Insights Into the Decoding Mechanism from Studies of Mutant Ribosomes

Dissertation

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By

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Abstract

In all cells, the 2.5 MDa, two-subunit complex known as the ribosome is responsible for translating each messenger RNA (mRNA) into a functional protein. To do so, the ribosome must correctly select the cognate aminoacyl-tRNA (aa-tRNA) specified by each three-nucleotide codon in the mRNA's coding sequence, and incorporate the attached amino acid into the peptide chain. The accuracy of this process is critical for cellular function; many potent antimicrobial agents act by promoting errors in aa-tRNA selection, and even moderate defects in translation fidelity contribute to several disease states in higher eukaryotes.

To investigate ribosome's role in the decoding mechanism, we performed a genetic screen for mutations in the 16S ribosomal RNA (rRNA) that increased errors during translation. This method identified 34 unique residue substitutions that cluster to distinct regions of the ribosome. The locations of these mutations primarily indicate that three regions are critical to high-fidelity decoding: 1) the ribosomal A site, 2) the h12/S4/S5 region on the 30S solvent face, and 3) intersubunit bridge B8, made by contacts between h8/h14 and L14/L19. We then characterized a representative subset of these mutations to determine the functional contributions of each of these critical regions. Our results show that mutations that disrupt bridge B8 decrease the fidelity of both
phases of decoding, initial selection and proofreading. These mutations that affect B8 increase misincorporation of near-cognate aa-tRNAs in a general manner, suggesting that disruption of the bridge is an important step in decoding for all codons. Mutations in the A site also decreased fidelity during both decoding phases. However, the effects of these mutations, particularly those in h34, were only apparent for specific aa-tRNA-codon combinations. This indicates that these A-site mutations operate through a distinct mechanism to those affecting B8, perhaps by creating spurious favorable contacts that stabilize non-canonical base paring in the decoding center. These results, and others presented below, reveal the important functional roles distinct regions of the ribosome play in maintaining the fidelity of the genetic code.
Dedication

This document is dedicated to my son Colin, who I hope never reads it or discovers what I was working on when we could have been playing firefighter.
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Chapter 1: Introduction

1.1 Protein assembly at the molecular level

In all cells, genetic information is encoded in ribonucleic acids (DNA and RNA) and must be translated into proteins that carry out most of the physical and chemical functions of life. The ribosome is the molecular factory that directs protein synthesis. To do so, the ribosome must carry out many steps analogous to assembly of goods in macro factories. It must engage the messenger RNA (mRNA), which encodes the protein, at the correct location to begin assembly. Then, it must gather the transfer RNAs (tRNA) that read each the nucleo-base codon of the mRNA, coordinate amino acid polymerization, facilitate movement from one codon to the next and terminate the process at the correct stop site. While these steps are similar to a factory assembly line, the ribosome does not have the advantage of robots or engineers to ensure quality control at each step. Instead, it must rely on chemistry and thermodynamic fluctuations to carry out these steps at the molecular level, and accordingly, through billions of years ribosome has evolved a complex structure to ensure the efficiency of translation.
1.1.1 Overview of the ribosome and the translation cycle

The ribosome is composed of two protein-RNA subunits. In bacteria, these total 70 Svedbergs (S) and ~2.5 megadaltons (MDa). The large 50S subunit, composed of the 5S and 23S ribosomal RNA (rRNA) and about 30 proteins, contains the amino acid polymerization active site. The 30S small subunit is composed of the 16S rRNA and about 20 proteins and contains the mRNA binding site. There are three binding sites for tRNA located on both subunits, the aminoacyl-tRNA or A site, the peptidyl-tRNA or P site, and the exit or E site. The two subunits are joined by 13 intersubunit bridges (B1a, B1b, B2a, B2b, B2c, B3, B4, B5, B6, B7a, B7b, B8) [1].

Translation occurs in four phases: 1) initiation, in which the ribosome complex assembles at the beginning of the gene, 2) elongation, where each codon is paired with an aa-tRNA and the attached amino acids are polymerized into a peptide chain, 3) termination, or release factor (RF) catalyzed polypeptide hydrolysis and release at a stop codon, and 4) recycling of the ribosomal subunits, or dissociation of the post-termination complex into free subunits ready to re-initiate the process [2].

1.1.2 Overview of the elongation cycle

The elongation phase can be subdivided into three steps (Figure 1). During decoding a ternary complex (TC) of elongation factor Tu (EF-Tu), aminoacyl-tRNA (aa-tRNA) and GTP (EF-Tu•aa-tRNA•GTP) binds to the ribosome. The aa-tRNA anticodon base pairs with the codon being translated in the 30S A site, which leads to hydrolysis of GTP by EF-Tu. The acceptor stem of the aa-tRNA is then released from EF-Tu and
moves into the 50S A site, where the ribosome catalyzes the peptidyl transfer of the growing peptide from the P-tRNA to the A site aa-tRNA. Then, during translocation elongation factor G (EF-G) catalyzes the movement of the P-tRNA to the E site, and the A-tRNA to the P site, and this advances the mRNA three nucleotides leaving an empty A site ready for the translation of the next codon. Through this cycle, each three-nucleotide codon is translated into its corresponding amino acid to generate a specific product from the gene.
1.1.3 The importance of accuracy in translation

Accurate selection of the correct, or cognate, aa-tRNA for each codon translated is critical for synthesis of the peptide chain according to the genetic code. The importance of decoding fidelity for all life is demonstrated by the myriad of defects that arise when translation errors are increased. Mistranslation can result in the accumulation of unfolded proteins and leave cells vulnerable to oxidative stress [3, 4]. Several potent antibiotics act
by binding the ribosome and increasing decoding errors [5]. An editing defect in alanyl-tRNA synthetase that increases errors in protein synthesis results in a neurodegenerative disease in mice [6]. Considering the consequences, it follows that the ribosome has evolved mechanisms to ensure the selection of cognate aa-tRNA during decoding.

1.2 The accuracy of protein synthesis in vivo

Attempts to quantify the frequency of translation errors in vivo have been complicated by the necessity for a reporter system allowing detection of such errors. Several studies have measured errors by tracking the incorporation of a particular amino acid into a protein that does not code for that amino acid. Using $^{35}$S labeled cysteine (cys), misincorporation of cys into E. coli flagellin was measured at approximately $10^{-4}$ [7]. Two additional studies adapting this technique to a variety of other E. coli proteins also measured cys misincorporation between $10^{-3}$ and $10^{-4}$ [8] [9]. Other studies have attempted to take advantage of physical changes to proteins induced by amino acid misincorporation. For the MS2 coat protein, a change in migration on isoelectric focusing gels induced by lysine (lys) incorporation at asparagine (asn), was used to measure lysine misincorporation at AAU (approximately $2 \times 10^{-3}$) and AAC ($\sim 2 \times 10^{-4}$) [10].

A more common approach for detecting errors has been to track mistranslation events that restore catalytic activity to an enzyme. By changing the codon of a catalytically critical residue to specify for another amino acid (missense mutation), enzymatic activity can be restored by misincorporation of the catalytic amino acid. However, the approach is limited by the degeneracy of the genetic code. Since a single
base change in the codon mostly specifies for an amino acid with similar chemical or physical properties, low levels of activity may persist and be difficult to distinguish from mistranslation. Nonetheless, this approach has resulted in several studies where misincorporation has been sensitively measured in ranges that agree with the experimental approaches mentioned above [11, 12].

Farabaugh and colleagues have carried out some of the most thorough applications of this approach. In *E. coli*, a dual luciferase reporter system, where activity of firefly luciferase requires misincorporation of lys at residue 529, was used to measure error frequencies at 14 near-cognate codons, differing from the cognate AAA or AAG codons by a single base change [13]. In this system, the average error frequency was $3.7 \times 10^{-4}$, however four codons (UAG, AGG, AGA, and AAU) had $>10$-fold higher suppression rates. When the same system was used to measure errors in *S. cerevisiae*, error rates were found to be ~3-fold lower, ranging from $4 \times 10^{-5}$ to $7 \times 10^{-4}$ and errors were more frequent at codons UAG and AGG [14]. These studies demonstrate that, at least for lysine, decoding errors are most detectable *in vivo* at specific codons, but overall levels of misincorporation are in the range determined by a broad range of experiments, $10^{-5}$-$10^{-3}$, [15].

1.3 Kinetic discrimination during decoding

In solution, the strength of codon-anticodon interaction is not sufficient to account for the accuracy of protein synthesis seen *in vivo*, as introduction of a single G-U mismatch would destabilize the helix less than ten-fold [16]. Therefore, the ribosome
must play an active role in increasing the stringency of decoding. Hopfield and Ninio independently proposed that the ribosome could increase the accuracy of aa-tRNA selection through a “kinetic proofreading” mechanism, where the expenditure of energy allows for multiple discrimination steps exploiting the substrate free energy difference [17, 18]. In the ribosome’s case GTP hydrolysis by EF-Tu would allow two separate selection steps, and near-cognate aa-tRNAs could be discriminated both before and after this functionally irreversible step. Indeed, near-cognate Leu-tRNA^{Leu} leads to a 10-fold increase in the amount of GTP consumed per peptide bond in poly-(U) synthesis assays and the stoichiometry is reduced by the error-promoting antibiotic streptomycin [19]. Additionally, the slowly hydrolyzable GTP-γS increases fidelity, as the theory predicts that allowing the ribosome greater time to reach equilibrium during decoding would increase accuracy [20].

The individual kinetic steps of the decoding reaction have been defined by correlating conformational changes (monitored by fluorescent labels) in the D-arm of the aa-tRNA and the active site of EF-Tu with the chemical steps of GTP hydrolysis and peptide bond formation (Figure 2) [21-24]. These experiments indicate that the ternary complex forms an initial interaction with the ribosome independent of the A site codon \((k_1, k_{-1})\) [25]. Following initial binding, codon recognition occurs in the A site \((k_2, k_{-2})\) [23]. Codon recognition leads to a conformational change in the active site of EF-Tu (GTPase activation, \(k_3\)) and subsequent hydrolysis of GTP [24]. Then EF-Tu releases the hydrolyzed inorganic phosphate and the aa-tRNA \((k_4)\) [26]. The aa-tRNA then either undergoes accommodation \((k_5)\), where the acceptor stem moves from its binding site on
EF-Tu into the peptidyl transferase center, or dissociates from the ribosome (rejection, $k_7$) [21, 22, 27].

Figure 2. A kinetic model of decoding. Based on work of Rodnina and colleagues. Cognate codon-anticodon pairing accelerates rates of steps shown as green arrows and decelerates rates shown as red arrows.

The accuracy of decoding is determined by the relative rates that a cognate aa-tRNA pass through each of these steps as compared to a near-cognate aa-tRNA. In line with the kinetic proofreading model, the functionally irreversible step of GTP hydrolysis delineates the initial selection phase (codon recognition, and GTPase activation) from the proofreading phase (accommodation, peptide bond formation), and the overall accuracy is the product of the accuracy of both phases. During initial selection, the rate of codon recognition ($k_2$) is uniform for both cognate and near-cognate aa-tRNA, although cognate complexes are more stable and dissociate slower from the recognition complex ($k_{-2}$) and during proofreading ($k_7$) [21, 27, 28]. However, the primary reason for the preferential
selection of cognate aa-tRNA is that cognate codon-anticodon pairing stimulates the forward rates of GTPase activation ($k_3$) and accommodation ($k_5$) [21, 27]. In high-fidelity conditions, these steps are up to 650-fold faster for cognate aa-tRNA than for near-cognate [27]. This mechanism suggests that the ribosome utilizes the free energy gained from codon-anticodon pairing to drive the productive incorporation of the aa-tRNA, allowing for both rapid and accurate decoding.

Now, single molecule techniques are being used to dissect the decoding mechanism ribosome by ribosome. Blanchard, Puglisi, Chu and coworkers have used aa-tRNAs labeled with FRET-probes to observe reversible aa-tRNA movement during the selection process [29] [28, 30]. These experiments have helped clarify models developed with bulk measurements. For instance, whereas bulk experiments have relied on non-hydrolyzable GTP analogues and other inhibitors to observe the transient codon recognition step preceding GTP hydrolysis, single molecule experiments readily show that a reversible FRET-state precedes transition into the GTPase active state. While the rate of transition into this state match $k_2$ in bulk measurements, dissociation from this state appears to be extremely rapid for both cognate (200 s$^{-1}$ versus 0.82 s$^{-1}$ for $k_2$ in bulk) and near-cognate (600 s$^{-1}$ versus 180 s$^{-1}$) ternary complexes, which suggests inhibitors used in previous experiments may have led to underestimates of the rates of dissociation from the codon recognition complex ($k_2$) [28]. More importantly, these methods also show that selectivity of the decoding process is largely determined by the higher probability of productive (forward) transitions for cognate complexes relative to
near-cognate, consistent with the higher rate constants for cognate reaction in ensemble studies [28, 30].

It is important to note that a large number of variables, including pH, temperature, and Mg2+ concentration, can have large effects on accuracy in vitro. A number of studies have attempted to interpret or reconcile these effects [31]. Therefore, it is likely that specific aspects of these mechanisms do not accurately represent the situation in the cell.

1.4 Structural basis for decoding

1.4.1 Structural recognition of the codon-anticodon interaction

 Upon binding of a cognate aa-tRNA to the ribosomal A site, three universally conserved nucleotides, G530, A1492, and A1493, rearrange to dock in the minor groove of the codon-anticodon helix (Figure 3A-B) [32]. At the first codon position, A1493 flips out from h44 and forms a Type I A-minor interaction (Figure 3C) [33]. The 2'OH and N1 of A1493 form hydrogen bonds with the 2'OH groups of the codon and anticodon, respectively. At the second position, A1492 flips from h44 and G530 switches from a syn to anti conformation. The 2'OH and N3 of A1492 interact with the 2'OH of the codon nucleotide, while the 2'OH and N3 of G530 form a similar interaction with the 2'OH of the anticodon nucleotide, and the two 16S bases form a hydrogen bond through their N1 positions (Figure 3D). These interactions at the first and second positions are specific for the 10.5 Å distance between the 2'OH groups of Watson-Crick base pairs, restricting the
geometry of codon-anticodon interactions. The contacts formed by the 16S residues contribute significantly to the free energy ($\Delta G$) of codon-anticodon pairing, explaining why cognate codon-anticodon binding in the A site is significantly more stable than in solution (e.g. In the ribosome, introduction of a first position G-U mismatch results in a $\Delta \Delta G > 12$ kJ/mol compared to the cognate A-U, whereas that substitution in solution would result in a $\Delta \Delta G < 3$ kJ/mol.) [34, 35]. Secondary interactions formed with G530 and A1492 by 16S rRNA and S12 further stabilize this arrangement (Figure 3D).

