In Vivo Translational Inaccuracy in *Escherichia coli*: Missense Reporting Using Extremely Low Activity Mutants of *Vibrio harveyi* Luciferase†

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ABSTRACT: A convenient, sensitive assay for measurement of in vivo missense translational errors is reported that uses luciferase activity generated by mistranslation of a gene encoding an inactive mutant α chain of the *Vibrio harveyi* enzyme. Mutations were introduced at α45 His, a position known to be highly intolerant of amino acids other than histidine. To normalize for any variations in expression level, the concentration of wild-type luciferase $\alpha\beta$ dimer was determined by a novel assay using co-refolding of active/wild-type β enzyme subunits with inactive α subunits in lysate with an excess of exogenously added active α subunits. Four His α45 missense mutants of luciferase encoded by leucine codons (CUC, CUU, CUG, and UUG) had histidine misincorporation rates of 2.0×10^{-6}, 1.3×10^{-6}, 9.0×10^{-8}, and 1.5×10^{-8} respectively, a variation of over 133-fold among synonymous codons. Any substantial contribution of mutation was ruled out by a Luria−Delbrück fluctuation test. The two leucine codons with the highest rates, CUU and CUC, have a single central-mismatch to the histidyl-tRNA^{QUG} anticodon. Aminoglycoside antibiotics known to enhance mistranslation increased the error rate of the CUC codon more than those of the CUU and CUG codons, consistent with the hypothesis that CUC codon mistranslation arises primarily from miscoding events such as the selection of noncognate histidyl-tRNA^{QUG} at the central position of the codon.

The transfer of genetic information into proteins is highly precise, but not flawless. Errors in DNA replication and transcription are respectively in the range from 10^{-6} to 10^{-10} (*1*−*3*) and 10^{-5} per base (*4*−*7*). The last and least accurate step, translation, has an average error frequency of about 10^{-4} per codon (*8*−*11*), as derived from a relatively small and heterogeneous collection of experimental measurements.

The products of missense errors are less easily detected and removed than those of premature termination, stop codon readthrough, and frameshift, all of which change the size of the protein product. The variant species resulting from missense errors can comprise a very diverse population, each having slightly different properties and each present at a low concentration (*12*), or there can be one or a few dominant inaccurate forms (*13*−*15*). Most mistranslated forms with a single amino acid substitution retain the overall structure, charge, and epitopes of the authentic molecule (*16*, *17*) and can be extremely difficult to remove on a preparative scale, as in pharmaceutical manufacturing.

Translational accuracy can be reduced during high-level expression of heterologous proteins. Codon bias (*15*, *18*−*20*) and poor regulation of amino acid biosynthetic pathways in the host strain can produce a high level of singly substituted derivatives. In some instances, however, the occurrence of these high-level errors can be decreased or eliminated by replacing rare codons or by adding supplemental amino acids (*14*, *21*).

Inactive Enzyme Mutants as Positive Reporters of Missense Errors. One promising approach to the measurement of in vivo missense errors is the use of an enzymatic reporter, such as the activity of β-lactamase mutants (*22*), the reduced specific activity and thermostability of β-galactosidase (*23*, *24*), or activity and heat-lability changes of β-galactosidase (*25*). The first in vivo measurement of a specific mistranslation event in an eukaryotic cell (*Saccharomyces cerevisiae*) used inactivating mutants of type III chloramphenicol acetyl transferase (*26*).

Constraints upon inactive enzyme mutants used as reporters of missense errors were described in our preliminary work with tryptophan synthetase (*27*). Useful reporters combine complete inactivation of wild-type enzyme by a single amino acid substitution and stability of the mutant form. Determination of absolute mistranslation rates requires not only sensitive measurement of the catalytic activity of the enzymatic missense reporter but also determination of the concentration of the reporter species in cell extracts. The use of inactive enzyme mutants as reporters of missense errors offers several advantages: convenience, avoidance of possible loss of mistranslated species during fractionation, and preselection of the type and position of nucleotide mismatch at the codon−anticodon interface, as well as the specific type of misincorporation.

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Bacterial Luciferase as a Reporter for Missense Errors. We have examined missense reporter systems based on luciferase, which is widely used as a reporter of gene expression (*28*−*31*). The successful expression of heterodimeric $\alpha\beta$ bacterial luciferase in *Escherichia coli* and *S. cerevisiae* as well as in mammalian and plant cells (*32*, *33*) suggests that reporter systems based on this enzyme could be broadly applicable. Moreover, the luciferase assay (*34*−*36*) features extreme sensitivity, linearity over many orders of magnitude, convenience, nondestructiveness, and adaptability to common scintillation counters.

The catalytic center of *Vibrio harveyi* luciferase has been localized on the α subunit (*37*, *38*), and a histidine residue was shown to be essential to the activity of the *V. harveyi* enzyme (*39*). Xin et al. (*40*) constructed and characterized single α chain mutations of *V. harveyi* luciferase at five conserved histidine residues (positions 44, 45, 82, 224, and 285) and found that position 45 was particularly intolerant of substitutions. Exhaustive mutagenesis of position 45 (*41*, *42*) suggested that, when substituted for histidine, all 14 amino acids tested reduced the activity of bacterial luciferase by at least 300-fold, with leucine reducing activity by ca.10^6-fold (see below). The five amino acids not tested are chemically highly distinct from histidine (i.e., no positive charges or cyclic side chains) and structurally similar to amino acids known to produce very low activity. For example, valine and isoleucine, which were not tested, are expected to be similar to leucine, which was among the least active. These amino acids therefore are not expected to contribute significant activity in a missense assay.

A missense assay based on a "one-active-form" strategy was developed using inactive leucine mutants at position 45 on the α subunit. Six leucine codons (CUU, CUC, CUA, CUG, UUA, and UUG) offer an attractive synonymous, low-residual-activity set differing in closeness to the nearest histidine codons (CAC and CAU). CUU and CUC have one central base difference, CUA and CUG have both central and wobble base differences, and UUA and UUG differ in all three positions from the two histidine codons. The codons used in this study (CUU, CUC, CUG, and UUG) represent at least one of each type. Aspartic acid codon CAG, with only a 3′ wobble position difference from the histidine codons, was also studied. The very low residual activity of the mutant reporter enzymes (as little as 10^{-11} of the native reporter enzyme) allows the accurate quantification of missense error frequencies in vivo with no background bioluminescence from the host.

The determination of luciferase expression levels was necessary to normalize the catalytic signal from the reporter by the luciferase expression level, similar to the stimulatory assay previously used for tryptophan synthetase-based mistranslation assays (*27*, *43*). While the maxi-cell technique (*22*), immunoassays, luciferase aldehyde-oxidation activity, and the luciferase/second reporter fusions recently employed by Shaw et al. (*44*) were considered, the capability of bacterial luciferase for subunit exchange under denaturation and refolding conditions (*45*) allowed a direct internal control on the expression level of luciferase in *E. coli* cell extracts. Since the luciferase α and β subunits are synthesized in equal quantities in both *V. harveyi* and *E. coli* (*46*, *47*), measuring the light emission after refolding in the presence of excess active wild-type α chains and formation of active dimers is equivalent to measuring the initial amount of α inactive chains in the lysate. This refolding assay permitted the measurement of luciferase expression levels in crude lysate. With the catalytic assay and the refolding assay used as an internal control for expression, we were able to calculate missense error rates from luciferase α45 mutants, which are primarily due to missense incorporation of histidine at this position.

Since the discovery by Gorini and Kataja (*48*) of a streptomycin-resistant strain bearing ribosomal protein mutations, aminoglycoside antibiotics have played an important role in the study of ribosome function. A major recent step forward in the elucidation of antibiotic mechanisms was the determination of ribosomal subunit crystal structures at resolutions of 3−8 Å (*49*, *50*). The aminoglycosides streptomycin, paromomycin, and neomycin were found to bind to the A-site decoding region of the ribosome, where they alter the codon−anticodon interaction as well as the energetics of the A-site domain closure around the anticodon stem loop (*51*−*54*). The effects of streptomycin, paromomycin, and neomycin on histidine misincorporation at position α45 of luciferase were studied. Since streptomycin, paromomycin, and neomycin selectively induce miscoding errors (not misacylation or mistranscription) by interfering with the selection of the aminoacyl-tRNA, an increase in error frequency in response to these antibiotics would provide evidence that leucine CUC or CUU codon missense errors derive primarily from miscoding.

