution of the code interactions, i.e., modifications that increase third-position wobble (degeneracy) decrease first-position error. Therefore, it seems likely that the effect of *miaA* on errors depends on the errors being studied.

The accuracy of protein synthesis is not maintained simply by the structure of each member of the translational apparatus but also by the balance of their concentrations within the cell. Altering the ratio of an aminoacyl-tRNA synthetase and its cognate tRNA can lead to misacylation (288), and overproducing glutamine-tRNAs in *S. cerevisiae* can lead to readthrough of stop codons (177, 249). We have also found that apparent misreading of glutamine codons as histidine can be seen only in cells that are overproducing His-tRNA (A. Ulrich and J. Parker, unpublished results). Similarly, lowering the concentration of EF-Tu in *E. coli* can lower the readthrough of UGA (306), and raising the concentration of RF1 or RF2, or both, decreases readthrough (191). However, EF-Tu is also involved in both selection and proofread-

ing (290, 293), and many of the effects noted with EF-Tu and RF1 are quite context specific (126, 127, 266, 306). It is likely that at least some of the mutations in the genes for RFs (62, 218, 250, 266) will yield considerable information on termination and readthrough.

Mutations that affect accuracy are also known to exist in eucaryotes and their organelles. They also have alterations in particular members of the translational apparatus such as ribosomal proteins (see, e.g., references 68 and 133) and rRNA (see, e.g., references 86, 176, and 317). There are also a variety of mutations in many organisms which seem to affect fidelity but are not yet characterized. These, no doubt, include genes for enzymes for more modified nucleotides, ribosomal proteins, and possibly new factors that may be involved in protein synthesis.

Despite the considerable amount of recent work on programmed errors in a variety of organisms, including *E. coli*, there is very little information on how these errors are affected by accuracy mutations. Once again, the exception is with stop codon readthrough and *rpsL* (78). Clearly, if RF2 is an essential protein, very restrictive *rpsL* mutations do not eliminate high-level frameshifting.

Stringent Response

We have seen that amino acid starvation can be an effective way to increase errors at the hungry codons. However, in *E. coli* this increase is much less dramatic in wild-type strains than in mutants defective in the stringent response. The stringent response is an apparently universal response of procaryotes to amino acid limitation (reviewed in reference 253). The stringent response in *E. coli* has been intensively studied, and there is an excellent, extensive, and recent review (39). The amino acid limitation is sensed through lowered levels of aminoacyl-tRNA and results in very widespread regulatory changes in the cell metabolism. The effectors of these changes, at least in *E. coli*, seem to be the guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp). It has been shown that in in vitro systems pppGpp is synthesized on ribosomes by a protein known as stringent factor in response to stalling at a codon with cognate but uncharged tRNA bound in the A site (112). It must also be remembered that the synthesis of (p)ppGpp is a specific response to the change in charging level of the limited amino acid. Since the average charging level of total tRNA in a normally growing cell is approximately 80% (175), limiting a single amino acid will have a marginal effect on the total level of uncharged tRNA. The stringent factor, which is present in the cell in small amounts relative to ribosomes (142, 237), is encoded by the *relA* gene. Mutations in this gene lead to the inability to synthesize these nucleotides in response to amino acid deprivation and thereby to the relaxed phenotype, as do mutations in the gene encoding ribosomal protein L11 (reviewed in reference 39). There is a breakdown pathway of pppGpp that leads to ppGpp and then to guanosine diphosphate, and ppGpp is present at much higher concentrations during starvation. Strains having a mutation in the *spoT* gene degrade ppGpp slowly and maintain high levels for some time after the amino acid limitation is reversed (166).

As discussed above, *relA* mutants misread at 10-fold-higher levels during amino acid starvation than wild-type cells do. This increased error level in relaxed cells has been found for frameshifting as well (89). Errors also increase in starved stringent cells, but not to the same extent (230). In vitro studies support the hypothesis that ppGpp may be most effective under limited amino acid limitation (256). There are stringent strains of *E. coli* that synthesize ppGpp but nonetheless have high levels of errors (228). The defects in these cells have not been identified.

If *relA* cells are tricked into making ppGpp by a stringent-factor-independent pathway, subsequent amino acid starvation does not lead to increased errors (89). This implies that ppGpp is directly involved in maintaining accuracy. O'Farrell (219) found that after the release of amino acid starvation in a *relA*$^+$ *spoT* mutant, the rate of protein synthesis remained low, as if ppGpp was acting as an inhibitor. Also, the number of polysomes seems to be reduced in wild-type cells during starvation (see above). Thus, a simple explanation for the decreased error level would be that the inhibition by ppGpp allowed the charging level of the cognate tRNA to increase to a level that would prevent misreading. However, the measurements of charging levels in starved *relA* mutants seem to be the same as those in wild-type strains (21, 244, 330), although these are difficult measurements and it is possible that critical isoacceptors could behave differently (219).