Figure 3. Structural changes in the ribosome during codon recognition. Binding of a cognate aa-tRNA to the apo-ribosome (A) causes G530, A1492, and A1493 to rearrange and dock into the codon-anticodon minor groove (B). These interactions at the 1st (C) and 2nd (D) base pair are specific for Watson-Crick codon-anticodon geometry. Interactions at the 3rd position (E) are less stringently monitored. The aa-tRNA must undergo a 30˚ distortion in its anticodon-stem and D-arm in order to reach the codon-recognition state. Figure adapted from [36].
The geometry of the third position interaction is less stringently monitored. The 2'OH of the codon is coordinated by the N2 of G530, as well as a Mg$^{2+}$ coordinated by G518 and P48 of S12 (Figure 3E). The anticodon nucleotide packs up against C1054, which protrudes from h34, although the precise nature of this interaction varies in several crystal structures (discussed further in Chapter 5). Unlike the interactions at the first and second positions, these third positions interactions do not depend on the inter-base distance, allowing for the variety of wobble interactions observed at this position [37].

Residue 34 of the tRNA, which is the anticodon contribution to the third position base pair, is frequently modified. These modifications are critical for the expanded base pairing allowed at the wobble position. Deamination of A34 to inosine (I) in tRNA$^{\text{Arg}}_{\text{ICG}}$ allows this tRNA to read codons CGU, CGC, and CGA. This last codon is unique because the modification promotes the formation of a purine-purine base pair between the Watson-Crick faces of both residues [38]. The stretched geometry of this pairing is accommodated by rearrangements in the anticodon backbone. Similarly, mnm$^5$U34 modification in tRNA$^{\text{Lys}}$ stabilizes G:U pairs by promoting base stacking with U35, compensating for the weaker hydrogen bonding between the codon and anticodon [39]. Modifications mnm$^5$s$^2$U34 in human tRNA$^{\text{Lys}}$, cmo$^5$U34 in tRNA$^{\text{Val}}$, and f$^5$C34 in tRNA$^{\text{Met}}$ allow Watson-Crick G:U and A:C pairs at codons AAG, GUG, and AUA respectively through keto-enol tautomerization of the anticodon base [40-42]. Additionally, the cmo$^5$U34 modification in tRNA$^{\text{Val}}$ stabilizes an unusual U:U pyrimidine-pyrimidine pair by forming an additional hydrogen bond between the carboxyl group of the modification and the N4 of the codon base [42]. Agmatidine
modification of C34 in tRNA^{Ile} also stabilizes A:C pairing through an additional hydrogen-bond formed by the modification, although in this case the bond is formed with the phosphate backbone of the upstream (P-site) codon [43].

The structure of the A site and the tRNA stabilize cognate codon-anticodon interactions, but it is less clear how the A site responds to non-cognate base pairing. Structures of the 30S subunit with an A site codon bound to a near-cognate anticodon stem loop suggest that these interactions do not promote the minor-groove docking of G530, A1492, and A1493, as well as other conformational changes throughout the subunit [35]. However, 70S ribosomes with near-cognate tRNA bound in the A site show that the three nucleotides are positioned in the minor-groove and that the mispaired nucleotides conform to Watson-Crick geometry, suggesting that the bases must undergo tautomerization to allow the arrangement [44]. Psudouridine modification at UGA, UAG, and UAA stop codons in eukaryotes allows efficient read-through by non-cognate elongator tRNA [45]. A recent crystal structure of ΨAG bound to tRNASerIGA shows that A:G and G:I pairs at the second and third position adopt highly unusual Watson-Crick:Hoogsteen geometry [46]. Surprisingly, in this arrangement the distance between the 2'OH groups of the second position nucleotides is 10.9 Å, not much different than the 10.5 Å seen for canonical base pairing at this position, allowing G530 and A1492 to adopt similar minor groove interactions as for cognate base pairs. Unfortunately, crystallography is unable to speak to the thermodynamic stability of any of these unexpected arrangements, so a critical unanswered question is how these phenomena
effect decoding and contribute to mistranslation and programmed stop codon read-through.

1.4.2 Structural basis of EF-Tu GTPase activation

The accelerated rate of EF-Tu-dependent GTP hydrolysis induced by cognate ternary complex indicates that codon-anticodon recognition in the A site must somehow be communicated to the EF-Tu active site, over 70 Å away. Insights into the structural changes that lead to GTPase activation primarily come from studies of ternary complexes stalled on the ribosome with the antibiotic kirromycin, which traps the complex by preventing release of the aa-tRNA after GTP hydrolysis [23]. Ribosome binding induces several conformational changes in the ternary complex. First, codon-anticodon interaction while the aa-tRNA acceptor stem is bound to EF-Tu requires adoption of the A/T state, where the tRNA is distorted ~30° through the anticodon-stem-loop and D-stem [47-49]. The A-site interactions with the anticodon region are nearly identical between this A/T-tRNA and the A/A-tRNA resulting from accommodation of the aa-tRNA into the peptidyl transferase center. The distorted tRNA is primarily stabilized by interactions in the A site and its interaction with EF-Tu. The only additional contacts between the ribosome and the aa-tRNA are between the acceptor stem A75 and A55 in h5, A67 and S12 residue Q74, part of the conserved QEH motif, and the D-loop and 23S residues A1067 and U1068 (Figure 4) [50]. The relatively sparse contacts outside of the A site are
likely important for rapid accommodation of aa-tRNA into the A/A site after release from EF-Tu.

Figure 4. Interactions between the ternary complex and the ribosome. Residues involved in contacts between the ribosome and aa-tRNA or the ribosome and EF-Tu are shown as spheres. Figure adapted from [50].

The structure of ribosome bound EF-Tu•kirromycin is similar to that in solution, save two significant rearrangements that may play a role in the ribosome's stimulation of
the GTPase activity [50, 51]. First, two loops in domain 2, composed of residues 219-226 and 256-273, extend into h5 of the 30S shoulder. These interactions facilitate the distortion of the 3' end of the aa-tRNA and the A75-h5 contact. These changes may facilitate the opening of the “hydrophobic gate” of EF-Tu, formed between the switch I and P-loop motifs, that prevents the access of hydrolytic water into the GTP active site. The larger conformational change that must occur is a 7 Å shift of domain I to prevent a steric clash with H95, otherwise known as the sarcin-ricin-loop (SRL) as it is the target of the ribosome inactivating toxins α-sarcin and ricin. The SRL itself does not appear to move to facilitate factor binding. However, there is significant rearrangement in the nearby inter-subunit bridge, B8, which causes a loss of several of the hydrogen bonding contacts between h14 and L14/L19 that contribute to the bridge [52].

The SRL is critical for GTP hydrolysis. Structures of the ternary complex stalled on the ribosome with a non-hydrolysable GTP analog show that the conserved H84 of EF-Tu is positioned to catalyze the reaction by activating a hydrolytic water molecule with its pros-nitrogen (Figure 5) [53]. The 5'-phosphate of A2662 in the SRL coordinates the tele-nitrogen of H84. Therefore, rather than acting like a GTPase activating protein (GAP), which actively participate in catalysis for non-ribosomal GTPases, the ribosome acts analogous to regulator of G-protein signaling (RGS) that promote GTP hydrolysis by stabilizing the GTPase active state [54]. Interestingly, while the SRL plays a nearly identical role in GTP hydrolysis for EF-G (as well as other ribosomal GTPases) [55-57], the effects of SRL modification are distinct for the two EFs. Ricin, which depurinates
A2662, has little effect on EF-Tu function, but inactivates EF-G, but α-sarcin, which cleaves the SRL between A2661 and A2662, primarily inhibits EF-Tu [58].

Figure 5. The chemical mechanism of GTP hydrolysis by EF-Tu on the ribosome. (A) Positioning of the hydrolytic water molecule by EF-Tu residues H84, G83, and T61, and coordination of H84 by 23S residue A2662. (B) Chemical structure diagram of the mechanism of catalysis as proposed from the structure in (A). Figure adapted from [53].
1.4.3 Structural interactions during accommodation

Following GTP hydrolysis, aa-tRNA is released from EF-Tu and, during the proofreading phase, either dissociates from the ribosome or is accommodated into the A/A site to participate in peptidyl transfer. To do this, the aa-tRNA must undergo a nearly 45° rotation around the anticodon stem loop and transverse the accommodation corridor into the 50S active site. Due to the large conformational changes involved and the lack of stable intermediates, high-resolution structures of this process have not been solved. However, an all atom molecular-dynamics (MD) simulation of accommodation shows that the aa-tRNA interacts with >20 residues on its way into the peptidyl transfer center (PTC) [59]. During simulated accommodation, the aa-tRNA breaks its contacts with the SRL and A1067, the acceptor stem tracks along H89 onto H92 just outside the PTC, and then the 3' end finally moves into the PTC. Refined simulations show that these interactions create energy barriers that could divide the accommodation process into defined steps, and that the aa-tRNA can reversibly move between these barriers, consistent with single molecule data [60].

Because relatively little experimental data exists regarding the structural basis of accommodation, several important details remain unclear. For example, if, as simulations suggest, accommodation is a multi-step process, which step is rate limiting? Moreover, is the same step rate limiting for both cognate and near-cognate aa-tRNA? Answers to these questions would help explain the basis for discrimination at this stage of the decoding process. Recent crystal structures of 70S complexes with bound cognate and non-cognate
A-site tRNAs suggest that a network of interactions formed between the ribosome and cognate aa-tRNA may not be present for non-cognate, potentially contributing to the discrimination [61]. As smFRET experiments gain increasing sensitivity and resolution, it is possible that these tools may help identify the discrimination steps.

1.5 Mutations affecting accuracy

X-ray crystallography, cryo-electron microscopy, and other techniques can elucidate intermediate states and interactions between the ribosome and other players in the decoding reaction. But the functional roles of these interactions can only be discerned circumstantially. One way to more directly probe the contributions of specific structural elements is to study the effects of altering those structures, either by analyzing elements that appear important in structural models by direct mutagenesis or by isolating and characterizing mutations that affect translational accuracy in vivo.

1.5.1 Tools for isolating and analyzing mutations affecting decoding

Many early insights into how the structure of the ribosome contributes to accurate decoding came from studies of the aminoglycoside antibiotic streptomycin, which induces cell death by binding the ribosome and dramatically increasing decoding errors [62, 63]. Bacterial resistance to the antibiotic readily arises from spontaneous mutations in $rpsL$ encoding the small subunit protein S12 [64, 65]. The effects of $rpsL$ streptomycin resistance (Str$^R$) mutations on decoding are typically opposite to those of the drug; these mutations reduce errors during translation and are termed hyper-accurate or restrictive.
Too much accuracy also appears to be detrimental to cell growth, and many of these mutations have moderate to severe growth defects [66]. In fact, several mutations are so detrimental that they no longer grow in the absence of the antibiotic [67]. The streptomycin dependent (StrD) phenotype can be relieved by compensatory mutations that spontaneously arise in rpsD and rpsE, coding for the 30S proteins S4 and S5. Again, these streptomycin independence (StrI) mutations by themselves often have the opposite decoding effects as the mutations for which they compensate, increasing translation errors, and thus are termed ribosomal ambiguity or ram [65, 67]. A straightforward model of these mutations balancing the tradeoffs between speed and accuracy was complicated when it was discovered that several StrI mutations in S4 were themselves hyperaccurate and resistant to streptomycin. However, these mutations have still provided important clues into the mechanism of aa-tRNA selection.

Screens for mutants resistant to other antibiotics have also uncovered ram and restrictive mutations in distinct regions of the ribosome. For example, spontaneous resistance to the aminoglycosides neamine and gentramycin led to the isolation of restrictive mutations in S17 and L6, respectively [68-70]. Mutations in EF-Tu leading to kirromycin resistance increase decoding errors and some relieve streptomycin dependence [71, 72]. Kirromycin itself has no effect on accuracy, so, like the hyperaccurate/StrI S4 mutations, the ram and KirR phenotypes appear to be unrelated. Similarly, a mutation in S5 isolated for cold-sensitivity also has pleotropic ram and assembly defective effects [73].

Isolation of mutations in rRNA is made considerably more difficult by the fact
that multiple copies of the *rrn* genes exist in most organisms. By expressing mutant copies of the genes in high copy plasmids, upwards of 50% of the cells ribosomes may contain the mutation, and several studies have utilized this method to tease out the mutations effects from the wild type copies they are mixed with [74]. More recently, an *E.coli* strain was constructed with all seven chromosomally encoded *rrn*’s deleted, allowing plasmid transcribed rRNAs to support all function of the cell [75, 76]. A similar strain has also been developed in *S. cerivisiae* [77]. Of course, these methods all require that the ribosomal mutants support the growth of the cell. To study more deleterious alterations, an affinity tagging system has been developed, where the MS2 stem loop has been inserted in either H98 of the 23S or h61 of the 16S [78]. The MS2 sequence has high affinity for the MS2 coat protein, allowing pull-down of the tagged ribosome for *in vitro* analysis.