While this paper was in preparation, Salas-Marco and Bedwell (*55*) demonstrated the use of a similar luciferase-based reporter system for monitoring missense and readthrough errors in *S. cerevisiae*. Their system instead employed firefly luciferase with mutations at two essential residue positions (His245 and Lys529) as the reporter. Expression levels were monitored by an upstream wild-type *Renilla* luciferase expressed from the same promoter in a bicistronic plasmid, with the two activities being measured independently by sequential luminometry. This approach allows a more efficient monitoring of expression level than the refolding assay used here, although the output is a somewhat less direct measure of the concentration of the reporter enzyme in cell extracts.

MATERIALS AND METHODS

Chemicals and Microorganisms. Yeast extract, tryptone, and casamino acids for culture media were from Difco Laboratories; DTT[1] and IPTG[1] were from United States Biochemical Corp.; DNAse I was obtained from New England Biolabs; RNAse A and ampicillin were from Boehringer Mannheim Biochemicals; and sodium chloride, urea, EDTA,[1] sodium phosphate, and potassium phosphate (monobasic and dibasic forms) were from EM Science. Decanal and copper(I) bromide were from Aldrich, and the BCA protein assay kit was from Pierce. Flavin mononucleotide, ethidium bromide, and most other chemicals were from

[1] Abbreviations: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EDTA, *N*,*N*,*N*′,*N*′-ethylenediaminetetraacetic acid; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactoside; BSA, bovine albumin serum; MWCO, molecular weight cutoff; OD, optical density; SA, specific activity; LB, Luria broth.

Sigma. BSA[1] was from Promega. *E. coli* strain JM101 (*supE thi D(lac-proAB)/=F′traD36 proA*$^+$ *proB*$^+$*lacI*^{q} *lacZ* Δ*M15]*) was used for phage infection and subcloning. Oligonucleotides for site-directed mutagenesis were from Genosys (Houston, TX).

Luciferase α45 Mutants. Mutant *luxA* genes generated by site-directed mutagenesis (*40*, *41*, *56*) were inserted into a high-copy-number plasmid pUC19, which was introduced into *E. coli*. Methods for plasmid DNA preparation, ligation, phage infection, M13 DNA preparation, subcloning, ligation, and transformations, were as done described by Sambrook et al. (*57*).

Crude Cell Extracts. Extracts of *E. coli* JM101 cells bearing pUC19 plasmid with *luxAB* genes (*58*−*60*) were prepared after 3−3.5 h of cultivation at 37 °C to an optical density at 600 nm (OD_{600}) of 0.6−0.8 in LB[1] or M9 minimal medium supplemented with 100 μg/mL ampicillin. The cells were induced with 0.1 mM IPTG at OD_{600} ca. 0.2. When an aminoglycoside study was performed, it was added to the culture medium at the time of *E. coli* mutant inoculation. Cells were collected by centrifugation at 3100*g* for 15 min; washed with 100 mM potassium phosphate, 1 mM EDTA, pH 7.0; and resuspended with 5 mL of buffer. Cell suspensions were lysed by sonication on ice with a $^1/_8$″ microtip probe on a W-380 sonicator (Heat Systems Ultrasonic, Inc.) for 1 min with a pulse duration of 1 s at 90% of maximum output. Protein concentration was determined using the Pierce BCA protein assay with BSA as a standard.

Bioluminescence Assay. Luciferase activity was measured in vivo and in vitro with a Turner Designs model TD-20e luminometer with a sensitivity of 8.52×10^5 quanta s^{-1} light $unit^{-1}$, based on a radiochemical standard prepared according to the method of Hastings and Weber (*36*). Luciferase activity was measured in vivo by adding 10 μL of 1% v/v decanal in ethanol to a 1 mL sample of growing culture and was stable over the measurement period. The in vitro activity of bacterial luciferase was measured after injecting decanal (to 25 μM) and reduced flavin mononucleotide (to 27 μM) into a mixture of enzyme and oxygenated buffer following the method of Hastings and Weber (*36*). Flavin reduction with Cu(I)Br was conducted following the protocol of Lei and Becvar (*61*). A higher decanal concentration (240 μM) was used for mutant enzymes due to their higher K_M values (*40*). The aldehyde was premixed with the flavin solution rather than with the luciferase, and the use of BSA as a protection agent against aldehyde inhibition was then omitted (*62*). For all activity assays, 100 mM potassium phosphate, 1 mM EDTA, pH 7.0 was used at room temperature (22 °C).

Determination of Reversion Rate of V. harveyi Luciferase Mutant Gene in E. coli. Luria and Delbrück (*63*) established the classical fluctuation test to demonstrate bacterial mutation; the test is also useful for estimating spontaneous mutation rates. Their estimate is given by

$$p_0 = e^{-aN} \quad \text{or} \quad a = -\ln(p_0)/N$$

where p_0 is the probability of having no mutations, a is the reversion rate per generation (detectable mutations/cell), and N is the number of cells. Experimentally, p_0 is determined by culturing a large number, T, of dilute subcultures from the same seed culture for a certain number of generations. A bulk culture from the same seed culture is grown simultaneously to obtain an average specific activity measurement. The number of subcultures, m, having a loss or gain of activity resulting from reversion is counted and the experimental estimate for p_0 is given by

$$p_0 = (T - m)/T$$

In this work, the reversion rate was measured using reversion to brightness of JM101 cells with plasmid pKK223-3 bearing *luxAB* containing an aspartic acid codon (GAC) at position 45 (*40*). Two sets of subcultures were prepared by inoculating ca. 100 cells into each of 24 tubes (set 1) and 25 tubes (set 2) of 1 mL of LB containing 50 μg/mL ampicillin and 0.5 mM IPTG. After incubation at 30 °C for 14.8 h for set 1 (18.5 population doublings) and 19.3 h for set 2 (24.1 population doublings), the reversion rate of each set was measured using the in vivo bioluminescence assay.

Purification of Dimeric Native Luciferase. Native luciferase was prepared from JM101 cells bearing pUC19 plasmid with *luxAB* genes, cultured for 24 h to an OD at 600 nm of ca. 3.0 in LB broth, harvested by centrifugation at 3000*g*, and lysed with 1% w/v lysozyme, 0.8 mg/mL RNAse A, and 0.8 mg/mL DNAse I in 50 mM potassium phosphate, 1 mM EDTA, 0.5 mM DTT, pH 7.0. Ammonium sulfate 40% and 80% saturation cuts of the centrifuged lysate (12 000*g*, 30 min) were performed on ice. Luciferase was concentrated in the 80% ammonium sulfate pellet and was stable in this form at −20 °C for over 1 year. The pellet was dialyzed at 4 °C using 12 kDa MWCO[1] Spectrum dialysis bags against 100 volumes of 100 mM potassium phosphate, 1 mM EDTA, 0.5 mM DTT, pH 7.0.

Anion-exchange chromatography of the dialyzed sample used 30 mL of Pharmacia Q-Sepharose Fast Flow in a 1 cm Bio-Rad column. A 10 column-volume gradient from 100 to 400 mM potassium phosphate, pH 7.0, in 1 mM EDTA, 0.5 mM DTT, with a flow rate of 2 column volumes per hour, was used to collect bound luciferase, which eluted near 300 mM. The presence of luciferase in the fractions was determined using the in vitro assay described above. Purity was determined by silver-stained SDS-PAGE[1] using Pharmacia PhastSystem, 8−25 SDS Phastgels. The α and β subunits of luciferase appeared at 37 and 41 kDa respectively. After concentration on an Amicon Centriprep-30 concentrator, the retentate was dialyzed against 100 mM potassium phosphate, 1 mM EDTA, 0.5 mM DTT, pH 7.0 and stored at −20 °C.

Purification of α Subunit. Wild-type luciferase α subunit used for expression level normalization was isolated from *E. coli* JM101 harboring a pUC19 plasmid bearing *luxA*. Cell growth, harvesting, and lysis were as described above. Cell lysate was dialyzed against 50 mM potassium phosphate, 1 mM EDTA, 0.5 mM DTT, pH 7.0. The white precipitate that appeared was collected by centrifugation (10 000*g*, 30 min, 4 °C); washed twice in 100 mM potassium phosphate, 1 mM EDTA; and dissolved with the addition of 8.3 mL of 8 M guanidine hydrochloride to the 5 mL suspension. A silver-stained polyacrylamide gel showed that the precipitate contained mostly the α subunit (ca. 85% pure) with the main contaminant being lysozyme. The α subunit was apparently soluble in the cell lysate before the dialysis and may have been precipitated by the decrease in salt concentration.