There are other possibilities for the action of ppGpp. Dix and Thompson (70) have detected increased proofreading in a polyuridylic acid system supplemented with ppGpp, possibly indicative of a general increase in accuracy caused by ppGpp. However, during isoleucine or serine starvation, we could detect no effect of ppGpp on the level of errors at asparagine codons (229), whereas during asparagine starvation there was a dramatic decrease (230). These data are consistent with a specific effect of ppGpp on the hungry codon, or at least with the idea that it is most effective there. Both Ninio (214) and Gallant (91) have proposed models in which ppGpp could affect misreading of only hungry codons. The Gallant model depends on the interaction of the uncharged tRNA and the hungry codon (91). However, increasing the level of $tRNA^{His}$ does not alter the level of misreading during histidine starvation (299). The Ninio model involves a ribosome which has both a low-accuracy and a high-accuracy state, with ppGpp regulating the switch from one state to the other. It has recently been reported that ribosomes do seem to have a memory, in that they continue to elongate slowly after having made an error (93), which is consistent with a two-state ribosome. It is clear that more information is necessary to determine exactly how ppGpp prevents the increase in translational errors seen in starved *relA* cells.

Protein Turnover

The bête noire of studying mistranslation is the possibility of rapid turnover of mistranslated protein. If this is so, the levels of errors actually measured in cells can be argued to be simply a minimum estimate, and a very heterogeneous one at that, since it can be presumed that some mistranslated protein will be stable and that some will not. Turnover of defective protein might be an effective means of recycling metabolites as well as offering protection from any harmful product. It has long been known that some proteins are unstable and that among these are many abnormal proteins (reviewed in references 100, 117, and 203). Abnormal proteins which might have relevance to mistranslation in normal cells include nonsense fragments (178), some missense proteins (17, 104, 337), and protein from cells with a generally higher rate of mistranslation caused by either a Ram phenotype or the addition of streptomycin (99, 245).

We have investigated the relative stability of total *E. coli* protein in which the level of asparagine-for-lysine or glu-

tamine-for-histidine errors was very high, above 10% (226). No relative instability was observed in stringent or relaxed cells either during amino acid starvation or during growth. In addition, this particular type of mistranslated protein was assembled into ribosomes in the same ratio as it was synthesized, indicating that protein containing these errors must be folded normally. I proposed that the errors that still occur at high frequency when reading the code are those that cause the least damage to the cell (226). However, the fact that some mistranslated protein can be turned over does not mean that more deleterious substitutions cannot be detected. The majority of missense-containing protein is stable (17, 337), and for most of the full-length, missense-containing proteins that are degraded more rapidly, the increase is not great enough to seriously affect error measurements in pulse-labeled cells.

The case for shorter peptides is quite different. Some of these are turned over very rapidly and therefore could prove quite difficult to detect (178). This is particularly relevant for estimating drop-off and frameshifts, which may occur early in the mRNA. It is possible that as many as 20% of the peptide bonds synthesized in normally growing cells are rapidly hydrolyzed (331). It could well be that this represents the hydrolysis of erroneous peptides made early in protein synthesis.

Cells have a variety of responses to environmental stress, such as the stringent response. In at least some of these responses, there is a specific enhancement in the turnover of aberrant protein. This is true of both the stringent response in bacteria and the universal heat shock response (reviewed in reference 181). In *E. coli* the adenosine triphosphate-dependent, heat-shock-inducible La protease plays a role in degrading abnormal proteins (46, 49). Other types of stress, including overproduction of foreign protein and the stringent response, also induce La (96, 105, 307), and, in addition, the enzyme seems to be activated by its substrates (308). Other adenosine triphosphate-dependent proteases must play a role in turnover of aberrant protein (193), possibly the recently discovered adenosine triphosphate-dependent protease Ti (129, 145). Another heat shock protein that seems to be involved is the product of the *dnaK* gene, which may aid in disaggregating clumps of aberrant protein or unfolding abnormally folded protein and thus generating substrate for other proteolytic enzymes (147).

The adenosine triphosphate-dependent ubiquitin pathway, which is important for abnormal protein turnover in eucaryotes (50), is also heat shock inducible (24). The finding that there are inducible pathways to degrade abnormal protein cannot be construed as evidence that these have evolved to protect cells from mistranslated protein. Denatured or misfolded protein does not have to be mistranslated. Conditions that increase misreading severalfold (streptomycin addition or Ram mutants) are likely to yield a considerable number of multiple errors in proteins. It is possible that this also occurs under physiologically relevant stress conditions, e.g., heat shock, but there is no strong evidence that this is so.