Secondary effects caused by mistranslated or mis-regulated proteins can complicate *in vivo* studies of mutant ribosomes, even those that support cellular function. A useful method for avoiding these complications is altering the 16S anti-Shine-Dalgarno region of the 16S rRNA, which directs the ribosome to the start codon [79]. This creates a subpopulation of specialized ribosomes that only translate genes bearing a complementary mutation in their Shine-Dalgarno sequence (Figure 6). This system has been used to study ribosome mutations with a variety of effects on translation, as well as create orthogonal expression systems that may be useful for synthetic biology [80-82].
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Figure 6. The specialized ribosome system. Ribosomes assembled with a plasmid-encoded 16S rRNA (Red) containing a "specialized" mutation in their anti-Shine-Dalgarno sequence (ASD*) specifically translate chromosomally encoded reporter mRNA containing a complimentary Shine-Dalgarno sequence (SD*) and do not translate endogenous mRNA containing the canonical Shine-Dalgarno sequence. Ribosomes assembled with chromosomally-encoded, wild type 16S rRNA are free to carry out translation of endogenous mRNA and do not interfere with translation of the reporter mRNA, which has a non-complimentary Shine-Dalgarno sequence. Figure adapted from N. Abdi.

1.5.2 Where accuracy mutations are found

Mutations affecting the accuracy of decoding have been identified at hundreds of residues across the ribosome, tRNA, and EF-Tu. However, the mutations appear to cluster at several distinct regions, implicating these sites in the decoding mechanism. Those mutations that have been shown to affect accuracy, either in vitro or in vivo are
shown in Table 1 and Table 2.

Most accuracy mutations in ribosomal proteins have been identified though selection experiments with streptomycin. Restrictive, StrR and StrD mutations have been isolated in S12, although one StrR mutation, K42R increases errors and relieves the StrD phenotype. Interestingly, StrR mutations generally cluster around K42, while StrD mutations cluster at or near P90. The reason for this distinction is unclear, and the two residues are spatially close to one another. Notably, D88, nearby P90, is an essential residue and the site of a β-methylthiolation modification that may be disrupted by some StrD mutations [83]. Streptomycin independent, ram mutations are found in S4, S5 and L19. The mutations in S4 and S5 generally map along the interface of the two proteins. Other methods have also identified mutations in S17, L6 and L7/L12, as mentioned above.
Unlike mutations in r-proteins, mutations in rRNA have been identified primarily through directed mutagenesis or selection for error phenotype rather than by streptomycin studies. Nevertheless, these mutations also cluster, and often in the vicinity of the r-protein mutations. In the 16S rRNA, mutations in h18 and h27, both found near S12, and, like the StrR and StrD mutations, are often restrictive. Near S4 and S5 are mutations in h12 and h21, and these display \textit{ram} phenotypes, similar to their protein mutation counterparts. Helices 8 and 14 are also the sites of many \textit{ram} mutations and form

Table 1. \textit{R}-protein mutations that alter decoding accuracy.

<table>
<thead>
<tr>
<th>r-protein</th>
<th>Mutation</th>
<th>AB phenotype</th>
<th>Accuracy(a)</th>
<th>Growth(b)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4</td>
<td>Q53P</td>
<td>Str(i),Str(n)</td>
<td>\textit{ram}</td>
<td>-</td>
<td>Bjorkman, et al. 1999</td>
</tr>
<tr>
<td>S4</td>
<td>Q53L</td>
<td>Str(i)</td>
<td>-</td>
<td>Bjorkman, et al. 1999</td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td>176(USA)</td>
<td>Str(i)</td>
<td>\textit{ram}</td>
<td>-</td>
<td>Andersson 1982</td>
</tr>
<tr>
<td>S4</td>
<td>I199N</td>
<td>Str(i)</td>
<td>\textit{ram}</td>
<td>-</td>
<td>Bjorkman, et al. 1999</td>
</tr>
<tr>
<td>S4</td>
<td>(\Delta)V200</td>
<td>Str(i),Str(h)</td>
<td>\textit{res}</td>
<td>-</td>
<td>Bjorkman, et al. 1999</td>
</tr>
<tr>
<td>S4</td>
<td>201(UAG)</td>
<td>Str(i)</td>
<td>\textit{res}</td>
<td>-</td>
<td>Bjorkman, et al. 1999</td>
</tr>
<tr>
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<td>K205T</td>
<td>Str(i)</td>
<td>\textit{ram}</td>
<td>-</td>
<td>Bjorkman, et al. 1999</td>
</tr>
<tr>
<td>S4</td>
<td>K205N</td>
<td>Str(i)</td>
<td>\textit{ram}</td>
<td>-</td>
<td>Bjorkman, et al. 1999</td>
</tr>
<tr>
<td>S5</td>
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<td>none</td>
<td>\textit{ram}</td>
<td>-</td>
<td>Kirth, et al. 2006</td>
</tr>
<tr>
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<td>Str(i)</td>
<td>\textit{ram}</td>
<td>-</td>
<td>Inaoka, et al. 2001</td>
</tr>
<tr>
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<td>K42A</td>
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<td>\textit{res}</td>
<td>-</td>
<td>Holberger and Hayes, 2009</td>
</tr>
<tr>
<td>S12</td>
<td>K42T</td>
<td>Str(h)</td>
<td>\textit{res}</td>
<td>+</td>
<td>Tubulekas and Hughes, 1993; Holberger and Hayes, 2009</td>
</tr>
<tr>
<td>S12</td>
<td>K42N</td>
<td>Str(h)</td>
<td>\textit{res}</td>
<td>-</td>
<td>Tubulekas and Hughes, 1993; Holberger and Hayes, 2009</td>
</tr>
<tr>
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<td>K42R</td>
<td>Str(i),Str(h)</td>
<td>\textit{ram}</td>
<td>-</td>
<td>Tubulekas and Hughes, 1993; Holberger and Hayes, 2009</td>
</tr>
<tr>
<td>S12</td>
<td>R53L</td>
<td>Str(h)</td>
<td>\textit{res}</td>
<td>-</td>
<td>Tubulekas and Hughes, 1993</td>
</tr>
<tr>
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<td>\textit{res}</td>
<td>nd</td>
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<td>Str(h)</td>
<td>\textit{res}</td>
<td>-</td>
<td>Holberger and Hayes, 2009</td>
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<td>S12</td>
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<td>Str(h)</td>
<td>\textit{res}</td>
<td>-</td>
<td>Holberger and Hayes, 2009</td>
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<td>S12</td>
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<td>P90F</td>
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<td>-</td>
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<td>P90Y</td>
<td>Str(h)</td>
<td>\textit{res}</td>
<td>-</td>
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</tr>
<tr>
<td>S17</td>
<td>H30P</td>
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<td>\textit{res}</td>
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<tr>
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<td>Gen(i)</td>
<td>\textit{res}</td>
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<tr>
<td>L7L12</td>
<td>(\Delta)38-42</td>
<td>none</td>
<td>\textit{ram}</td>
<td>+</td>
<td>Kisesborn, et al. 1986</td>
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<tr>
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<td>\textit{ram}</td>
<td>-</td>
<td>Maisnier-Patin, et al. 2007</td>
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<td>L19</td>
<td>Q40L</td>
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<td>L19</td>
<td>Q40H</td>
<td>Str(i)</td>
<td>\textit{ram}</td>
<td>-</td>
<td>Maisnier-Patin, et al. 2007</td>
</tr>
</tbody>
</table>

\(a\): \textit{ram} indicates nonsense or missense suppression > control; \textit{res} indicates nonsense or missense suppression; both indicates \textit{ram} or \textit{res} phenotype depending on reporter used; \textbf{bold text} indicates effects \( \geq 5\)-fold change from control

\(b\): + indicates no significant growth effects; - indicates \( \leq 50\% \) decrease in doubling time; -- indicates > \(50\% \) decrease in doubling time; nd indicates not determined
intersubunit bridge B8 with L19. Mutations have also been found in 23S rRNA helices H80, H89, and H92, as well as the SRL (H95).
<table>
<thead>
<tr>
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<th>Mutation</th>
<th>Location</th>
<th>Accuracy</th>
<th>Growth</th>
<th>Source</th>
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<td>5' end</td>
<td>res</td>
<td>--</td>
<td>Pinard, et al. 1993</td>
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<tr>
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<td>h21</td>
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<td>h22</td>
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<td>h23</td>
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<td>+</td>
<td>McCloy, et al. 2010</td>
</tr>
<tr>
<td>16S</td>
<td>C1054A</td>
<td>h34</td>
<td>ram</td>
<td>-</td>
<td>McCloy, et al. 2010</td>
</tr>
<tr>
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<td>h34</td>
<td>ram</td>
<td>--</td>
<td>McCloy, et al. 2010</td>
</tr>
<tr>
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<td>ram</td>
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<tr>
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<td>h34</td>
<td>ram</td>
<td>nd</td>
<td>McCloy, et al. 2010</td>
</tr>
<tr>
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<td>h44</td>
<td>ram</td>
<td>nd</td>
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<td>ram</td>
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<td>h80</td>
<td>ram</td>
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<tr>
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<td>ram</td>
<td>--</td>
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<tr>
<td>23S</td>
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<td>h80</td>
<td>ram</td>
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<td>O'Connor and Dahlberg 1995</td>
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<tr>
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<td>H89</td>
<td>ram</td>
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<td>O'Connor and Dahlberg 1995</td>
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<tr>
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<td>H89</td>
<td>ram</td>
<td>-</td>
<td>O'Connor and Dahlberg 1995</td>
</tr>
<tr>
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<td>H89</td>
<td>ram</td>
<td>-</td>
<td>O'Connor and Dahlberg 1993</td>
</tr>
<tr>
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<td>H95</td>
<td>ram</td>
<td>+</td>
<td>O'Connor and Dahlberg 1996</td>
</tr>
<tr>
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<td>H95</td>
<td>res</td>
<td>--</td>
<td>Shi, et al. 2011</td>
</tr>
<tr>
<td>23S</td>
<td>G2861C</td>
<td>H95</td>
<td>res</td>
<td>nd</td>
<td>Melançon, et al. 1992</td>
</tr>
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<td>H95</td>
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<tr>
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<td>G2865C</td>
<td>H95</td>
<td>res</td>
<td>--</td>
<td>Shi, et al. 2011</td>
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<tr>
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<td>C2666U</td>
<td>H95</td>
<td>ram</td>
<td>+</td>
<td>O'Connor and Dahlberg 1996</td>
</tr>
</tbody>
</table>

Table 2. Ribosomal RNA mutations that alter decoding accuracy.

- *ram* indicates nonsense or missense suppression > control; *res* indicates nonsense or missense suppression; *both* indicates *ram* or *res* phenotype depending on reporter used; *bold text* indicates effects ≥ 5-fold change from control.
- *+* indicates no significant growth effects; *--* indicates ≤ 50% decrease in doubling time; *-* indicates > 50% decrease in doubling time; *nd* indicates not determined.
- *nd* indicates data not determined.
1.6 What do mutations reveal about the decoding mechanism?

1.6.1 The rearrangement of three A site nucleotides initiates the decoding reactions

The minor-groove interactions formed by 16S nucleotides G530, A1492, and A1493 are highly specific for Watson-Crick interactions at the first and second position of the codon-anticodon helix [32]. But, do these interactions serve to increase the binding specificity for the cognate substrate or do they begin a larger rearrangement that leads to the increased rates of GTP hydrolysis and accommodation? Cochella, et al. addressed this question by making mutations of each of the three bases in the affinity tagged 16S rRNA construct. Mutations to these residues presumably abolish the ability of these residues to form the codon-anticodon minor groove interactions. Ribosomes containing any of the mutations displayed severe defects in both the EF-Tu-dependent GTP hydrolysis and peptide bond formation [84]. The rates are partially rescued by the antibiotic paromomycin [84], which binds in h44 and forces A1492 and A1493 to flip out as they would upon binding of a cognate anticodon [32]. Interestingly, the rates are also rescued by streptomycin [84]. This antibiotic binds h44, but not in a way that forces A1492 and A1493 from the helix [85]. However, in 30S crystals, streptomycin does induce A1492 and A1493 to dock into the minor-groove of near-cognate ASLs, an interaction not seen in 30S crystals without antibiotic [35, 85]. The behavior of the A site mutants indicates that it is the rearrangement of the nucleotides that is critical for the rate enhancement and not the binding energy derived from the additional hydrogen bonds formed in the minor-groove.
1.6.2 How does A site rearrangement trigger GTP hydrolysis?

If minor-groove interactions in the A site contribute to the rate enhancement caused by cognate codon-anticodon pairing, how is this change communicated to the distal sites of the EF-Tu active site and the accommodation corridor? Based on structures of 30S subunits with cognate or near-cognate ASLs bound in the A site and the classically isolated ram and restrictive mutations, Ogle, et al. proposed the first hypothesis for the structural basis of the mechanism [35]. In the 30S crystals, a cognate ASL induced the rearrangement of the minor-groove triad, but also caused a larger rearrangement of the subunit, where the head and shoulder domains rotated inward toward the platform domain. On the other hand, near-cognate ASLs failed to induce either of these conformational changes. This “domain closure” movement could promote the formation of key shoulder contacts with the ternary complex that lead to the activation of EF-Tu [50]. Ram mutations located at the interface of S4 and S5 supported this model. A number of salt bridges between these proteins are broken in the “closed” conformation and the loss or disruption of these contacts could allow shoulder movement. While the ram mutations in S4/S5 are located at the interface the shoulder rotates from, the restrictive mutations in S12 are located at the area the rotation moves towards, consistent with their opposite effects on decoding (Figure 7) [16].
Figure 7. The domain closure model. The cartoon at right represents a cross section of the 30S subunit taken from the crystal structure at left. Red arrows show the direction of shoulder movement induced by cognate codon-anticodon interaction in 30S crystals. Proteins S4, S5, and S12 are shown in pink, blue, and orange respectively. The model proposes that shoulder rotation stimulates EF-Tu-dependent GTP hydrolysis by promoting shoulder contacts with the ternary complex, which binds at the shoulder and platform domains on the interface side of the subunit [50]. Ram mutations are proposed to promote shoulder rotation, while restrictive mutations are proposed to inhibit closure. Figure adapted from [16].