Denaturation/Refolding Assay for Expression Level. Expression levels of α chain mutant luciferases were measured

by activating their wild-type β chains by co-refolding with excess exogenous α wild-type chain (*47*). Equal volumes of cell extract (or pure luciferase solution) and 10 M urea in 100 mM potassium phosphate, pH 7.0, were mixed in 0.5 mL microcentrifuge tubes and incubated at room temperature for 30 min. The tubes were then kept on ice and used within 1 h. The refolding assay was characterized at room temperature and at 4 °C; refolding at 4 °C is much slower, but somewhat more complete (see below).

A fixed amount of urea-denatured purified α subunit and four varying amounts of a given urea-denatured extract sample were mixed. The final volume was adjusted to 50 μL with 5 M urea. The 50 μL sample was then diluted with 2 mL of recovery buffer (1% w/v BSA in 100 mM potassium phosphate, 1 mM EDTA, 1 mM DTT, pH 7.0) and mixed by vortexing. After dilution, the final concentrations of the wild-type α subunit and urea were 15 μg/mL and 0.125 M, respectively. For the standard assay, the diluted samples were refolded at room temperature for 2 h. After refolding, four 50 μL aliquots of refolding mixture were diluted into 1 mL of assay buffer (100 mM potassium phosphate, 1 mM EDTA, pH 7.0), and the recovered catalytic activity was measured as described above, in quadruplicate. The light emission was plotted against concentration of total cellular protein; the slope gave the specific activity of the recovered enzyme in light units/(μg of protein/mL).

Determination of the Mistranslation Rate. The specific activity of the mutant or wild-type enzyme for each cell extract was determined by the ratio of catalytic activity (light units/(μg/mL)) to the luciferase fraction (μg of luciferase/μg of total protein) determined by the calibration curve described above. The expression for observed specific activity (SA) is given by

$$\text{observed SA}_{\text{mutant}} = \text{SA}_{\text{wild-type}}\, f_{\text{wild-type}} + \sum \text{SA}_i\, \text{f}_i$$

where $f_{\text{wild-type}}$ is the fraction of mistranslated species to wild-type, and f_i is the fraction of each of the other translated species *i*. In a typical case, for a SA_i of ca. 10^{-4} light units/μg of luciferase (*40*), $\text{SA}_i/\text{SA}_{\text{wild-type}}$ is on the order of 10^{-8} and therefore yields the fraction of species mistranslated to histidine as $f_{\text{wild-type}} = \text{SA}_{\text{mutant}}/\text{SA}_{\text{wild-type}}$.

RESULTS

Reversion Rate of Mutant V. harveyi Luciferase Gene in E. coli. Spontaneous reversion of the mutant reporter *luxA* genes, if it occurred at a frequency of about 10^{-6} or higher, would invalidate the measurement of translational errors, because the signal due to mutation would dominate that due to mistranslation (*64*). The estimated average mutation rate from two different Luria−Delbrück experiments with the GAC mutant construct was found to be 5.5×10^{-10}, which is negligible compared to the average mistranslation rates found in this work.

Bioluminescence Assay. The measured catalytic rate of bacterial luciferase has the advantage of being linear over a wide range of enzyme concentrations, from femtomolar to micromolar. It is not significantly affected by the presence of *E. coli* protein contained in cell extracts spiked with luciferase, as shown in Figure 1. The specific activity under the assay conditions used calculated by linear regression of data between 5 and 200 ng/mL is 7.47 light units/(ng of wild-type/mL).

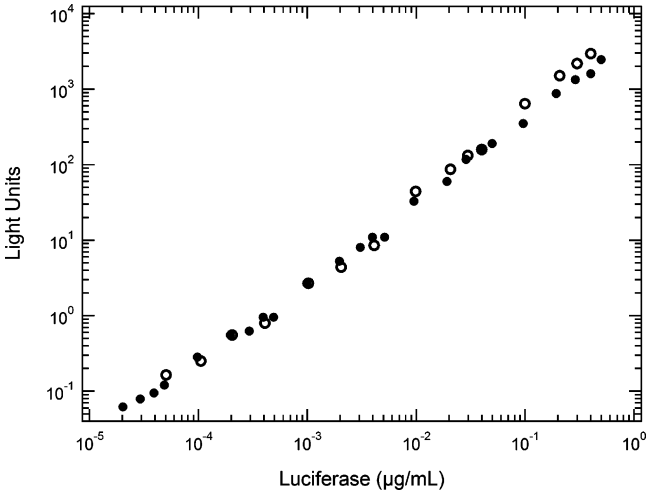

FIGURE 1: Activety of purified luciferase (●) and luciferase in a synthetic extract of *E. coli* JM101 harboring backbone plasmid pUC19 (○) spiked with varying concentrations of luciferase protein.

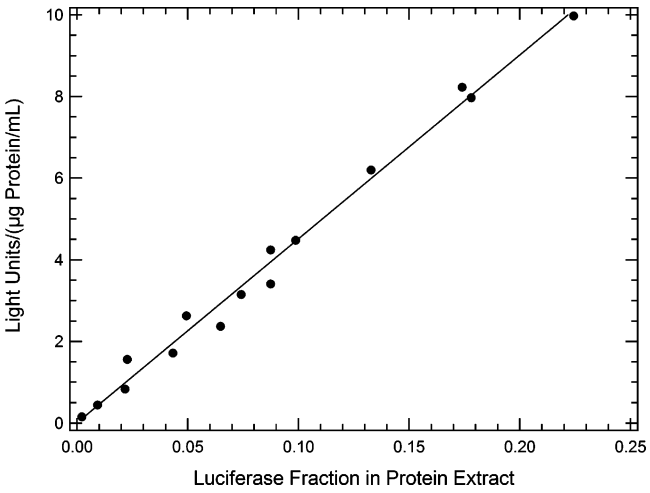

FIGURE 2: Refolding assay calibration curve showing luciferase specific activity (light units/(μg of total protein/mL)) as a function of luciferase fraction of total protein in luciferase-spiked extracts of *E. coli* JM101 harboring backbone plasmid pUC19.

Refolding Assay. A calibration curve relating light intensity to luciferase content was obtained using the standard refolding protocol and known percentages of pure $\alpha\beta$ luciferase spiked into extracts of *E. coli* JM101 harboring the backbone plasmid pUC19. The recovered specific activities are plotted in Figure 2 against the fraction of luciferase in the extract. Recovered specific activities are proportional to the luciferase level in extracts containing up to 20% luciferase, as shown. All cell extracts in this work gave activities corresponding to an expression level lower than 20% of total protein.

Expression levels of α-mutant luciferase were measured by refolding and activating their wild-type β chains (present in 1:1 molar ratio to mutant α) with excess exogenous wild-type α chains. While loss of activity with denaturant is very rapid, the later-recovered activity of luciferase was found to decrease with longer incubation of the denatured protein sample in urea, perhaps due to unfolding of individual subunits. Shorter denaturation times, however, may not allow for complete subunit interchange with the $\alpha^{\text{mutant}}\beta^{\text{wild-type}}$

inactive forms and, therefore, may have a reduced chance for a β subunit to recombine with an active α chain during the refolding step. For the assay of interest here, to allow the $\alpha^{mutant}\beta^{wild-type}$ protein to completely unfold and the $\beta^{wild-type}$ to refold with the added excess $\alpha^{wild-type}$, a 30 min urea incubation was chosen, similar to the denaturation time chosen by Ziegler et al. (*65*).

The recovery of activity depends on temperature and increases with the refolding time until a maximum is reached. In agreement with the results of Friedland and Hastings (*66*), the refolding is faster but less complete at higher temperatures. Ninety percent recovery of activity with pure luciferase was reached after 110 h at 4 °C and after 2 h at 23 °C. Refolding at 23 °C was also linear with time until it reached a maximum value. A lag phase of about 20 h was seen at 4 °C and has been shown by Baldwin et al. (*45*) to be due to slow (first-order) steps in the refolding of both subunits. To rapidly achieve a high yield, a period of 2 h at room temperature was selected for the refolding assay.

A large excess of the wild-type α chain was used to ensure that recovery of activity was independent of the concentration of the α chain. Since all of the cell extracts had luciferase expression of 20% or less of the total cellular protein production (2 μg/mL), 15 μg/mL (353 nM) of exogenously added $\alpha^{wild-type}$ was used. Below this concentration, the molar ratio of $\alpha^{wild-type}/\alpha^{mutant}\beta^{wild-type}$ is too low and the recovery of activity becomes dependent on the amount of $\alpha^{wild-type}$ added as well as the amount of $\alpha^{mutant}\beta^{wild-type}$ reporter enzyme.