CONCLUSION

The ribosome clearly has options available to it that many scientists rarely consider. High-level frameshifts can be programmed into mRNAs, as can readthrough of stop codons, even including the insertion of novel amino acids. Many cells and cellular parasites seem to take advantage of these and other translational alternatives to solve specific regulatory or functional problems. But what of error in reading the average mRNA? Our first consideration must be that in the average mRNA the codons and their contexts are apparently under strong selection pressure, some of which almost assuredly involves accuracy. Therefore, consider an *E. coli* mRNA encoding a protein that contains 300 amino acid residues and that occurs at 500 monomers per cell. Transcriptional errors may occur at frequencies too high to ignore. If the average transcriptional error occurs at a frequency of 10^{-4}, almost 1 of 10 mRNAs for the protein will have some defect, and 10 mRNAs will be required to make 500 copies of the protein (148). It would be futile to present arguments on what errors are most likely, but a base-pair insertion or deletion would result in a very high cost to the cell unless the mRNA is rapidly degraded. However, over 90% of the mRNA is error free (possibly considerably more than this if the transcriptional errors are lower). Only these error-free mRNAs will be considered, and it is assumed that there are 500 initiation events and, further, that errors will not occur preferentially at or soon after initiation. Each of these assumptions increases the likelihood of obtaining a perfect protein. This will be offset by uncertainties about individual error frequencies. In a normally growing cell, the chances of a ribosome frameshifting or prematurely terminating at a particular codon could be of the order of 10^{-4} (see above). If so, a ribosome reading this mRNA has a probability of about 97% of reaching the stop codon and a possibility of perhaps only 0.01% of reading through to the next (since a typical stop codon is UAA). If half of the frameshift or drop-off protein is rapidly hydrolyzed, possibly 8 of the protein monomers existing in the cell should be truncated versions of the 485 copies of full-length protein. Missense errors seem to occur at frequencies of 5×10^{-3} to ca. 1×10^{-5} and depend very much on both the particular substitution being considered and the context of the codon involved. If the frequency of the average error per codon in the completed monomer was of the order of 5×10^{-4} (a number very similar to that estimated by Ellis and Gallant [76]), a typical protein has a 14% chance of having a missense error as a result of misreading. Therefore, of the 485 full-length monomers, 68 have an error. Certainly, some proteins will have more and some less, depending on their sequence.

In discussing missense errors, I discussed the fact that experiments had to be designed specifically to find them. If over 10% of a given population of protein contains an error, would that be likely to have been detected by other means? As Kurland and Gallant pointed out in their review (163), truly random amino acid substitutions at a frequency above 10^{-3} (a higher estimate than I have used) should lead to subpopulations of proteins identifiable by inactivation kinetics, and such populations are not found (90). However, the genetic code seems to be designed in such a way that missense errors will not give random substitutions but, rather, conservative substitutions, which would be quite difficult to detect.

More important, however, is the question of whether any information is available that would allow us to determine whether the error levels in cells are near the tolerable upper limit. It is very difficult to determine how much error an organism can tolerate, but, certainly, *E. coli* can be grown for hundreds of generations with error levels at least 10 times normal (90), and Ram mutants do not have higher death rates (141). If all error levels were increased uniformally, frameshifting and/or termination would now be at 10^{-3}, and thus the probability of completing our average protein drops to about 75%. There is no evidence that *E. coli* is energy

limited, so the wastage may be tolerable, but there must certainly be very few naturally occurring high-level frameshift or drop-off errors in essential genes. Quite possibly, essential and large proteins have mRNAs which tightly control frameshift errors. The possibility of making a perfect protein would decrease dramatically, to about 22%. This could be so if the essential residues of most proteins are encoded in such a way (both codon and context) that they are not misread at high frequencies. However, it seems very likely that there is no need for the remainder of the protein to have a perfect sequence. Even closely related organisms have significant numbers of differences in the sequences of their proteins. Recently, it has been shown that many single-amino-acid changes in β-galactosidase have no demonstrable selective effect (67). However, wild-type strains of *E. coli* show a remarkable conservation of amino acid sequence in *trpB* (202).

The situation is less clear for other organisms, because far less information is available. There is no clear evidence that translational error plays a role in physiological processes such as aging (reviewed in reference 262). The ribosomes of higher eucaryotes could be more accurate on a typical mRNA, but they exhibit the full range of alternative reading strategies, and some occur at very high levels. There are many unanswered questions about the control of translational fidelity for both eucaryotes and procaryotes. However, it is clear that accuracy, as defined by a scientist with a codon table, is not set at some fixed and negligible level but is controlled by interactions between the mRNA and the rest of the translational apparatus, which can be wonderfully specifically adjusted.

ACKNOWLEDGMENTS

I thank many colleagues who have generously shared information and ideas, particularly G. Björk, A. Böck, J. Gallant, and C. G. Kurland. My thanks also for the hospitality and stimulation afforded by C. Squires and C. G. Kurland and their colleagues. The literature cited was arbitrarily limited, and I apologize for all omissions. I thank Amy Ulrich for her help and suggestions and E. P. Parker for reading the manuscript.

Research in my laboratory has been supported by Public Health Service grant GM25855 from the National Institutes of Health.

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