The domain closure model is consistent with much of the mutational and kinetic data, but it is inconsistent with several important observations. For one, a number of restrictive mutations have been isolated in S4 at the S5 interface [86]. Additionally, it is not clear if domain closure occurs in the 70S ribosome. Crystals of 70S complexes show
the ribosome already in the closed state [87-89]. If anything, ternary complex binding appears to move the shoulder outward in order to fit EF-Tu in the factor binding site [50, 53].

Recent insight into this puzzle comes from crystal structures of 70S ribosomes containing one of two 16S ram mutations, G299A in h12, near S4/S5, and G347U in h14. Both of these mutations increase the rate of EF-Tu-dependent GTP hydrolysis during decoding (see Chapter 2) [52, 81]. G347U distorts h14, and leads to a loss of several hydrogen bonds between h14 and L14 /L19, which make up inter-subunit bridge B8 (Figure 8A-B) [52]. This disruption is similar to that seen in 70S structures with bound ternary complex, indicating that disruption of the bridge is a critical step in activation of EF-Tu for GTP hydrolysis [50, 52, 53]. More interestingly, G299A does little to change the near-by h12/S4/S5 structure, but instead causes a similar disruption of B8 to that of G347U or the ternary complex (Figure 8C) [52]. This shows that bridge B8 can be disrupted allosterically, without requiring shoulder movement, and that B8 and h12 are linked. These observations raise the possibility that the S4/S5 mutations also act by allosterically influencing B8 rather than impacting domain closure.
Figure 8. Effects of mutations G299A and G347U on the structure of h14. These mutations cause a similar disruption of bridge B8. (A) B8 contacts between h14 and L14/L19 of the large subunit. Mutations G347U (B – green structure) and G299A (C – blue structure) lead to the displacement of h14 away from L14/L19, disrupting the hydrogen bonding network. Figure adapted from [52].
1.6.3 An active role for aa-tRNA in decoding fidelity

One of the most striking features of the ribosome bound ternary complex is the 30° bend the aa-tRNA must undergo in order to simultaneously interact with both EF-Tu and the A site codon. What role does this distorted conformation have in decoding? Some studies have suggested that the bent conformation is critical for activation of EF-Tu for GTP hydrolysis [48, 90]. A G24A mutation in the D-arm of tRNATrp increases stop codon readthrough [91]. Kinetic studies of this mutant tRNA show that it increases the rates of both EF-Tu-dependent GTP hydrolysis and accommodation on near-cognate codons [92]. It may be that this, as well as other mutations in the anticodon stem with similar kinetic effects, may promote the bent conformation of the ternary complex, as these mutations occur along the distorted region [47, 50, 93]. However, crystal structures of 70S ribosomes with G24A or A9C (another error prone mutant) Trp-tRNATrp ternary complexes stalled with kirromycin suggest that instead, these base changes promote interactions between the aa-tRNA and shoulder region [94].

1.6.4 Mutations can have distinct effects on different phases of decoding

Because cognate aa-tRNA increases the rates of both EF-Tu-dependent GTP hydrolysis and accommodation, an important unanswered question is: how are these two distinct phases activated by the same A site interactions? Are they linked by a common mechanism? Zaher and Green studied two accuracy mutations, rpsD14, a ram mutation that results in a C-terminal truncation of S4, and rspL141, a restrictive mutation that results in a K42N alteration in S12. Surprisingly, these two mutations appear to have
distinct effects on decoding. The restrictive mutation has small effects on the rate of EF-Tu-dependent GTP hydrolysis during initial selection, but significantly increases proofreading of a near-cognate Leu-tRNA [95]. On the other hand, the rpsD14 ram mutation leads to a large increase in GTP hydrolysis for near-cognate Leu-tRNA, while having no effect on accommodation or proofreading. This result suggests that the two phases of decoding can be controlled by distinct mechanisms.

However, other ram and restrictive mutations appear to be able to affect both phases of decoding. Streptomycin dependent mutations lead to large decreases in the rate of GTP hydrolysis and increased rejection of aa-tRNA during proofreading [96]. Furthermore, in Chapters 2 and 3, we show that ram mutations that affect bridge B8 result in defects in both initial selection and proofreading. Also in Chapter 3, we show that some mutations have effects on proofreading only in certain translation contexts.

Advances in ribosome crystallography have provided incredible insights into the mechanism of translation. However, continued study of how mutations in the structure affect translation can still help elucidate the functional roles specific elements of the ribosome in decoding and other steps in protein synthesis.
Chapter 2: Missense suppressor mutations in 16S rRNA reveal the importance of helices h8 and h14 in aminoacyl-tRNA selection

2.1 Introduction

In order to faithfully translate the genetic code, the ribosome must select the correct (cognate) aminoacyl-tRNA (aa-tRNA) from the total cellular pool of aa-tRNAs in each round of elongation. This process, termed aa-tRNA selection or decoding, is facilitated by elongation factor Tu (EF-Tu), a three-domain GTPase that forms a ternary complex with GTP and aa-tRNA. Studies by Wintermeyer, Rodnina, and colleagues have led to the following model for decoding (for review, see[97]) The ternary complex initially interacts with the ribosome in a codon-independent manner, primarily through contacts to the 50S subunit. Then codon–anticodon pairing occurs in a 30S A site, which leads to activation of the GTPase domain of EF-Tu and GTP hydrolysis. Hydrolysis of GTP causes a large conformational change in the factor that leads to release of the aa-tRNA. The acceptor end of aa-tRNA then moves into the 50S subunit A site (a step termed accommodation), which is followed by a rapid peptide bond formation.

It has long been recognized that the thermodynamic stability of a cognate codon–anticodon interaction versus one with a single base-pair mismatch (near-cognate) cannot
account for the accuracy of protein synthesis (for review, see [16, 97]). One way the translation machinery achieves high fidelity is by discriminating twice against near-cognate tRNA, once before and once after the functionally irreversible GTP hydrolysis step. This proofreading mechanism, though, is not maximally exploited to ensure high fidelity. Instead, the ribosome additionally employs an induced-fit mechanism in which cognate aa-tRNA accelerates two steps of the process—activation of EF-Tu for GTP hydrolysis and accommodation. Because this induced-fit mechanism increases rates for cognate aa-tRNA specifically, the ribosome can decode mRNA with both accuracy and speed.

The kinetic data imply that cognate codon–anticodon pairing induces a conformational change in the ribosomal complex that activates EF-Tu. Structural studies show that when cognate tRNA binds the ribosomal A site, three universally conserved 16S rRNA nucleotides (G530, A1492, and A1493) reorder to dock into the minor groove of the codon–anticodon helix [32, 35]. This local rearrangement is accompanied by rotations of the 30S head and shoulder domains toward the subunit interface, conformational changes collectively described as “domain closure.” Ramakrishnan and colleagues proposed that domain closure plays a central role in the induced-fit mechanism by promoting interactions between the ternary complex and ribosome that are critical for EF-Tu activation [16]. This domain closure model is supported by the observation that error-inducing antibiotics, such as paromomycin, stabilize the closed conformation of the ribosome and enhance forward rate constants for near-cognate tRNA [98] [35]. Also, a number of ribosomal mutations that cause miscoding localize along the
interface of S4 and S5 [99], ribosomal proteins that separate during domain closure [32]. These mutations are expected to destabilize the open state, decreasing the energy barrier for domain closure in the presence of near-cognate tRNA, consistent with their miscoding phenotypes.

While several lines of evidence support the domain closure model, some functional data are difficult to reconcile. In one study, certain mutations at the S4-S5 interface were found to make ribosomes hyperaccurate rather than error prone [86]. In a separate study, the effects of several S4 mutations on the level of S4-S5 binding were assessed using a yeast two-hybrid assay, and no correlation to translational accuracy was observed [100]. These unexpected data suggest that our understanding of aa-tRNA selection remains incomplete.

Genetic studies of ribosomal RNA have generally been hampered by the fact that most model organisms have multiple copies of the rRNA genes. In this work, using a specialized ribosome strain to bypass this issue, we have isolated and characterized a number of mutations in 16S rRNA that decrease the fidelity of translation elongation. Our findings strongly implicate shoulder movement in aa-tRNA selection and uncover the role of helices h8 and h14 in regulating GTP hydrolysis by EF-Tu.

2.2 Results and Discussion

2.2.1 Isolation of missense and nonsense suppressor mutations in 16S rRNA

Using a specialized ribosome system described previously [82], a classical genetic approach was taken to identify mutations in 16S rRNA that decrease the fidelity of
translation elongation. Two screens were performed, one for missense suppressors and the other for nonsense (UGA) suppressors. To identify missense suppressors, we used indicator strain KLF4001, in which codon 461 of the reporter SD*(5′-AUCCC-3′)-lacZ gene was changed from GAA (Glu) to GAT (Asp). Glutamate 461 of β-galactosidase contributes to the active site, and an Asp substitution at this position abolishes enzymatic activity [101, 102]. Hence, the production of active β-galactosidase in the strain KLF4001 depends on misreading of the near-cognate GAU by Glu-tRNA. To identify nonsense suppressors, an indicator strain (KLF2723) containing TGA (stop) in place of TGG (Trp) at codon 585 of lacZ was used. In this strain, production of full-length LacZ requires the read-through of UGA. The two screens were done in an analogous way. Plasmid pKF207 encoding specialized 16S rRNA (ASD*: 5′-GGGGU-3′) was mutagenized by propagation in XL1-Red (Stratagene) and transformed into the appropriate indicator strain. Transformants were then screened on plates containing X-gal and arabinose for those expressing increased levels of β-galactosidase.
Table 3. Mutations in 16S rRNA that increase misreading of sense and stop codons.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Location</th>
<th>Misense</th>
<th>Nonsense</th>
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<tr>
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<td>5' end</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>A151G</td>
<td>h8</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>C153U</td>
<td>h8</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>G158A</td>
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<td>1</td>
<td>0</td>
</tr>
<tr>
<td>G159A</td>
<td>h8</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
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<td>h8</td>
<td>9</td>
<td>3</td>
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<tr>
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<td>h8</td>
<td>8</td>
<td>3</td>
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<td>0</td>
</tr>
<tr>
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<tr>
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<td>h12</td>
<td>15</td>
<td>5</td>
</tr>
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<td>0</td>
</tr>
<tr>
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</tr>
<tr>
<td>G1491A</td>
<td>h44</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

*ins indicates insertion; Δ, deletion.

*Isolates were only considered independent if they originated from separate preparations of mutagenized pKF207.

*In this case, codon 461 of lacZ was changed from GAA to GAT. Production of active β-galactosidase requires misreading of GAA by Glu-RNA.

*In this case, codon 585 of lacZ was changed from TGG to TGA. Production of active β-galactosidase requires read-through of UGA.

*Insertion of A after C345.
The results of these screens are shown in Table 3. A number of mutations were isolated in both screens, as was expected since ribosomes defective in aa-tRNA selection should increase misreading both GAU and UGA. With the exception of G886A, these mutations were distinct from those identified in an earlier screen for defects in initiation fidelity [82]. Nearly all of the mutations obtained were transitions. This bias seems to be due to the use of XL1-Red, because transversions engineered at several of these positions confer strong suppressor phenotypes that would have been easily detected in the screens (see below). Of the 34 mutations recovered, at least six (G886A, U911C, C1054U, C1200U, C1469U, and G1491A) were shown previously to influence the fidelity of elongation [103].
To verify that each mutation in Table 3 conferred a suppressor phenotype, site-directed mutagenesis was used to introduce each mutation into pKF207 de novo. The resulting plasmids were transformed into the appropriate indicator strain(s), and in each case, missense and/or nonsense suppression was confirmed. Then, the effects of these and a number of additional mutations on decoding fidelity in vivo were quantified (Figure 9). To estimate the frequency of misreading (i.e., missense errors), the activity of β-galactosidase translated from lacZ (GAA 461 GAT) relative to that from lacZ (control) was determined for each of the mutant ribosomes. An analogous approach was taken to estimate the frequency of UGA read-through (i.e., nonsense errors) for a subset of the mutant ribosomes (Figure 10). In general, the quantified error rate correlated with the number of times a given mutation was isolated in the corresponding screen (Table 3).
Figure 9. Effects of 16S rRNA mutations on missense suppression. Values on the leftward axis correspond to the relative levels of β-galactosidase translated from SD*-lacZ (control) mRNA by each of the mutant ribosomes (as indicated). Data represent the mean ± SEM from three or more independent experiments. Values on the rightward axis reflect the missense error rate, calculated as the level of active β-galactosidase produced from SD*-lacZ (GAA 461 GAT) divided by that from SD*-lacZ (control). For the wild-type specialized ribosomes, this quotient was 0.0013 ± 0.00008. The data shown correspond to the normalized quotient of two mean ± SE from three or more independent experiments. Mutations analyzed include those identified in the screens and those engineered. Prefixes “ins” and “Δ” denote insertion and deletion, respectively.
2.2.2 Elements of 16S rRNA in the fidelity of translation

The suppressor mutations clustered to distinct regions of 16S rRNA (Figure 11). Three mutations (A7G, G299A, A300G) mapped to the solvent side of the subunit near
the S4-S5 interface, where a number of protein mutations affecting fidelity have been previously localized (Figure 11B, D) [86, 99, 104]. Of these, G299A conferred the strongest phenotypes, increasing both missense and nonsense errors by approximately 10-fold (Figure 9, Figure 10). Structural studies show that G299 normally pairs with the Hoogsteen face of G566, while the adjacent nucleotide A300 contacts U565 and C564 [88]. Nucleotides 564–566 are part of the 560 loop (nucleotides 557–566), which contains several sharp turns in its backbone and physically links the 5′ and central domains of 16S rRNA. O6 of G299 and O1P of G558 are involved in coordinating a magnesium ion that appears to stabilize the compressed fold of the 560 loop. Mutations A7G, G299A, and A300G are each predicted to destabilize these interactions between h12 and the 560 loop. Because these interactions lie where the shoulder domain interfaces with the remainder of the subunit, these mutations may increase miscoding by promoting domain closure, as has been proposed for S4/S5 mutations [16]. Pyrimidine substitutions were engineered at position 299, and these gave considerably weaker phenotypes than did G299A (Figure 9). The fact that G299A confers the strongest phenotype might be explained by the electrostatic repulsion between N6 of the introduced A and the bound Mg$^{2+}$, which may destabilize the h12-560 loop interaction to the largest degree.
Figure 11. Where the mutations map. An overview of the locations of the mutations (depicted in red) on the tertiary structure of 16S rRNA (PDB 2WRN) viewed from the interface (A) and solvent (B) perspective. Large red arrows in panel A indicate movements of 30S head and shoulder (SHDR) domains with respect to the platform (PF) during domain closure. (C) Model of the ternary complex bound to the 70S ribosome with kirromycin (Protein Data Bank [PDB] 3FIH and 3FIK). The modeled switch 1 motif of EF-Tu is shown in green. (D) Solvent view of the 30S subunit (PDB 2AVY) showing mutations found in h12 and h21. Mutations isolated in proteins S4 and S5 [99] are in pink. (E) View of the 30S A site (PDB 2WRN). (F) Closer view of the complex shown in panel C, showing locations of mutations found in h8, h14, and h44 (PDB 3FIH and 3FIK). Mutations in protein L19 that decrease fidelity [105] are indicated in turquoise.
Three other mutations (U598C, G606A, and C634U) mapped nearby in h21 (Figure 11D), and each of these caused a relatively modest (two- to fourfold) increase in miscoding (Figure 9). From the platform domain, h21 spans across the backside of the subunit, and its terminal loop docks with elements of the shoulder domain (including h4, S4, and S16) [88]. Because h21 provides a structural support to the shoulder domain, it is plausible that these mutations in h21 affect shoulder rotation and it thereby confers their effects on decoding. Interestingly, U598 and C634 are located adjacent to His30 in S17, which is the location of a neamine resistance mutation that leads to a hyperaccurate phenotype [68, 70].