Missense Error Frequencies of Luciferase Mutants with Synonymous Codons at Position α45. Since mutations at position α45 profoundly reduce the activity of luciferase (*41*, *42*), differing specific activities of mutants bearing synonymous codons must be largely due to incorporation of a native histidine at position 45. Four luciferase mutants with synonymous leucine codons at position α45 had error frequencies varying from 2.9×10^{-6} to 1.5×10^{-8} (Table 1). The CUG and UUG codons gave the lowest activity and have the most base differences (two and three, respectively) from the native histidine codons (CAC, CAU). The CUC and CUU codons, which have only one central base difference from the histidine codons, gave 14−133-fold higher mistranslation rates, supporting the hypothesis that the CUC and CUU codons are mistranslated to histidine, although with very low error frequencies of $(1.3-2.0) \times 10^{-6}$.

Errors such as misacylation of cognate tRNA and mistranscription of mRNA occur prior to translation, and the observed error frequency is a combination of these error sources along with mistranslation. Misacylation of the leucyl-tRNAs, e.g., triggered by depletion of the required amino acid, would likely result in similar activity for the four mutants. Moreover, the specific activity of the CUC mutant was not significantly different in rich LB medium and in minimal M9 medium with or without 1 g/L casamino acids (results not shown). Therefore, the large activity differences among the four leucine mutants arise primarily from either miscoding or mistranscription, and not misacylation. One way to distinguish these possible error sources is by the use of error-promoting antibiotics that selectively promote miscoding.

Table 1: Histidyl-tRNA Anticodon (3′-GUQ-5′) (Mis)Matching with Various Codons at Position α45 of Luciferase along with Their Specific Activities (±Standard Deviation) and Error Frequencies[d]

codon/ anticodon mismatching	SA ± SD (light units/ μg of luciferase)	error frequency $SA_{mutant}/SA_{wild-type}$
histidine 5′-CAC-3′ 5′-CAU-3′	7790 ± 1420	
glutamine 5′-CAg-3′	37 ± 12	4.1×10^{-3} [a]
proline 5′-CcU-3′	1.06 ± 0.19	1.4×10^{-4} [c]
leucine 5′-CuC-3′	0.015 ± 0.004	2.9×10^{-6} [b]
leucine 5′-CuU-3′	0.010 ± 0.005	1.3×10^{-6}
aspartic acid 5′-gAC-3′	1.48 ± 0.27	2.0×10^{-4} [a]
tyrosine 5′-uAC-3′	0.050 ± 0.009	6.6×10^{-6} [c]
leucine 5′-Cug-3′	0.00070 ± 0.00030	9.0×10^{-8}
lysine 5′-aAa-3′	0.167 ± 0.030	2.2×10^{-5} [a]
arginine 5′-aga-3′	1.22 ± 0.22	1.6×10^{-4} [c]
leucine 5′-uug-3′	0.00012 ± 0.0006	1.5×10^{-8}
alanine 5′-gcc-3′	0.0040 ± 0.0007	5.3×10^{-7} [a]

[a] Average results from Xin et al. (*40*) and Li et al. (*41*) for purified luciferase mutants, which differ on average by 61%. [b] Average results from the present work and Li et al. (*41*), which differ on average by 61%. [c] Results from Li et al. (*41*) for purified luciferase mutant protein. [d] Lowercase nucleic acid bases indicate a mismatch with the histidyl-tRNA anticodon. All measurements were made at least in triplicate.

Effects of Streptomycin, Neomycin, and Paromomycin on the Activity of the Leucine (CUC) Mutant. The effects of streptomycin, neomycin, and paromomycin on the culture-density-normalized specific activity of the CUC mutant were determined in preliminary experiments, and the effects of streptomycin on the specific activity of three different leucine mutants was studied using the in vitro expression assay. An approximately linear relationship was observed between antibiotic concentration and the culture-density-normalized activity of cultures grown for 2 h in the presence of the antibiotics. The slopes obtained for streptomycin, paromomycin, and neomycin were 0.35, 1.36, and 3.5 (light units/OD_{600})/μM antibiotic, respectively, and at a 2 μg/mL concentration, the increase in catalytic activity was 10-, 53-, and 68-fold, respectively. Control experiments confirmed that 3.5 μM streptomycin decreases total protein and luciferase expression levels by 50%, but does not alter the specific activity of wild-type luciferase expressed in antibiotic-treated cells (not shown). The sensitivity of the CUC mutant's activity to all three miscoding-enhancing antibiotics suggests that histidine misincorporation events arise in large part due to noncognate tRNA anticodon−codon interactions.

As illustrated by Figure 3, the activities induced by neomycin and paromomycin were larger and more similar to each other than those induced by streptomycin. Paromomycin and neomycin are known to impair codon−anticodon recognition in the first control in tRNA selection (*49*, *52*, *67*, *68*) and favor mismatching at the central position of the

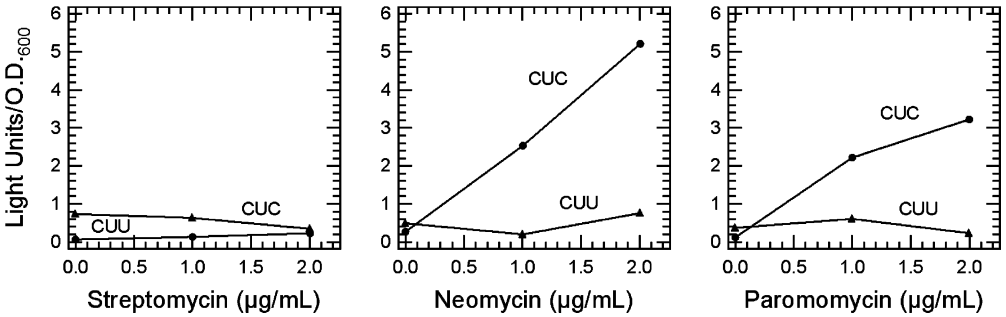

FIGURE 3: Influence of aminoglycoside antibiotic concentration on the light emission of *E. coli* expressing α45 leucine (CUC and CUU) mutant luciferases. A 1% inoculum of an overnight culture of *E. coli* JM101 cells harboring the plasmid pUC19 with mutant *luxAB* was inoculated into LB with 50 μg/mL ampicillin and 0−5 μg/m streptomycin, neomycin, or paromomycin. The light emission (light units), as well as the optical density at 600 nm (OD_{600}), was measured after 2 h of growth. The ratio light units/OD_{600} is plotted as a function of antibiotic concentration.

codon in poly-UUU programmed ribosomes. The large activity increase observed for the CUC mutant in the presence of neomycin, which arises from miscoding at the central base, is consistent with these observations. Furthermore, the weaker effect of streptomycin on the CUC mutant is consistent with the fact that streptomycin does not promote errors at the middle position of the codon, but rather at the 5′ and 3′ positions by interfering with proofreading (*49*, *69*, *70*).

Effects of Streptomycin and Neomycin on Histidine Misincorporation of the Glutamine (CAG) Mutant. Miscoding errors are most tolerable and most likely at the degenerate 3′ wobble position, where they are less likely to lead to an amino acid substitution. The glutamine codons (CAG and CAA) differ from those for histidine (CAC and CAU) solely at the wobble position. The misincorporation of histidine at the CAG codon is indeed 2500-fold higher than that of the central-mismatched leucine codon (CUC) used in the previous experiment. We estimated an error frequency of 4.8×10^{-3} for the glutamine codon (CAG) using the in vitro assay. This value is comparable to the ratio of mutant activity to wild-type activity (3.3×10^{-3}) measured by Li et al. (*41*) using purified α45 histidine → glutamine enzyme (giving an average error frequency of 4.1×10^{-3} in Table 1), which indicates that the lysate assay can produce values of the error frequency matching those found with purified mutant enzyme.

To confirm that the observed activity was not due to the residual activity of the mutant protein, and to test if the activity of glutamine codon (CAG) construct could be due to miscoding, the culture-density-normalized activity of the glutamine mutant was measured in the presence of streptomycin and neomycin. As shown in Figure 4, the presence of 2.5 μM streptomycin or neomycin increased the catalytic activity of the mutant by 12- and 3.5-fold, respectively. Streptomycin enhanced misreading at the CAG codon (with a possible wobble mismatch) more than neomycin. The different responses of the CAG mutant to both antibiotics suggest that streptomycin and neomycin do not affect the miscoding event at the wobble position in the same manner. Together with the results for the CUC mutant described above, our results confirm the findings of Brakier-Gingras et al. (*67*, *68*, *71*) that streptomycin promotes outside-position miscoding and neomycin promotes middle-position miscoding.

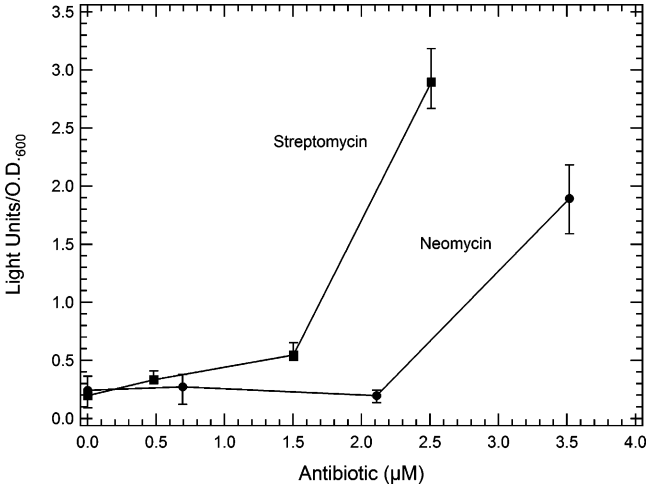

FIGURE 4: Influence of aminoglycoside antibiotics on the light emission of *E. coli* expressing α45 glutamine (CAG) mutant luciferase. A 1% inoculum of an overnight culture of *E. coli* JM101 cells harboring the plasmid pUC19 with mutant *luxAB* was inoculated into LB with 50 μg/mL ampicillin and 0−3.5 μM aminoglycoside. The light emission (light units), as well as the optical density at 600 nm (OD_{600}), was measured after 2 h of growth. The ratio light units/OD_{600} is plotted as a function of antibiotic concentration.

Effect of Streptomycin on the Error Frequency of Synonymous Codons by in Vitro Expression Level Measurement. The effect of antibiotics observed on the leucine (CUC) codon using culture-density normalization were further investigated using the better-controlled in vitro refolding assay for expression level. It was shown that the error frequencies of synonymous leucine codons (Table 1) in the absence of the antibiotics differed by 14−133-fold. As shown in Figure 5, 2 μg/mL streptomycin increased the error frequency of three leucine codons from 3.4-fold for the CUG mutant (with a two-base difference from the $tRNA^{His}$ anticodon) to 4.6-fold for the CUU mutant to 18.2-fold (versus 7-fold observed above with optical density normalization) for the CUC mutant (both with one base difference). The differences in responsiveness of these mutants to streptomycin confirm that synonymous codons differ in translational accuracy. The greater responsiveness of the CUC codon may arise from its greater ability to bind to noncognate tRNAs (in this case histidine tRNA) in the first step of translation (*72*). The addition of streptomycin, which is known to enhance misreading through the second proof-

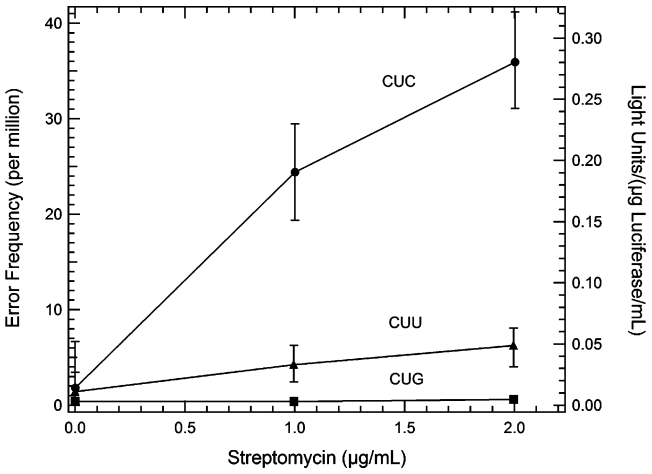

FIGURE 5: Influence of streptomycin on error frequencies of leucine CUU, CUC, and CUG luciferase mutants as measured by the in vitro refolding assay for luciferase expression. *E. coli* JM101 cells harboring the plasmid pUC19 with *luxAB* with corresponding mutations on position 45 of the *luxA* gene were grown overnight. Cell extracts were generated after 2 h of growth from a 1% v/v inoculum of the overnight culture in liquid LB with 50 μg/mL ampicillin. Streptomycin was added to a final concentration of 0−2 μg/mL at the time of inoculation. The catalytic activity and refolding assays were performed on the extracts, and the specific activity was calculated for each mutant and each antibiotic concentration.

reading step, will enhance the acceptance of errors from the first step.

DISCUSSION

Error Frequencies of Synonymous Codons. The error frequencies of the leucine codons varied by up to 133-fold (Table 1). Misacylation was not the main error source because (1) the cells were not starved for leucine, (2) culture on various growth-rate-determining media did not produce detectable differences in error frequency with the CUC codon, and (3) misacylation should favor amino acids that are structurally related (*73*) or smaller in size. A histidine-to-leucine miscoding event especially with the CUC and CUU codons would result from the selection of noncognate histidyl-tRNA^{QUG} (tRNA^{His}). This type of miscoding event would be similar to those described by Parker et al. (*8*, *74*) and Parker and Precup (*19*), in which two synonymous asparagine and phenylalanine codons were misread by noncognate lysine and leucine tRNAs, respectively. In that case, however, mistranslation occurred under starvation conditions and at the wobble position.

The error frequency with the UUG codon was extremely low (1.5×10^{-8}). Since this codon has a three-base mismatch with the histidyl-tRNA^{QUG} anticodon (Table 1), a miscoding event with tRNA^{His} appears improbable. The UUG codon is read by tRNA$_4^{Leu}$ and tRNA$_5^{Leu}$ (anticodon NAA), which are not very abundant (<3% of total tRNA) in *E. coli* (*75*, *76*), so misreading at this codon may be enhanced by shortage of cognate tRNAs. A one-base mismatch is more plausible with one of the phenylalanine anticodons GAA or AAA (mismatch at the 3′ wobble position) or with the CUG leucine anticodon by tRNA$_1^{Leu}$, which is the most abundant leucyl-tRNA. The other possible one-base miscoding events are with the tryptophan tRNA^{CCA} anticodon or with the serine tRNA^{CGA} anticodon. The possible selection of leucine, phenylalanine, tryptophan, or serine tRNAs, however, would have all resulted in inactive enzyme molecules (*41*). The activity seen with the UUG codon, therefore, may well arise from mistranscription, which is typically on the order of 10^{-5} or less (*4*−*7*).

The CUG codon differs by two bases from the tRNA^{His} anticodon and gave an extremely low error frequency (9.0×10^{-8}). A misreading of the CUG codon by tRNA^{His} would involve U·U and Q·G mismatches at the second and third positions, as shown in Table 1. The CUG codon is the most preferred leucine codon in *E. coli* and its tRNA (tRNA$_1^{Leu}$) is the most abundant leucyl-tRNA (*75*−*78*). The combination of codon bias with the improbable mismatch of two bases with the histidyl-tRNA anticodon would reduce the occurrence of leucine-to-histidine miscoding events at the CUG codon. Furthermore, the weak response of the CUG mutant to the presence of streptomycin also indicates that only a small fraction of the misincorporation rate comes from miscoding. Similarly to the UUG codon, the errors observed with the CUG codon may arise primarily from mistranscription. The error frequencies with the CUU and CUC codons (1.3×10^{-6} and 2.9×10^{-6}, respectively) were 14−87-fold greater than the other two leucine codons. CUC and CUU are less frequently used leucine codons in *E. coli*, and tRNA$_2^{Leu}$, whose anticodon NAG recognizes both, is ca. one-fifth as abundant as leucyl-tRNA$_1^{CAG}$ (*76*−*78*). Misreading errors may be caused by a shortage of tRNA$_2^{Leu}$, particularly since it is about one-third as abundant as histidyl-tRNA, which could misread both the CUC and CUU codons with a possible U·U mismatch at the middle position (*75*, *79*).