Another group of mutations (ΔU420, G423A, and G424A) mapped in or adjacent to the UUCG tetraloop that caps h16 at the “top” of the shoulder domain (Figure 11A-C), and these mutations increased miscoding by two- to threefold (Figure 9). Deletions of 1 and 2 base pairs (bp) were engineered to shorten h16. Both of these truncations increased miscoding by about threefold, although the larger deletion was more deleterious to overall translation (Figure 9). The tetraloop of h16 forms a contact with S3, a protein of the head domain. When the shoulder rotates during domain closure, this contact is partially disrupted as indicated by a 16% reduction of buried surface area [32]. Hence, these mutations are predicted to destabilize the open state of the 30S subunit, which may explain their miscoding phenotypes.
Two mutations mapped to h27 (G886A and U911C) (Figure 11A), and each is predicted to change the G886-U911 wobble pair to a Watson–Crick pair. This region was previously implicated in translational fidelity [106], but the idea of a dynamic “switch” between two alternative base-pairing arrangements in this region has been ruled out [107]. These mutations lie at the junction of the major domains of the subunit, near the streptomycin-binding site.

Mutation C1054U was isolated four times in the nonsense suppressor screen (Table 3), consistent with a similar screen done in the past [108]. C1054U was not recovered as a missense suppressor, which was explained by the fact that the mutation decreased the misreading of GAU by twofold (Figure 9). Mutations C1054A and C1054G, on the other hand, increased misreading by 20- and 40-fold, respectively. These purine substitutions also increased UGA read-through, although C1054A conferred the larger error rate in that case (Figure 10). Structural studies have shown that C1054 contributes to the A site of the 30S subunit, contacting nucleotide 34 of tRNA and residues of release factors RF1 and RF2 [32, 88, 109-111]. The ability of mutations at position 1054 to increase read-through of stop codons is well established [108, 112-116], but whether these A-site mutations increase the frequency of the missense errors has been less clear. Murgola and colleagues failed to see effects of these mutations on misreading of several codons in trpA [117]. However, the relationship between the misreading error rate and the concentration of competing cognate aa-tRNA [13] was unappreciated at that time, and the ability of ribosomal mutations to suppress those particular missense mutations was not demonstrated. Murgola and coworkers did see effects of 1054
mutations on the activities of several suppressor tRNAs that varied depending on the ribosomal mutation, suppressor tRNA, and/or trpA mutation [117]. Those experiments, involving missense suppression in the presence of a suppressor tRNA, essentially assess a competition between two cognate tRNAs and, hence, speak little to the question of decoding fidelity. Our data unambiguously show that C1054A and C1054G can decrease the fidelity of decoding in vivo (Figure 9). The strong effects of C1054A and C1054G on both GUA misreading and UGA read-through suggest that these mutations may be generally detrimental to decoding fidelity. For example, a purine at 1054 may stabilize the near-cognate tRNA and/or reduce the energetic cost of domain closure in the presence of the near-cognate tRNA. Alternatively, C1054 may normally contribute to the uniformity of aa-tRNA selection [93, 118], in which case mutations at this position would be expected to increase the rate of decoding for certain tRNAs and decrease the rate for others. This imbalance would lead to variable effects on missense suppression, which could explain the available data. Further experiments will be necessary to investigate these possibilities.

Three other mutations (C1200U, C1203U, and G1491A) localized near the A site (Figure 11E). Two of these (C1200U and G1491A) were isolated in previous screens for nonsense suppressors [108, 114] [117]; and conferred larger effects on UGA read-through than on GAU miscoding (Figure 9, Figure 10). The latter observation suggests that C1200U and G1491A inhibit RF2-dependent termination or enhance misreading by Trp-tRNA more than misreading by Glu-tRNA. All three mutations are predicted to perturb the A site in some way and thereby confer their phenotypes [32, 88]. N4 of
C1200 donates a hydrogen bond to O1P of nucleotide 1055. The U substitution at 1200 precludes this interaction, potentially altering the conformation of the adjacent A-site nucleotide C1054. Nucleotide C1203 normally pairs with G1057, forming one of two Watson–Crick pairs that lie between loops (1054–1055 and 1200–1202) that bulge from opposite strands of h34. Mutation C1203U replaces this Watson–Crick pair with a wobble G–U pair, which may alter the position of nearby nucleotides such as C1200 and C1054. Nucleotide G1491 pairs with C1409 and lies adjacent to A-site nucleotides A1492 and A1493. These adenines rearrange when cognate tRNA binds the A site, moving out of h44 to dock into the minor groove of the codon– anticodon helix [32]. A1492 and A1493 also undergo substantial rearrangement upon release factor binding, although the resulting conformation is distinct from that induced by tRNA [109, 110] [111]. Mutation G1491A disrupts the 1409–1491 pair, which may affect these conformational changes of A1492 and A1493 important for decoding and/or termination.

Nearly half of the mutations mapped to either h8 or h14 (Table 3). These helices interact with each other near the EF-Tu binding site and contribute to intersubunit bridge B8 (Figure 11F). Mutations with the strongest phenotypes were those of A160, A161, and G347 (Figure 9). These residues are located at the interface between the two helices, suggesting that disruption or destabilization of this interface is responsible for the observed fidelity defects. Helix 14 contacts L19 and L14 of the 50S subunit to form bridge B8 [1] [88]. Previous work identified two residues of L19 important for elongation fidelity [105]. One of these residues, Q40, lies right next to the h14 contact site (Figure 11F). The other residue, G104, also lies near the subunit interface, where L19 contacts
h44 (Figure 11F). Our missense suppressor screen identified two h44 mutations, A1430G and C1469U, which lie right in this vicinity (Table 3; Figure 11F). The latter mutation was isolated previously as a suppressor of streptomycin dependence and was shown to increase misreading in vitro [119].

2.2.3 Role of h8 and h14 in aa-tRNA selection

Recent cryo-EM studies provided evidence that EF-Tu interacts directly with h14 of 16S rRNA at the h8–h14 junction [47] [49]. In those complexes, the antibiotic kirromycin was used to stabilize the ternary complex on the ribosome in a conformation believed to resemble the GTPase-activated state. On the ribosome, the switch 1 motif of the GTPase domain is repositioned away from the switch 2 and P-loop motifs in order to contact h14. This appears to open a hydrophobic “gate” that lies between the catalytic residue His84 and GTP in the ground (nonactivated) state. These data raised the possibility that the interaction between h14 and switch 1 is important for activating GTP hydrolysis during decoding [49]. To investigate this possibility, we constructed mutations that decrease or increase the helix length by 1 or 2 bp. The 2-bp deletion (h14Δ2), which should disrupt the putative switch 1 contact, decreased overall ribosome activity, but by only fivefold (Figure 9). Furthermore, ribosomes carrying h14Δ2 were able to support growth of an *Escherichia coli* Δ7 prrn strain (see below). The fact that the h14Δ2 ribosomes retained substantial activity argues against an essential role for h14 in activating EF-Tu. In contrast, lengthening h14 by 2 bp completely abolishes translation activity in vivo (Figure 9). Normally, the end of h14 butts up against L19 and L14 to
form bridge B8 (Figure 11F). A 2-bp extension of h14 is predicted to push the subunits apart in this region, a condition that appears to be intolerable for translation.

Figure 12. Effects of mutations in helices h8 and h14 on initial selection. 70S initiation complexes (70SIC) programmed with either cognate cognate UUU (closed symbols) or near-cognate CUU (open symbols) in the A site were rapidly mixed with EF-Tu•[γ-32P]GTP•Phe-tRNA\(^\text{Phe}\), and rates of GTP hydrolysis were determined. Wild type indicated by ●/○ and solid lines; G347U, ■/□ and long dashed lines; h14Δ2, [diamond]/[diamond with plus] and medium dashed lines; and h8Δ3, ▲/[big up triangle, open] and short dashed lines. (A) Examples of time courses at 2 µM 70SIC. Data were fit to a single exponential function to obtain the apparent rates of GTP hydrolysis (kapp). Apparent rates for near-cognate tRNA (B) and cognate tRNA (C) plotted versus [70SIC]. Data were fit to the equation kapp = k\text{GTP max} • [70SIC]/(K1/2 + [70SIC]), yielding the parameters shown in Table 2.

Although active, h14Δ2 ribosomes were found to be error prone, misreading GAU at a rate 10-fold higher than that of control ribosomes (Figure 9). We also constructed 2- and 3-bp deletions in h8 (h8Δ2 and h8Δ3), each of which should disrupt its contact to h14 but still allow potential interactions between h14 and EF-Tu to form. These mutant
ribosomes exhibited phenotypes very similar to the h14Δ2 mutant (Figure 9), suggesting that disruption of the h8–h14 interface is sufficient for loss of fidelity.

How does disruption of the h8–h14 interface affect aa-tRNA selection? To address this question, we introduced mutations h14Δ2, h8Δ3, and G347U into an E. coli Δ7 prrn strain, purified the corresponding mutant 70S ribosomes, and analyzed their activities in the initial selection phase of decoding (i.e., EF-Tu-dependent GTP hydrolysis) (Figure 8). Mutation G347U was included because it had the largest decoding defect among the collection of h8/h14 mutations (Figure 9). Previous work by Rodnina and colleagues indicates that initial selection can be represented by a three-step kinetic model, in which initial binding of the ternary complex (step 1) is followed by codon recognition (step 2), which is followed by GTPase activation/GTP hydrolysis (step 3) [22] [21, 27] [120]. The hydrolysis event itself is very rapid and so can be grouped with the GTPase activation in the scheme. In the near-cognate case, step 3 is much slower than step 2 and hence is rate limiting for the overall reaction. In the cognate case, steps 2 and 3 occur at comparable rates; hence, each is partially rate limiting for the overall reaction. Ribosome complexes programmed with either UUU (cognate) or CUU (near-cognate) in the A site (at various concentrations ≥0.5 µM) were rapidly mixed with EF-Tu•[γ-32P]GTP•Phe-tRNA^Phe (<0.3 µM), and the rate of single-turnover GTP hydrolysis was determined. Apparent rates were plotted versus ribosome concentration, and the data were fit to obtain parameters $k_{GTPmax}$, the maximal rate of GTP hydrolysis, and $K_{1/2}$, the concentration of ribosomes at which half-maximal rate was observed. We found that each mutation stimulated GTP hydrolysis, increasing $k_{GTPmax}$ by five- to eightfold in the near-
cognate case and by two- to threefold in the cognate case (Figure 12; Table 4). In the framework of the Rodnina model, these data suggest that the mutations increase $k_3$ (GTPase activation). The large effects on $k_{GTPmax}$ seen in the near-cognate case can only be explained with an increase in $k_3$. In the cognate case, a similar increase in $k_3$ would be expected to increase $k_{GTPmax}$ more modestly and increase $K_{1/2}$, and both of these effects are observed. Effects on step 2 (e.g., an increase in $k_2$ and/or decrease in $k_{-2}$) could also contribute to the observed increases in $k_{GTPmax}$, so additional experiments will be necessary to elucidate more precisely how initial selection is perturbed by these mutations. Nonetheless, these data are clearly inconsistent with the idea that h14 helps activate the GTPase domain of EF-Tu. Instead, these data suggest that h14 acts with h8 to negatively regulate GTP hydrolysis and thereby increase the stringency of decoding.