Error Frequencies of Other Codons with a Single Mismatch to Histidine. The glutamine (CAG) mutant gave by far the highest error frequency measured (*40*, *41*, *80*); the histidine misincorporation rate of the CAG codon is about 2500-fold higher than that of the "most active" leucine mutant (CUC). A histidine-for-glutamine translational error is relatively probable on the basis of the wobble hypothesis (*81*). The CAG codon is more likely to be mispaired to the histidine tRNA^{QUG} anticodon than any other anticodon. Ulrich et al. (*81*) observed a histidine misincorporation for glutamine in glutamine-starved cells with a normal level of tRNA^{His} and an enhanced level of substitutions with an overproduction of tRNA^{His}. Lu et al. (*82*) observed the reverse event, a glutamine-for-histidine substitution in a recombinant methionyl granulocyte colony stimulating factor expressed in *E. coli*. They also observed three variant isoforms of this protein resulting from misincorporation of histidine at three glutamine residues. Similarly, O'Farrell (*83*) found that histidine starvation of *relA* error-prone mutants caused high levels of glutamine-for-histidine substitutions, apparently due to a third-position misreading by glutamyl-tRNA. Other types of mispairing at the 3′ wobble position have been observed. For example, two asparagine codons (AAU and AAC) were misread by lysine tRNA (*74*) to induce lysine-for-asparagine misincorporation error frequencies of 2×10^{-3} and 4×10^{-4}, respectively, which are comparable to the error frequency obtained with the glutamine (CAG) mutant (4.8×10^{-3}) in the present study.

The mistranslation rate of the aspartic acid GAC mutant (2.0×10^{-4}) and the proline CCU mutant (1.4×10^{-4}) are comparable to the previously estimated average mistranslation rate. As shown in Table 1, a single mismatch of G·G or U·C by tRNA^{His} could take place at the 5′ and middle codon

positions, respectively. The error frequency of the proline CCU mutant was 2 orders of magnitude higher than that of the leucine mutants (CUC and CUU), even though the codon/anticodon mismatch would be at the same (middle) position. It is possible that a U·C misreading of a cytosine at the middle position (in the case of proline) is more frequent than a U·U misreading of uracil (in the case of leucine).

Another single-mismatch codon is that of the tyrosine (UAC) mutant. The error frequency of this codon (6.6×10^{-6}) was 20 times lower than those of the GAC and CCU mutants discussed above, but it has the same order of magnitude as the misincorporation rates of the CUU and CUC mutants. Interestingly, the error frequency is similar to the histidine-to-tyrosine misincorporation rate of 5×10^{-6} measured at position 195 of type III chloramphenicol acetyl transferase expressed in *E. coli* (*26*, *40*). The relatively low histidine misincorporation rate with the tyrosine mutant may be due to a possible miscoding at the wobble position, which would result in early termination. Presumably, these defective forms would have been eliminated during purification of the enzyme; therefore, the error frequency would be underestimated.

Error Frequencies with Two or Three Mismatches to Histidine. The error frequencies of double-mismatch codons (lysine AAA and alanine GCC) and triple-mismatch codons (arginine AGA) were determined from the previous work of Xin et al. (*40*) and Li et al. (*41*), respectively. On the basis of the proceeding discussion, a miscoding event resulting in histidine incorporation at position 45 is unlikely for these three codons, but mistranscription or misacylation could still take place. The histidine misincorporation rate for the alanine codon (GCC) with two codon−anticodon mismatches is very low (0.53×10^{-6}) and similar to that of the histidine-for-alanine rate measured at position 195 of chloramphenicol acetyl transferase in *E. coli* (1×10^{-6}) (*26*, *84*). The authors suggest that a mistranscription event is most likely the source of the misincorporation (as paromomycin had little effect on the error rate). The apparent error frequencies observed with the lysine (AAA) and arginine (AGA) mutants (2.2×10^{-5} and 1.6×10^{-4}, respectively) are unexpectedly high, especially when compared to the other codons with double mismatches (alanine GCC and leucine CUG). However, these basic amino acids may be partially functional at position 45 in place of histidine. Most reporter systems will not afford a perfect one-active, 19-totally inactive reporter, and replacement of the active wild-type histidine by basic lysine or arginine might well be expected to give high residual activity relative to other amino acids (though the activity is only 0.002% and 0.016% of the wild-type, respectively). It is most important to have one choice that gives very low activity, and the various leucine codons give up to 10 000-fold lower residual activity than lysine or arginine. In addition to possibly high residual activity of basic amino acid substitutions, the AGA codon in *E. coli* is relatively rare and frequently mistranslated (*23*, *77*), and the very low abundance of $tRNA^{Arg}$ in *E. coli* could result in the depletion and misselection of a noncognate histidyl-tRNA.

Salas-Marco and Bedwell found in their firefly luciferase reporter system that synonymous arginine codons (CGC and CGA) have a 3.5-fold difference in basal mistranslation rates, which are enhanced to approximately 8-fold in the presence of paromomycin (200 μg/mL), consistent with our observations. Additionally, they found only two of 15 mutants tested with paromomycin (200 μg/mL) had an increase in misincorporation rate greater than 4.3-fold, while the majority of these 15 mutants responded less than 1.5-fold. The effects of ribosomal protein mutations (S9B-D94N and L12 depletion) were also investigated and showed mistranslation enhancement trends similar to that of paromomycin, suggesting that these factors share a common influence on the ribosome decoding mechanism. Finally it was also shown that paromomycin has a greater effect on stop codon readthrough than on misincorporation; all six tetranucleotide termination signals tested showed a readthrough enhancement greater than 14-fold in the presence of 200 μg/mL paromomycin.

CONCLUSIONS

The replacement of histidine at position 45 of the α subunit by most amino acids (*27*, *40*, *42*, *56*) profoundly inactivates the *V. harveyi* luciferase enzyme, producing a nearly ideal "one-active" missense reporter as described by Cornut and Willson (*27*). The catalytic activity is linear over a wide range of luciferase concentrations (5−200 ng/mL). This and similar assays could be used to study the mechanisms of other antibiotics or miscoding-enhancing agents on the ribosome and to help understand how ribosome variants, expression levels, codon usage and frequency, growth phase and medium composition, amino acid starvation, and tRNA levels influence missense error rates for specific codons. In this assay, however, a change in the codon context, i.e., by changing surrounding codons (CAC at position 44; UUC at position 46 on *luxA*), would be more problematic, since His α44 is another essential amino acid of the α subunit of *V. harveyi* luciferase. This type of assay might also be useful in optimizing the manufacture of therapeutic proteins by high expression of heterologous genes under conditions that may promote missense errors, such as intermittent glucose feeding and starvation (*85*), or in emerging in vitro translation methods (*86*). *V. harveyi* and other luciferases can be expressed in a variety of hosts (*87*). Transgenic mice expressing luciferase raise the possibility of measuring species-, organ-, or development-specific translational accuracies (*88*) and investigating the therapeutic effects of mistranslation promoting agents on genetic diseases (*89*).

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REFERENCES

1. Goodman, M. F., and Branscomb, E. W. (1986) in *Molecular Processes: Its Control and Relevance to Living Systems* (Kirkwood, T. B. L., Rosenberger, R. F., and Galas, D. J., Eds.) p 191, Chapman and Hall, London, New York.
2. Kirkwood, T. B. L., Rosenberger, R. F., and Galas, D. J. (1986) *Molecular Processes: Its Control and Relevance to Living Systems*, Chapman and Hall, London, New York.
3. Drake, J. W. (1969) Comparative rates of spontaneous mutation, *Nature 221*, 1132.