We propose that intersubunit bridge B8 normally acts to counter inward rotation of the 30S shoulder domain, a conformational change critical for GTPase activation. Any mutations in h8, h14, and L19 that compromise the bridge would reduce the energetic cost of inward shoulder rotation, allowing higher rates of GTP hydrolysis in the absence of a cognate codon–anticodon interaction and hence higher error rates. The idea that small changes in intersubunit distance in this region play a key role in decoding is supported by structural studies [16] and our observation that a 2-bp extension of h14 results in complete loss of translation activity (Figure 9). Mutations in h44 and L19, which lie at the section of bridge B6 closest to B8 (Figure 11F), are also likely to reduce constraints on shoulder rotation, and hence their effects on fidelity can also be rationalized with this simple model.
The ratio of cognate $k_{\text{GTPmax}}/K_{1/2}$ to near-cognate $k_{\text{GTPmax}}/K_{1/2}$ was calculated to estimate the effects of mutations h14Δ2, h8Δ3, and G347U on the fidelity of initial selection (Table 4). For control ribosomes, this ratio was 34, in line with previous studies [27] [118]. The mutations reduced this ratio by four- to ninefold, generally consistent with their fidelity defects in vivo. These estimated effects on the initial selectivity, though, cannot completely account for the decoding defects observed in vivo, and the trend h14Δ2 > h8Δ3 > G347U seen in vitro differs from that (G347U > h14Δ2 ≈ h8Δ3) seen in vivo (Table 4). These differences may stem from the different tRNA species involved in the assays and/or additional effects on the proofreading stage of decoding.

<table>
<thead>
<tr>
<th>Ribosomes</th>
<th>Cognate $k_{\text{GTPmax}}$ (sec$^{-1}$)</th>
<th>Cognate $K_{1/2}$ (µM)</th>
<th>Near-cognate $k_{\text{GTPmax}}$ (sec$^{-1}$)</th>
<th>Near-cognate $K_{1/2}$ (µM)</th>
<th>Initial selectivity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>54 ± 3</td>
<td>1.5 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td>1.8 ± 0.6</td>
<td>34</td>
</tr>
<tr>
<td>G347U</td>
<td>130 ± 10</td>
<td>2.8 ± 0.5</td>
<td>15 ± 1</td>
<td>2.5 ± 0.4</td>
<td>7.7</td>
</tr>
<tr>
<td>h14Δ2</td>
<td>140 ± 10</td>
<td>5.4 ± 0.6</td>
<td>12 ± 4</td>
<td>1.8 ± 1</td>
<td>3.9</td>
</tr>
<tr>
<td>h8Δ3</td>
<td>93 ± 6</td>
<td>2.7 ± 0.3</td>
<td>11 ± 1</td>
<td>1.8 ± 0.5</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Values and their standard errors were calculated from the curve fits shown in Figure 4. $^a(Cognate \, k_{\text{GTPmax}}/K_{1/2})/(Near\,-\,cognate \, k_{\text{GTPmax}}/K_{1/2})$.

Table 4. Kinetic parameters for EF-Tu-dependent GTP hydrolysis on control and mutant ribosomes
The ability of these ribosomal mutations to stimulate EF-Tu raises the question of whether translation elongation is increased as a consequence. Our data do not address this question. The steady-state rate of product yield (e.g., translation activity in Figure 9) is normally limited by initiation rather than elongation. An increase in the elongation rate would be predicted to decrease the density of ribosomes on the lacZ mRNA with little or no effect on the overall rate of product yield [121] [122]. Experiments that directly measure the elongation rate will be necessary to determine which mutations affect the speed of translation [123] [124].

Ribosomes harboring G347U, h14Δ2, or h8Δ3 were able to support growth in the Δ7 prrn strain, although the rate of growth in these strains was reduced by 15%, 33%, and 14%, respectively (data not shown). These data show that E. coli can tolerate quite low translational fidelity, at least when grown on rich media in the laboratory. Interestingly, we have constructed Δ7 prrn strains with mutations C1054A (18% reduced growth rate) and C1054U (6% reduced growth rate) but were unsuccessful in our attempts to make Δ7 prrn (C1054G) [2]. It is tempting to speculate that C1054G, which causes a 40-fold increase in misreading by Glu-tRNA (Figure 9), decreases the accuracy of decoding below the minimal threshold required for viability.

2.2.4 Importance of h5 in translation

The data described above provide functional evidence that movement of the shoulder domain plays a critical role in the accuracy of decoding. Clues to how shoulder rotation may activate the GTPase domain of EF-Tu come from a recent 3.6 Å resolution
crystal structure of the ternary complex bound to the ribosome [50]. This complex was stabilized by kirromycin and hence is quite analogous to those solved by cryo-EM; however, in the crystals, the switch 1 region of EF-Tu was completely disordered. A number of specific conformational changes in tRNA and EF-Tu could be inferred by the high-resolution co-crystal structure. When the cognate ternary complex binds the ribosome, the tRNA adopts a distorted conformation—its anticodon stem bends and exhibits less helical twist, while its 3′ end rearranges to make specific contacts with h5 of the shoulder domain of the 30S subunit. Two regions of domain 2 of EF-Tu (residues 256–273 and 219–226) also change position in order to interact with h5 of the shoulder. It was proposed that these conformational changes, stabilized by the shoulder in the closed state, disrupt an interaction between switch 1 and the 3′ end of tRNA to open the hydrophobic gate and activate the GTPase domain of EF-Tu [50]. The effects of the mutation G222D of EF-Tu lend support to this model [125]. This mutation, which is predicted to disrupt the contacts between domain 2 of EF-Tu and h5 of 16S rRNA, specifically blocks the GTPase activation step of decoding [125].
Figure 13. Effects of mutations in helix 5 on translation activity in vivo. (A) Values correspond to the relative levels of β-galactosidase translated from SD*-lacZ (control) mRNA by each of the mutant ribosomes (as indicated). The background level of β-galactosidase, determined from cells lacking specialized ribosomes (vector), is approximately 1%. Data represent the mean ± SEM from three or more independent experiments. (B) Interaction of h5 with domain 2 of EF-Tu and the 3′-CCA end of the A/T-tRNA (PDB ZWRN). The location of a G222D mutation, which impairs GTPase activation [125], is indicated in green.

To investigate the potential importance of h5 in decoding, we targeted several nucleotides that contact the residues of EF-Tu and/or tRNA. Base substitutions were
generated at positions 55, 357, and 367, and their effects on translation activity were quantified using our specialized ribosome system (Figure 13). Most of these mutations reduced ribosome activity to background levels. Some residual activity (2%–6% of the control) was seen for certain substitutions at positions 357 and 367, but in no case was ribosome activity high enough to assess the fidelity of elongation. These data show that these h5 nucleotides, particularly A55, are critical for translation, in line with the model proposed by Ramakrishnan and coworkers [50]. It is important to point out, though, that these data reflect the overall translation activity as opposed to the decoding step in isolation. Further experiments will be necessary to determine the degree to which these h5 mutations influence decoding and other steps in protein synthesis.

2.3 Conclusions

Here, we report the first screen for missense suppressor mutations in 16S rRNA. Most of the mutations mapped near interfaces between the 30S shoulder domain and other parts of the ribosome, strongly implicating shoulder movement in the molecular mechanism of decoding. The largest cluster of mutations localized to helices h8 and h14, which contact each other to form the 30S portion of bridge B8. While ribosomes carrying a 2-bp extension of h14 were completely inactive, those with a truncation of either h14 or h8 were error prone and exhibited elevated rates of GTP hydrolysis by EF-Tu. These data argue against a critical role for h14 in activating EF-Tu and suggest instead that h14 acts with h8 to negatively regulate GTP hydrolysis and thereby increase the stringency of aa-tRNA selection.
2.4 Materials and Methods

2.4.1 Bacterial strains

Indicator strains KLF4001, KLF2723, and KLF2674 carry the lacZ reporter in single copy on the chromosome and were constructed from parental strain CSH142 [F-ara Δ(gpt-lac)5] as described previously [80] [126]. In these strains, lacZ is preceded by the alternative Shine–Dalgarno (SD) sequence 5’-ATCC-3’ (SD*) and is under transcriptional control of a consensus P_{an} promoter. In KLF4001, lacZ carries a missense mutation at codon 461 (GAA to GAT). In KLF2723, lacZ contains a UGA stop at codon 585. In KLF2674, lacZ contains no mutations (control strain). These indicator strains were made recA− by P1 transduction using donor strain JC14604 [Δ(recA-srl)306, srlR301 Tn10] obtained from the E. coli Genetic Stock Center (Yale University). E. coli Δ7 prrn strains were made using SQZ10 as described previously [126].

2.4.2 Engineered mutations

The precise nature of the deletion mutations constructed in 16S rRNA are as follows: h8Δ2bp, Δ(154,155,166,167); h8Δ3bp, Δ(154,155,157,164,166,167); h14Δ1bp, Δ(340,349); h14Δ2bp, Δ(340,341,348,349); h16Δ1bp, Δ(418,425); and h16Δ2bp, Δ(417,418,425,426). For the insertion mutations in h14, either one (h14ins1bp) or two (h14ins2bp) additional C–G pairs were added in the middle of the helix.

2.4.3 Genetic screens
The genetic screens were performed as described previously [82], except that indicator strains KLF4001 and KLF2723 were used. Mutator strain XL1-Red (Stratagene) was employed for the mutagenesis. Although the results of the screens suggested a bias for transitions in the mutagenesis, we did not perform a thorough analysis of the method with regard to mutation frequency or positional distribution.

2.4.4 β-Galactosidase assays

β-Galactosidase activity was measured as described previously [80] [82], using the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) for experiments involving missense suppression and chlorophenol red-β-D-galactopyranoside (CPRG; Sigma) for those involving nonsense suppression. ONPG was chosen in the former case because the E461D mutant of LacZ cannot hydrolyze ONPG [127] but has residual activity on other substrates [101] that could potentially mask miscoding events in our experimental system. This was not an issue for nonsense suppression (because the truncated LacZ is inactive on all substrates), so CRPG was used for increased sensitivity.

2.4.5 Kinetic assays

GTP hydrolysis measurements were performed as described [118], except that reactions were carried out in polymix buffer [128]. The mRNAs were the same as those used in a previous study [129].
Chapter 3: Distinct functional classes of ribosomal ambiguity mutations in 16S rRNA

3.1 Introduction

Translation of the genetic code is highly accurate (error rate of $10^{-3}$-10^{-6}$), and the ribosome plays an active role in achieving this level of fidelity [130]. Decoding occurs through a two-stage selection process. During initial selection, aminoacyl-tRNA (aa-tRNA) binds the ribosome as part of a ternary complex (TC) with elongation factor Tu (EF-Tu) and GTP. Interactions between the codon and anticodon in the 30S A site lead to activation of EF-Tu and GTP hydrolysis. Then, during the proofreading stage, the aa-tRNA either moves into the A/A site (a step termed accommodation), where it can participate in peptide bond formation, or dissociates from the ribosome. Discrimination of cognate versus near-cognate aa-tRNA relies on a number of key steps in the process [21, 27, 28]. Cognate codon-anticodon pairing not only stabilizes aa-tRNA at both stages of decoding but also increases the forward rate constants for GTPase activation / GTP hydrolysis and accommodation. Acceleration of these forward steps in response to codon-anticodon pairing allows translation to be both accurate and fast.

High-resolution structures of ribosomal complexes show that cognate codon-anticodon pairing induces three universally-conserved residues in the 16S rRNA (G530,
A1492 and A1493) to rearrange and dock into the minor groove of the codon-anticodon helix [32, 35, 53]. The contacts formed by these 16S nucleotides require Watson-Crick base pair (bp) geometries at the first two positions of the codon-anticodon helix. The geometry of the third base pair is less stringently monitored, allowing for example the formation of wobble pairs. These structures help explain how the ribosome increases the binding specificity for cognate aa-tRNA [35, 120, 131]. How these interactions in the 30S A site stimulate GTP hydrolysis and subsequent accommodation remains less clear.

We previously isolated many ribosomal ambiguity (ram) mutations in the 16S rRNA [81]. These mutations increase errors during translation elongation and cluster in several distinct regions of the ribosome. Based on the number of isolates obtained and the strength of phenotypes conferred, three regions stand out as most critical for decoding fidelity: (1) the A site; (2) helix h12, which lies on the solvent side of the subunit near S4 and S5; and (3) helices h8 and h14, which constitute the 30S portion of intersubunit bridge B8. In line with this assessment, ram mutations isolated in r-proteins map to the S4/S5 interface and to L19, a component of the 50S portion of B8 [86, 99, 104, 132, 133].

Analysis of a subset of these 16S ram mutations has shown that bridge B8 increases the stringency of decoding by negatively regulating GTPase activation [52, 81]. Mutations predicted to disrupt bridge B8 (i.e., h8Δ3, a 3-bp truncation of h8; h14Δ2, a 2-bp truncation of h14; and G347U in h14)—as well as mutation G299A in h12—increase the rate of EF-Tu-dependent GTP hydrolysis, particularly in the near-cognate case. Crystal structures of mutant 70S ribosomes show that either G347U or G299A induces a
distortion of h8/h14 that disrupts B8 [52]. These conformational changes in h8/h14 are virtually identical to those seen in co-crystal structures of wild-type 70S ribosomes bound with TC (stabilized with either kirromycin or GDPCP) [50, 52, 53], suggesting that G347U and G299A act, at least in part, by promoting formation of the GTPase-activated state. Together these observations indicate that disruption of bridge B8 is a critical aspect of GTPase activation and that h12 is allosterically linked to B8 [52].

In this study, we characterize a larger subset of ram mutations in 16S rRNA, comparing their effects on initial selection, proofreading, RF2-dependent termination, and overall miscoding in multiple contexts. Our data show that bridge B8 contributes to both initial selection and proofreading and that mutations in or near the A site influence decoding in a codon-anticodon-dependent manner.