4. Parker, J. (1989) Errors and alternatives in reading the universal genetic code, *Microbiol. Rev. 53*, 273−298.
5. Rosenberger, R. F., and Hilton, J. (1983) The frequency of transcriptional and translational errors at nonsense codons in the lacZ gene of *Escherichia coli*, *Mol. Gen. Genet. 191*, 207−212.
6. Rosenberger, R. F., and Foskett, G. (1981) An estimate of the frequency of in vivo transcriptional errors at a nonsense codon in *Escherichia coli*, *Mol. Gen. Genet. 183*, 561−563.
7. Springgate, C. F., and Loeb, L. A. (1975) On the fidelity of transcription by *Escherichia coli* ribonucleic acid polymerase, *J. Mol. Biol. 97*, 577−591.
8. Parker, J., Johnston, T. C., Borgia, P. T., Holtz, G., Remaut, E., and Fiers, W. (1983) Codon usage and mistranslation. In vivo basal level misreading of the MS2 coat protein message, *J. Biol. Chem. 258*, 10007−10012.
9. Ellis, N., and Gallant, J. (1982) An estimate of the global error frequency in translation, *Mol. Gen. Genet. 188*, 169−172.
10. Edelmann, P., and Gallant, J. (1977) Mistranslation in *E. coli*, *Cell 10*, 131−137.
11. Loftfield, R. B., and Vanderjagt, D. (1972) The frequency of errors in protein biosynthesis, *Biochem. J. 128*, 1353−1356.
12. Davies, I. A. (1990) Testing criteria, *Nature 345*, 658.
13. Forman, M. D., Stack, R. F., Masters, P. S., Hauer, C. R., and Baxter, S. M. (1998) High level, context dependent misincorporation of lysine for arginine in *Saccharomyces cerevisiae* a1 homeodomain expressed in *Escherichia coli*, *Protein Sci. 7*, 500−503.
14. Bogosian, G., Violand, B. N., Jung, P. E., and Kane, J. F. (1990) In *The Ribosome: Structure, Function, and Evolution*, p 549, American Society of Microbiology, Washington, DC.
15. Seetharam, R., Heeren, R. A., Wong, E. Y., Braford, S. R., Klein, B. K., Aykent, S., Kotts, C. E., Mathis, K. J., Bishop, B. F., Jennings, M. J., Smith, C. E., and Siegel, N. R. (1988) Mistranslation in IGF-1 during over-expression of the protein in *Escherichia coli* using a synthetic gene containing low frequency codons, *Biochem. Biophys. Res. Commun. 155*, 518−523.
16. Reidhaar-Olson, J. F., and Sauer, R. T. (1990) Functionally acceptable substitutions in two alpha-helical regions of lambda repressor, *Proteins 7*, 306−316.
17. Kurland, C. G., and Ehrenberg, M. (1987) Growth-optimizing accuracy of gene expression, *Annu. Rev. Biophys. Biophys. Chem. 16*, 291−317.
18. Sorensen, H. P., Sperling-Petersen, H. U., and Mortensen, K. K. (2003) Production of recombinant thermostable proteins expressed in *Escherichia coli*: Completion of protein synthesis is the bottleneck, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 786*, 207−214.
19. Parker, J., and Precup, J. (1986) Mistranslation during phenylalanine starvation, *Mol. Gen. Genet. 204*, 70−74.
20. Johnston, T. C., Borgia, P. T., and Parker, J. (1984) Codon specificity of starvation induced misreading, *Mol. Gen. Genet. 195*, 459−465.
21. Kane, J. F. (1995) Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*, *Curr. Opin. Biotechnol. 6*, 494−500.
22. Toth, M. J., Murgola, E. J., and Schimmel, P. (1988) Evidence for a unique first position codon−anticodon mismatch in vivo, *J. Mol. Biol. 201*, 451−454.
23. Davis, B. D. (1987) Mechanism of bactericidal action of aminoglycosides, *Microbiol. Rev. 51*, 341−350.
24. Branscomb, E. W., and Galas, D. J. (1975) Progressive decrease in protein synthesis accuracy induced by streptomycin in *Escherichia coli*, *Nature 254*, 161−163.
25. Hughes, D. (1991) Error-prone EF-Tu reduces in vivo enzyme activity and cellular growth rate, *Mol. Microbiol. 5*, 623−630.
26. Stansfield, I., Jones, K. M., Herbert, P., Lewendon, A., Shaw, W. V., and Tuite, M. F. (1998) Missense translation errors in *Saccharomyces cerevisiae*, *J. Mol. Biol. 282*, 13−24.
27. Cornut, B., and Willson, R. C. (1991) Measurement of translational accuracy in vivo: Missense reporting using inactive enzyme mutants, *Biochimie 73*, 1567−1572.
28. Schultz, D. W., and Yarus, M. (1990) A simple and sensitive in vivo luciferase assay for tRNA-mediated nonsense suppression, *J. Bacteriol. 172*, 595−602.
29. Williams, T. M., Burlein, J. E., Ogden, S., Kricka, L. J., and Kant, J. A. (1989) Advantages of firefly luciferase as a reporter gene: Application to the interleukin-2 gene promoter, *Anal. Biochem. 176*, 28−32.
30. Olsson, O., Koncz, C., and Szalay, A. A. (1988) The use of the *lux*A gene of the bacterial luciferase operon as a reporter gene, *Mol. Gen. Genet. 215*, 1−9.
31. Engebrecht, J., Simon, M., and Silverman, M. (1985) Measuring gene expression with light, *Science 227*, 1345−1347.
32. Aflalo, C. (1990) Targeting of cloned firefly luciferase to yeast mitochondria, *Biochemistry 29*, 4758−4766.
33. Brasier, A. R., Tate, J. E., and Habener, J. F. (1989) Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines, *Biotechniques 7*, 1116−1122.
34. Holzman, T. F., and Baldwin, T. (1983) Reversible inhibition of the bacterial luciferase catalyzed bioluminescence reaction by aldehyde substrate: Kinetic mechanism, *Biochemistry 22*, 2838−2846.
35. Becvar, J. E., and Hastings, J. W. (1975) Bacterial luciferase requires one reduced flavin for light emission, *Proc. Natl. Acad. Sci. U.S.A. 72*, 3374−3376.
36. Hastings, J. W., and Weber, G. (1963) Total quantum flux of isotropic sources, *J. Opt. Soc. Am. 53*, 1410−1415.
37. Cline, T. W., and Hastings, J. W. (1972) Mutationally altered bacterial luciferase. Implications for subunit functions, *Biochemistry 11*, 3359−3370.
38. Meighen, E. A., Nicoli, M. Z., and Hastings, J. W. (1971) Functional differences of the nonidentical subunits of bacterial luciferase. Properties of hybrids of native and chemically modified bacterial luciferase, *Biochemistry 10*, 4069−4073.
39. Cousineau, J., and Meighen, E. (1976) Chemical modification of bacterial luciferase with ethoxyformic anhydride: Evidence for an essential histidyl residue, *Biochemistry 15*, 4992−5000.
40. Xin, X., Xi, L., and Tu, S.-C. (1991) Functional consequences of site-directed mutation of conserved histidyl residues of the bacterial luciferase alpha subunit, *Biochemistry 30*, 11255−11262.
41. Li, H., Ortego, B. C., Maillard, K. I., Willson, R. C., and Tu, S.-C. (1999) Effects of mutations of the alpha His45 residue of *Vibrio harveyi* luciferase on the yield and reactivity of the flavin peroxide intermediate, *Biochemistry 38*, 4409−4415.
42. Xin, X. (1992) Elucidation of structural basis for the light-emitting activity of bacterial luciferase by site-directed mutagenesis. Ph.D. Thesis in Biochemical and Biophysical Sciences, University of Houston, Texas.
43. Cornut, B. (1990) Measurement of translational accuracy in *Escherichia coli*. MS Thesis in Chemical Engineering, University of Houston, Texas.
44. Shaw, R. J., Bonawitz, N. D., and Reines, D. (2002) Use of an in vivo reporter assay to test for transcriptional and translational fidelity in yeast, *J. Biol. Chem. 277*, 24420−24426.
45. Baldwin, T. O., Ziegler, M. M., Chaffotte, A. F., and Goldberg, M. E. (1993) Contribution of folding steps involving the individual subunits of bacterial luciferase to the assembly of the active heterodimeric enzyme, *J. Biol. Chem. 268*, 10766−10772.
46. Waddle, J. J., Johnston, T. C., and Baldwin, T. O. (1987) Polypeptide folding and dimerization in bacterial luciferase occur by a concerted mechanism in vivo, *Biochemistry 26*, 4917−4921.
47. Tu, S.-C., Makemson, J. C., Becvar, J. E., and Hastings, J. W. (1977) Bacterial luciferase subunits are synthesized in equal quantities, *J. Biol. Chem. 252*, 803−805.
48. Gorini, L., and Kataja, E. (1964) Phenotypic repair by streptomycin of defective genotypes in *E. coli*, *Proc. Natl. Acad. Sci. U.S.A. 51*, 487−493.
49. Ogle, J. M., and Ramakrishnan, V. (2005) Structural insights into translational fidelity, *Annu. Rev. Biochem. 74*, 129−177.
50. Carter, A. P., Clemons, W. M., Brodersen, D. E., Morgan-Warren, R. J., Wimberly, B. T., and Ramakrishnan, V. (2000) Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics, *Nature 407*, 340−348.
51. Ogle, J. M., Carter, A. P., and Ramakrishnan, V. (2003) Insights into the decoding mechanism from recent ribosome structures, *Trends Biochem. Sci. 28*, 259−266.
52. Rodnina, M. V., Daviter, T., Gromadski, K., and Wintermeyer, W. (2002) Structural dynamics of ribosomal RNA during decoding on the ribosome, *Biochimie 84*, 745−754.
53. Pape, T., Wintermeyer, W., and Rodnina, M. V. (2000) Conformational switch in the decoding region of 16S rRNA during aminoacyl-tRNA selection on the ribosome, *Nat. Struct. Biol. 7*, 104−107.
54. Fourmy, D., Recht, M. I., Blanchard, S. C., and Puglisi, J. D. (1996) Structure of the A site of *Escherichia coli* 16S ribosomal RNA complexed with an aminoglycoside antibiotic, *Science 274*, 1367−1371.

55. Salas-Marco, J., and Bedwell, D. M. (2005) Discrimination between defects in elongation fidelity and termination efficiency provides mechanistic insights into translational readthrough, *J. Mol. Biol. 348*, 801−815.
56. Ortego, B. C. (1994) In vivo measurement of translational accuracy in *Escherichia coli:* Missense reporting using inactive mutants of *Vibrio harveyi* luciferase. Ph.D. Thesis in Chemical Engineering, University of Houston, Texas.
57. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) In *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press.
58. Meighen, E. A. (1988) Enzymes and genes from the *lux* operons of bioluminescent bacteria, *Annu. Rev. Microbiol. 42*, 151−176.
59. Miyamoto, C. M., Graham, A. D., Boylan, M., Evans, J. F., Hasel, K. W., Meighen, E. A., and Graham, A. F. (1985) Polycistronic mRNAs code for polypeptides of the *Vibrio harveyi* luminescence system, *J. Bacteriol. 161*, 995−1001.
60. Cohn, D. H., Mileham, A. J., Simon, M. I., Nealson, K. H., Rausch, S. K., Bonam, D., and Baldwin, T. O. (1985) Nucleotide sequence of the *lux*A gene of *Vibrio harveyi* and the complete amino acid sequence of the alpha subunit of bacterial luciferase, *J. Biol. Chem. 260*, 6139−6146.
61. Lei, B. F., and Becvar, J. E. (1991) A new reducing agent of flavins and its application to the assay of bacterial luciferase, *Photochem. Photobiol. 54*, 473−476.
62. Lei, B., Cho, K. W., and Tu, S.-C. (1994) Mechanism of aldehyde inhibition of *Vibrio harveyi* luciferase. Identification of two aldehyde sites and relationship between aldehyde and flavin binding, *J. Biol. Chem. 269*, 5612−5618.
63. Luria, S. E., and Delbrock, M. (1943) Mutations of bacteria from sensitivity to virus resistance, *Genetics 28*, 491−511.
64. Ninio, J. (1991) Connections between translation, transcription and replication error-rates, *Biochimie 73*, 1517−1523.
65. Ziegler, M. M., Goldberg, M. E., Chaffotte, A. F., and Baldwin, T. O. (1993) Refolding of luciferase subunits from urea and assembly of the active heterodimer. Evidence for folding intermediates that precede and follow the dimerization step on the pathway to the active form of the enzyme, *J. Biol. Chem. 268*, 10760−10765.
66. Friedland, J., and Hastings, J. W. (1967) The reversibility of the denaturation of bacterial luciferase, *Biochemistry 6*, 2893−2900.
67. Grise-Miron, L., Noreau, J., Melancon, P., and Brakier-Gingras, L. (1981) Comparison of the misreading induced by streptomycin and neomycin, *Biochim. Biophys. Acta 656*, 103−110.
68. Noreau, J., Grise-Miron, L., and Brakier-Gingras, L. (1980) Comparison of the action of streptomycin and neomycin on the structure of the bacterial ribosome, *Biochim. Biophys. Acta 608*, 72−81.
69. Johnston, T. C., and Parker, J. (1985) Streptomycin-induced, third-position misreading of the genetic code, *J. Mol. Biol. 181*, 313−315.
70. Recht, M. I., Douthwaite, S., and Puglisi, J. D. (1999) Basis for prokaryotic specificity of action of aminoglycoside antibiotics, *EMBO J. 18*, 3133−3138.
71. Brakier-Gingras, L., and Phoenix, P. (1984) The control of accuracy during protein synthesis in *Escherichia coli* and perturbations of this control by streptomycin, neomycin, or ribosomal mutations, *Can. J. Biochem. Cell. Biol. 62*, 231−244.
72. Dix, D. B., Thomas, L. K., and Thompson, R. C. (1990) In *The Ribosome: Structure, Function, and Evolution*, p 530, American Society of Microbiology, Washington, DC.
73. Fersht, A. R. (1986) in *Molecular Processes: Its Control and Relevance to Living Systems* (Kirkwood, T. B. L., Rosenberger, R. F., and Galas, D. J., Eds.) p 67, Chapman and Hall, London, New York.
74. Parker, J., Johnston, T. C., and Borgia, P. T. (1980) Mistranslation in cells infected with the bacteriophage MS2: Direct evidence of Lys for Asn substitution, *Mol. Gen. Genet. 180*, 275−281.
75. Dong, H., Nilsson, L., and Kurland, C. G. (1996) Co-variation of tRNA abundance and codon usage in *Escherichia coli* at different growth rates, *J. Mol. Biol. 260*, 649−663.
76. Ikemura, T. (1992) in *Transfer RNA in Protein Synthesis*, p 436, CRC Press, Boca Raton.
77. Nakamura, Y., Gojobori, T., and Ikemura, T. (2000) Codon usage tabulated from international DNA sequence databases: Status for the year 2000, *Nucleic Acids Res. 28*, 292.
78. Buckingham, R. H., and Grosjean, H. (1986) in *Molecular Processes: Its Control and Relevance to Living Systems* (Kirkwood, T. B. L., Rosenberger, R. F., and Galas, D. J., Eds.) p 96, Chapman and Hall, London, New York.
79. Ikemura, T. (1981) Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes, *J. Mol. Biol. 146*, 1−21.
80. Woese, C. (1967) The present status of the genetic code, *Prog. Nucleic Acid Res. Mol. Biol. 7*, 107.
81. Ulrich, A. K., Li, L. Y., and Parker, J. (1991) Codon usage, transfer RNA availability and mistranslation in amino acid starved bacteria, *Biochim. Biophys. Acta 1089*, 362−366.
82. Lu, H. S., Fausset, P. R., Sotos, L. S., Clogston, C. L., Rohde, M. F., Stoney, K. S., and Herman, A. C. (1993) Isolation and characterization of three recombinant human granulocyte colony stimulating factor His → Gln isoforms produced in *Escherichia coli, Protein Expr. Purif. 4*, 465−472.
83. O'Farrell, P. H. (1978) The suppression of defective translation by ppGpp and its role in the stringent response, *Cell 14*, 545−557.
84. Lewendon, A., Murray, I. A., Shaw, W. V., Gibbs, M. R., and Leslie, A. G. (1994) Replacement of catalytic histidine-195 of chloramphenicol acetyltransferase: Evidence for a general base role for glutamate, *Biochemistry 33*, 1944−1950.
85. McNulty, D. E., Claffee, B. A., Huddleston, M. J., Porter, M. L., Cavnar, K. M., and Kane, J. F. (2003) Mistranslational errors associated with the rare arginine codon CGG in *Escherichia coli*, *Protein Expr. Purif. 27*, 365−374.
86. Yang, J., Kanter, G., Voloshin, A., Michel-Reydellet, N., Velkeen, H., Levy, R., and Swartz, J. R. (2005) Rapid expression of vaccine proteins for B-cell lymphoma in a cell-free system, *Biotechnol. Bioeng. 89*, 503−511.
87. Greer, L. F., 3rd, and Szalay, A. A. (2002) Imaging of light emission from the expression of luciferases in living cells and organisms: A review, *Luminescence 17*, 43−74.
88. Doyle, T. C., Burns, S. M., and Contag, C. H. (2004) In vivo bioluminescence imaging for integrated studies of infection, *Cell. Microbiol. 6*, 303−317.
89. Maurel, M. C., Biard, B., Moulinier, C., Braz, D., Nugier, J., Chaumas, I., Reboud-Ravaux, M., and Decout, J. L. (2002) RNA-acting antibiotics: In-vitro selection of RNA aptamers for the design of new bioactive molecules less susceptible to bacterial resistance, *J. Pharm. Pharmacol. 54*, 1019−1031.

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