3.2 Results

To better understand the roles of distinct regions of the ribosome in decoding, we selected a subset of mutations for further analysis. These mutations were chosen based on their phenotypes in the cell and their location in the ribosome (Figure 14). Three mutations in the h8/h14 region (h8Δ3, h14Δ2, and G347U) directly destabilize bridge B8 (Figure 14A, D). Mutation G299A is located in h12, on the solvent side of the subunit near the S4/S5 interface (Figure 14B-C), and destabilizes bridge B8 allosterically [52]. All four of these mutations increase both missense and nonsense suppression in vivo [81]. Mutation G886A is located in h27 (Figure 14A), near the binding sites of several error-promoting antibiotics [134]. While conferring more modest effects on missense and
nonsense suppression [81], G886A was the only mutation to be additionally identified in a screen for translation initiation errors [82]. Four mutations (C1054U, C1054A, C1200U, and G1491A) are located in or near the A site (Figure 14E) and have variable effects on missense and nonsense suppression. With our reporters, C1054U acts as a nonsense (UGA) suppressor but exhibits a restrictive (hyperaccurate) phenotype for Glu-tRNA misincorporation [81]. C1200U and G1491A increase nonsense suppression to a greater degree than missense suppression, while C1054A strongly enhances both missense and nonsense suppression [81]. In the ribosome, C1054 sticks out of h34 and packs against nucleotide (nt) 34 of the anticodon of A-tRNA (Figure 14E). C1200 forms a base triple with A1055 and U1205 in h34 and forms a hydrogen bond with the phosphate of A1055. G1491 lies on the opposite side of the A site, next to A1492 and A1493 in h44 (Figure 14E).
Figure 14. Locations of ram mutations in 16S rRNA. Tertiary structure of the 16S rRNA, viewed from the subunit interface (A) and solvent (B) perspectives, and in the context of the 70S ribosome with bound ternary complex (C). Red spheres indicate positions of ram mutations. Small subunit proteins other than S4, S5, and S12 have been computationally removed for clarity. SHDR, shoulder domain; PF, platform domain. (D) Zoomed-in view of intersubunit bridge B8. Hydrogen bonds that are lost upon bridge disruption are shown as dashed lines. (E) Zoomed-in view of the 30S A site showing the interactions of various 16S rRNA residues with A-tRNA and mRNA. For clarity, h18 and S12 are omitted from the foreground of this view. Gray, 16S rRNA; tan, 23S/5S rRNA; magenta, unlabeled 50S proteins; other features, as indicated. Figure based on PDB files 2WRN, 2WRO, 2WDG, and 2WDI.
3.2.1 Effects of 16S ram mutations on initial selection

As mutations h8Δ3, h14Δ2, G347U, and G299A all cause defects in initial selection [52, 81], we tested whether the A-site ram mutations similarly affected EF-Tu-dependent GTP hydrolysis. Control and mutant 70S initiation complexes (70SICs), programmed with either a cognate UUU or near-cognate CUU codon in the A site, were rapidly mixed with EF-Tu•[γ-32P]GTP•Phe-tRNA^{Phe} under single-turnover conditions, and the reactions were quenched at various time points. Apparent rates obtained from individual time courses were plotted as a function of 70SIC concentration, and the data were fit to a hyperbolic function to determine the $k_{cat}$ and $K_M$ parameters (Table 5). Like previously-characterized ram mutations [52, 81], those in or near the A site increased $k_{cat}$ for both cognate and near-cognate reactions, without strongly impacting $K_M$ (Table 5). The effect on $k_{cat}$ was considerably larger in the near-cognate case, reducing the selectivity of the reaction substantially. C1054U appeared somewhat unique in that the mutation failed to increase $k_{cat}$ in the cognate case. Nevertheless, each 16S ram mutation tested thus far causes similar defects in initial selection (Table 5).
3.2.2 Effects of 16S ram mutations on RF2 function

Certain A-site mutations (C1054U, C1200U, G1491A) were found previously to confer a notably stronger phenotype for nonsense (UGA) suppression than missense suppression [81], raising the possibility that these mutations act largely by impairing RF2 function. To test this, we measured the rate of single-turnover RF2-catalyzed hydrolysis of Ac[^35S]Met-tRNA^{Met} bound to the P site of 70S ribosomes programmed with a UAA codon in the A site (Table 6), as described previously [135]. (The stop codon UAA was chosen for technical reasons—an mRNA with AUG UGA would create GUG in the +2 frame, which could potentially compete with AUG for Ac[^35S]Met-tRNA^{Met} pairing in the P site.) Mutations C1054U, C1200U and G1491A all increased $k_{cat}$ and $K_M$ modestly,
resulting in similar 2- to 3-fold decreases in $k_{\text{cat}}/K_M$. By contrast, mutation C1054A reduced $k_{\text{cat}}$ and increased $K_M$, resulting in a 9-fold drop in $k_{\text{cat}}/K_M$, consistent with earlier studies [112, 136]. While this substantial termination defect of C1054A probably contributes to the high-level UGA readthrough seen in vivo, C1054A also confers strong missense suppression, indicative of a large decoding defect [81]. The small effects of C1054U, C1200U, and G1491A on RF2-dependent termination do not readily explain the differential effects of these mutations on nonsense and missense suppression in the cell.

<table>
<thead>
<tr>
<th>Ribosome</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)$^a$</th>
<th>$k_{\text{cat}} / K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.14 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>7.0</td>
</tr>
<tr>
<td>C1054U</td>
<td>0.21 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>3.0</td>
</tr>
<tr>
<td>C1054A</td>
<td>0.08 ± 0.01</td>
<td>0.10 ± 0.03</td>
<td>0.8</td>
</tr>
<tr>
<td>C1200U</td>
<td>0.21 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td>2.6</td>
</tr>
<tr>
<td>G1491A</td>
<td>0.21 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>3.5</td>
</tr>
</tbody>
</table>

$a$ Calculated as described in Devaraj et al. 2010

Table 6. Kinetics of RF2-dependent peptide release for mutant ribosomes.

3.2.3 Effects of 16S rram mutations on proofreading

To determine which 16S mutations influenced proofreading, we measured the extent of GTP hydrolysis versus peptide bond formation in single-turnover decoding reactions (Table 7). EF-Tu•[γ$^{32}$P]GTP•Phe-tRNA$^{\text{Phe}}$ was mixed with 70SICs containing P-site [$^{35}$S]fMet-tRNA$^{\text{fMet}}$ and either codon UUU or CUU in the A site. After 30 s incubation at room temperature, the reaction was quenched, and the extent of both
[\gamma^{32}\text{P}]\text{GTP} \text{hydrolysis and} [^{35}\text{S}]\text{fMet-Phe formation was quantified. The ratio of peptide}
\text{bonds formed to GTP molecules hydrolyzed (dip/Pi) was then calculated (Figure 11).}
\text{This ratio is proportional to the probability of successful aa-tRNA accommodation after}
\text{GTP hydrolysis. Finally, the fidelity of the proofreading phase of decoding (F_p) was}
\text{estimated as} F_p = \frac{(\text{dip}/\text{Pi})_{\text{cognate}}}{(\text{dip}/\text{Pi})_{\text{near-cognate}}} \text{(Table 7), essentially as described [27].}

Figure 15. Effects of 16S \textit{ram} mutations on proofreading. Normalized ratio of dipeptides
\text{formed per GTP hydrolyzed by ternary complexes containing either Phe-tRNA (A) or}
\text{Tyr-tRNA (B) mixed with 70SICs programmed with either a cognate (white bars) or}
\text{near-cognate (black bars) A-site codon. This ratio is proportional to the probability of}
\text{successful accommodation of the aa-tRNA. The sequence of the codon used is indicated}
\text{above both graphs.}
<table>
<thead>
<tr>
<th>Ribosome</th>
<th>Phe-tRNA&lt;sub&gt;Phe&lt;/sub&gt;</th>
<th></th>
<th></th>
<th>Tyr-tRNA&lt;sub&gt;Tyr&lt;/sub&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.09 ± 0.00</td>
<td>11.0</td>
<td>1.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04 ± 0.01</td>
<td>25.0</td>
</tr>
<tr>
<td>G299A</td>
<td>0.95 ± 0.03</td>
<td>0.26 ± 0.01</td>
<td>3.7</td>
<td>0.74 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>2.6</td>
</tr>
<tr>
<td>h8Δ3bp</td>
<td>1.01 ± 0.03</td>
<td>0.30 ± 0.02</td>
<td>3.4</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>h14Δ2bp</td>
<td>0.85 ± 0.03</td>
<td>0.33 ± 0.02</td>
<td>2.6</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>G347U</td>
<td>1.00 ± 0.10</td>
<td>0.45 ± 0.04</td>
<td>2.2</td>
<td>0.90 ± 0.04</td>
<td>0.25 ± 0.03</td>
<td>3.6</td>
</tr>
<tr>
<td>G886A</td>
<td>0.83 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>4.4</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>C1054U</td>
<td>0.83 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>20.8</td>
<td>0.67 ± 0.06</td>
<td>0.19 ± 0.03</td>
<td>3.5</td>
</tr>
<tr>
<td>C1054A</td>
<td>0.92 ± 0.07</td>
<td>0.10 ± 0.01</td>
<td>9.2</td>
<td>0.78 ± 0.03</td>
<td>0.38 ± 0.01</td>
<td>2.1</td>
</tr>
<tr>
<td>C1200U</td>
<td>1.18 ± 0.29</td>
<td>0.32 ± 0.02</td>
<td>3.7</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>G1491A</td>
<td>0.88 ± 0.07</td>
<td>0.16 ± 0.02</td>
<td>5.5</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Ratio of cognate/near-cognate product formation (dip/Pi) as defined by Gromadski and Rodnina, 2004

<sup>b</sup> All other data normalized to this reference as described in Materials and Methods

nd Data not determined

Table 7. Effects of mutations on proofreading

Small variations in prepared TC made calculating absolute values of dip/Pi nontrivial, hence we chose to normalize all dip/Pi values to that of the cognate control reaction, which was included in every experiment. For each mutant ribosome programmed with a cognate codon, dip/Pi was near 1 (Figure 15, Table 7). Consistent with previous work [27], control ribosomes programmed with a CUU codon in the A site gave a dip/Pi value of 0.09, indicating that rejection of Phe-tRNA<sub>Phe</sub> (Phe-tRNA) at the proofreading stage is 11 times more likely in the presence of this first position codon-anticodon mismatch (F<sub>P</sub> = 11). Ribosomes with G299A, h8Δ3, h14Δ2, or G347U all displayed proofreading defects, with F<sub>P</sub> values decreased to 3.7, 3.4, 2.6, and 2.2, respectively. All of these mutations disrupt bridge B8 [52, 81], indicating that B8 regulates not only initial selection but also proofreading. In contrast, substitutions of A-
site residue C1054 showed no obvious defects in proofreading of Phe-tRNA. C1054A ribosomes gave an $F_P$ value similar to that of the control, while C1054U ribosomes gave an $F_P$ value of 21, indicating somewhat hyperaccurate proofreading. Mutation C1200U led to a proofreading defect, reducing $F_P$ to 3.7, similar to that of B8-disrupting mutations. Another A-site mutation, G1491A, conferred a smaller proofreading defect, indicated by an $F_P$ value of 5.5 (Table 7).

The unique behavior of C1054A and C1054U ribosomes prompted us to repeat the assay for another aa-tRNA, Tyr-tRNA$^{Tyr}$ (Tyr-tRNA), and ribosomes programmed with a cognate UAC or near-cognate UAG codon (Table 7). In this case, UAG-programmed control ribosomes rejected Tyr-tRNA 25 times more often than those programmed with the cognate UAC codon. Again, G299A and G347U ribosomes displayed proofreading defects, with $F_P$ values of 2.6 and 3.6, respectively. C1054A and C1054U ribosomes also displayed defects in proofreading of Tyr-tRNA, with respective $F_P$ values of 3.5 and 2.1, even though these ribosomes retained faithful proofreading in the Phe-tRNA case described above. Thus, substitutions of C1054 appear to differentially influence proofreading, depending on the particular tRNA and/or codon.

3.2.4 Effects of 16S ram mutations on miscoding in various contexts

The differences in proofreading by the C1054-substituted ribosomes raised the possibility that certain ram mutations promote only some types of errors. To investigate this, we measured apparent rates of miscoding with four different aa-tRNAs: Phe-tRNA$^{Phe}$, Tyr-tRNA$^{Tyr}$, Lys-tRNA$^{Lys}$, and Glu-tRNA$^{Glu}$. Various ribosome complexes
programmed with a near-cognate codon were mixed with each TC under conditions that facilitate rapid reassembly of TC [137], giving aa-tRNA multiple chances for misincorporation.

In control ribosomes, the apparent rate of miscoding varied dramatically depending on the nature of the tRNA and codon-anticodon mismatch (Figure 16, note scales of Y axes). First-position mismatches generally produced slower rates of misincorporation than third-position mismatches. However, there was one excursion from this pattern—misincorporation of Lys-tRNA^{Lys} (Lys-tRNA) occurred at higher frequency in response to GAA ($8.5 \pm 1.1 \times 10^{-3}$ s$^{-1}$) than to AAC ($2.9 \pm 0.4 \times 10^{-3}$ s$^{-1}$). Notably, the rate of misincorporation for Glu-tRNA^{Glu} (Glu-tRNA) at codon AAA (first-position mismatch) was more than 10,000-fold lower than at GAU (third-position mismatch) (Figure 17D, L), exemplifying the wide range of rates observed in this set of experiments.
Figure 16. Rates of misincorporation in control ribosomes differ depending on the aa-tRNA and mismatch. Control 70SICs (0.2 μM) were mixed with TC (2 μM) as described in Materials and Methods. Apparent rate of miscoding measured in each context is shown [± standard error of the mean (SEM)]. Each tRNA used is indicated along the X-axis and color coded for clarity. The codon programmed in the A site is also indicated on the X-axis beneath each bar.

Mutation G347U increased misincorporation to the greatest degree in almost all cases, from 3- to 38-fold, and the magnitude of its effects did not follow any discernible pattern with regard to first-position or third-position mismatches (Figure 17). Mutation G299A similarly increased misincorporation, in every case but one (Tyr-tRNA with the
codon CAC, Figure 17B), and showed no obvious bias between first and third position mismatches. These data suggest that G299A and G347U enhance miscoding in a general way.

<table>
<thead>
<tr>
<th>Phe-tRNA (3'-AAG-5')</th>
<th>Tyr-tRNA (3'-AUG-5')</th>
<th>Lys-tRNA (3'-Uummm-5')</th>
<th>Glu-tRNA (3'-CUuumm-5')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CUC</strong></td>
<td><strong>CAC</strong></td>
<td><strong>GAA</strong></td>
<td><strong>AAA</strong></td>
</tr>
<tr>
<td><strong>UUA</strong></td>
<td><strong>UAA</strong></td>
<td><strong>AAC</strong></td>
<td><strong>GAC</strong></td>
</tr>
<tr>
<td><strong>UUG</strong></td>
<td><strong>UAG</strong></td>
<td><strong>AAU</strong></td>
<td><strong>GAU</strong></td>
</tr>
</tbody>
</table>

Figure 17. Effects of *ram* mutations on miscoding in various contexts. Bars are color coded by mutation as indicated. The aa-tRNA used in each reaction is as follows (indicated at the top of each column): Phe-tRNA\(^{Phe}\) A, E, I; Tyr-tRNA\(^{Tyr}\) B, F, J; Lys-tRNA\(^{Lys}\) C, G, K; Glu-tRNA\(^{Glu}\) D, H, L. The codon programmed in the A site of each ribosome complex is indicated above each graph, with the mismatched base colored red. Each Y-axis indicates the apparent misincorporation rate and each bar is the average of \(\geq 3\) replicates with the exception of * AAU and GAU, which were measured once on a KinTek RQF-3. Error bars indicate \(\pm\) SEM or, for AAU and GAU, \(\pm\) fitting error.
Mutation C1054A, on the other hand, increased miscoding in a codon-anticodon-dependent manner (Figure 17). C1054A modestly increased misincorporation of first-position mismatched Phe-tRNA (4-fold), Lys-tRNA (4-fold), and Glu-tRNA (2-fold), and had no significant effect on first-position mismatched Tyr-tRNA (Figure 17A-D). However, C1054A dramatically increased misincorporation for several third-position mismatched tRNAs (Figure 17E-H). Interestingly, the magnitude of the C1054A effects correlate with the identity of the mismatched base pair at the third position. Phe-tRNA and Tyr-tRNA both form A-G mismatches with UUA- and UAA-programmed ribosomes, respectively, and C1054A increased misincorporation in these cases by 14- and 18-fold (Figure 17E-F). For Lys-tRNA with an AAC codon and Glu-tRNA with a GAC codon, both forming C-mnm5s2U mismatches, C1054A increased misincorporation by 27- and 54-fold (Figure 17G-H). These same two tRNAs create U-mnm5s2U wobble mismatches with AAU and GAU codons, and the observed increase was again similar but smaller, 2- and 5-fold (Figure 17K-L). Finally, for G-G wobble mismatches, C1054A had the smallest effects, decreasing misincorporation for Phe-tRNA on UUG by 2-fold and increasing misincorporation for Tyr-tRNA on UAG by 2-fold (Figure 17I-J).

Like C1054A, mutation C1200U influenced decoding in a context-dependent manner (Figure 17). C1200U modestly increased misreading for first-position mismatched aa-tRNA, and strongly increased misincorporation for certain third-position mismatched reactions, depending on the identity of the third-position mismatch. The contexts where C1200U had the greatest effects were G-G pairings for Phe-tRNA and Tyr-tRNA, (Figure 17I-J) followed by A-G pairings for the same two tRNAs (Figure
17E-F), then C-mnm$_5$s$_2$U (Figure 13G-H) followed by U-mnm$_5$s$_2$U pairings for Lys-tRNA and Glu-tRNA (Figure 17K-L). This pattern of G-G > A-G > C-mnm$_5$s$_2$U > U-mnm$_5$s$_2$U is clearly distinct from the C-mnm$_5$s$_2$U > A-G > U-mnm$_5$s$_2$U > G-G pattern seen with C1054A.

Mutations G886A and C1054U increased misincorporation in several cases, although the effects were small (Figure 17). Mutation G1491A had little or no effect on misincorporation, at least for the contexts tested in Figure 17.

3.3 Discussion

In this study, we provide evidence that _ram_ mutations in 16S rRNA fall into two functional classes—those that generally increase miscoding and those that influence decoding in codon-anticodon-dependent manner. Mutations in the former group include G299A and G347U, which lie away from the A site and are known to disrupt bridge B8 [52, 81]. These mutations consistently increase misreading, regardless of context. Mutations in the latter group include C1054A and C1200U, which are predicted to alter the structure of the 30S A site. These mutations cause high-level misreading in a manner that depends on the tRNA and/or nature of the mismatch.

C1054 contributes to the 30S A site, packing against nt 34 of the anticodon of A-tRNA. This interaction is seen whether tRNA occupies the A/T site or A/A site, suggesting that the contact is maintained throughout most of the decoding process [32, 50, 53, 89]. The base of C1200 is oriented to form a hydrogen bond with the 5’-phosphate of A1055, stabilizing the position of C1054. Hence, mutations C1054A and
C1200U are predicted to alter ribosomal contacts to nt 34 of A-tRNA, directly or indirectly. We propose that these mutations allow spurious favorable interactions to form, which stabilize near-cognate aa-tRNA in certain contexts. Because base-stacking interactions involving purines are generally more stable than those involving pyrimidines [138], an introduced purine at position 1054 may promote base stacking with nt 34 of A-tRNA, particularly for certain mismatched or non-Watson-Crick base pairs. Precedent for this idea comes from observed stacking between C1054 and base 34 of A-tRNA in crystal structures of ribosomes containing the ASL of tRNA^{\text{Arg}}_{\text{ICG}} [38]. When ASL^{\text{Arg}}_{\text{ICG}} is bound to a CGC codon, forming a Watson-Crick cytosine-inosine (C-I) bp at the wobble position, C1054 packs normally against the ribose of I34. However, when ASL^{\text{Arg}}_{\text{ICG}} is bound to a CGA codon, the purine-purine A-I pair widens the codon-anticodon helix at the third position, and C1054 is reoriented to stack with the inosine base. In an analogous way, the geometry of an A-G mismatch at third position may allow for favorable base stacking between G34 of Phe-tRNA or Tyr-tRNA and A1054, explaining the high misreading rate for C1054A ribosomes in such contexts. Mutations C1054A and C1054G confer much stronger miscoding phenotypes in vivo than C1054U [81], as one would expect if spurious stacking of 16S rRNA base 1054 and tRNA base 34 is responsible for the higher rates of miscoding. We had hoped to include C1054G in this study but were unable to generate the corresponding E. coli Δ7 prrn strain, perhaps because the decoding defects conferred by the mutation are too large [81].

In both bacteria and yeast, substitutions of C1054 cause variable suppression phenotypes, depending on the particular reporter constructs employed [81, 113, 116, 117,
The basis of this “phenotypic heterogeneity” has been a long-standing puzzle in the field. Here, we show that the effects on decoding of C1054A (and to a lesser degree C1054U) vary depending on the codon and anticodon involved. This helps to explain the complex suppression patterns conferred by mutations of C1054 in the cell.

Nucleotide G1491 is immediately adjacent to the universally conserved A1492 and A1493, which dock into the minor groove of the codon-anticodon helix. While G1491 itself is not highly conserved [143], mutation G1491A leads to high-level nonsense suppression, with a considerably smaller effect on missense suppression [81]. High UGA read-through does not seem to be due to a termination defect, as G1491A ribosomes are not particularly compromised in RF2 function (Table 6). G1491A does have significant effects on initial selection (Table 5), and to a lesser extent proofreading (Figure 15; Table 7), for Phe-tRNA. Puzzlingly, though, G1491A has little or no effect on the overall rate of misincorporation for any of the 10 contexts tested (Figure 17A-J), even in the case of Phe-tRNA misreading CUC (Figure 17A). We propose that G1491A strongly enhances miscoding only in certain contexts (e.g., Trp-tRNA misreading of UGA, Phe-tRNA misreading of CUU), none of which were part of the Figure 17 dataset. Consistent with this suggestion, a wider preliminary screen of contexts revealed that misreading of AAC by Tyr-tRNA is substantially increased (> 20-fold) in G1491A ribosomes. Further work will be needed to elucidate the effects of G1491A on decoding.

There is considerable interest in methods to incorporate unnatural amino acids into proteins. Among the most successful of these are translation systems that employ orthologous aa-tRNA / aa-tRNA synthetase pairs in combination with nonsense and/or
frameshift suppression [144]. Our finding that the A-site *ram* mutations impact decoding in a codon-anticodon-dependent fashion raises the possibility that the corresponding mutant ribosomes might prove useful in these protein engineering systems. For example, a particular A-site *ram* mutation could substantially increase the efficiency of orthologous aa-tRNA incorporation events without causing a problematic reduction in overall translation fidelity. In line with this idea, selection experiments for 16S rRNA mutations that specifically enhance quadruplet decoding by Ser-tRNA$_{UCU}$ identified mutations of nucleotides 1195-1197 [145], and A1196 normally contacts C1054 [89].

In control ribosomes, apparent rates of miscoding by Phe-tRNA, Tyr-tRNA, Lys-tRNA, and Glu-tRNA in response to a number of near-cognate codons varied over a wide range, four orders of magnitude (Figure 17). With one exception (see below), the frequency of misincorporation was higher for third- than first-position mismatches. These data are in line with more comprehensive analyses of Lys-tRNA miscoding *in vitro* and *in vivo* [13, 137], and argue against the idea that kinetic effects of single mismatches are similar regardless of their position [120]. The exceptional case in our dataset is that of Lys-tRNA, which seems to misread GAA at higher frequency than AAC, in contrast to previous reports [13, 137]. In retrospect, we noticed that the nucleotide following GAA in the mRNA for the experiment of Figure 17C is G. Hence it is possible that the higher frequency of incorporation in this case stems from a +1 frameshift of fMet-tRNA$_{fMet}$ (to pair with UGG, one base different from the cognate start codon UUG) and presentation of the cognate Lys codon AAG in the A site. Consistent with this possibility, earlier toeprinting experiments show that ribosome complexes with P-site tRNA$_{fMet}$ paired to
m291 (codons 1-2: AUG UUU) give a toeprint at position +16, whereas those with tRNA\textsuperscript{Met} paired to m292 (codons 1-2: AUG GUA) give toeprints at +16 and +17 [146]. Importantly, the experiment of Figure 17C is the only one in which this putative +1 frameshift event would present a cognate codon in the A site. Thus, we are confident that the measured incorporation rates for the other 11 contexts reflect misreading rates. Worth noting here is that, in our dataset, misreading of GAU by Glu-tRNA and of AAU by Lys-tRNA occurred at highest frequencies (Figure 17K-L), and the corresponding missense mutations are among the few such mutations that are clearly suppressible in the cell [13, 81].

In the proofreading stage of decoding, aa-tRNA is either accommodated into the A/A site or rejected from the ribosome. Cognate codon-anticodon pairing speeds accommodation and slows rejection [21, 28], greatly increasing the probability of productive aa-tRNA incorporation. While molecular dynamics simulations provide plausible paths for aa-tRNA transit from A/T to A/A sites [60], how the ribosome impacts these tRNA movements that ultimately determine the selectivity of proofreading remains unclear. In this study, we find that mutations that disrupt bridge B8 (G299A, h8\Delta3, h14\Delta2, and G347U) reduce the stringency of not only initial selection but also proofreading. The proofreading defects of these mutant ribosomes, indicated by substantially higher levels of dipeptide formation in the near-cognate case, must stem from an increased rate of accommodation and/or a decreased rate of rejection. How might B8 influence proofreading? One possibility is that B8 affects proofreading indirectly by promoting EF-Tu\textbulletGDP dissociation. As discussed above, formation of the GTPase-
activated state (with A/T aa-tRNA) involves disruption of B8. After GTP hydrolysis, EF-Tu undergoes a conformational change and dissociates from the ribosome. Reformation of B8 during these latter steps of decoding may facilitate release of EF-Tu•GDP. If so, mutations that disrupt B8 would be predicted to slow EF-Tu•GDP dissociation. As bound EF-Tu would present a steric obstacle to aa-tRNA release [50, 53], a slower rate of EF-Tu•GDP release should effectively shunt aa-tRNA toward the productive pathway. Another possibility is that B8 allosterically destabilizes aa-tRNA and thereby increases the stringency of proofreading. Mutations that disrupt B8, then, would be predicted to stabilize aa-tRNA during the A/T-to-A/A transition and hence increase the probability of successful accommodation. Mutation G299A (in h12) allosterically disrupts B8 [52], so there is precedent for the idea that B8 is conformationally linked to distal ribosomal sites. Clearly, further experiments are needed to test these hypotheses and elucidate the molecular mechanism through which B8 tunes proofreading.

Ram mutations away from the A site cluster largely to two regions— bridge B8 and the h12/S4/S5 region [81, 86, 99, 104, 132, 133]. The recent finding that mutation G299A in h12 allosterically disrupts B8 raises the question of whether other ram mutations in the h12/S4/S5 region act in a similar way [52]. Zaher and Green (2010) studied the effects of mutation rpsD12 on decoding in some detail [147]. This is a frameshift mutation that causes a C-terminal truncation of S4, effectively removing numerous contacts at the S4-S5 interface. They found that ribosomes harboring this S4 truncation exhibit defects in initial selection but not in proofreading, suggesting a functional distinction between rpsD12 and G299A. One caveat here, though, is that Zaher
and Green analyzed Leu-tRNA misreading of UUC as the near-cognate case, whereas we have used Phe-tRNA misreading of CUU. Clearly, it will be worthwhile to directly compare G299A, *tpsD12*, and other mutations in the h12/S4/S5 region to determine if and how their modes of action differ.

### 3.4 Materials and Methods

#### 3.4.1 Reagents

All experiments were done in polymix buffer [146, 148], unless otherwise noted. Purified *E. coli* MRE600 tRNAs (tRNA\(^{\text{Met}}\), tRNA\(^{\text{Met}}\), tRNA\(^{\text{Phe}}\), tRNA\(^{\text{Tyr}}\), tRNA\(^{\text{Lys}}\), and tRNA\(^{\text{Glu}}\)) were purchased from Chemical Block (Moscow, Russia), and aminoacylated as described [149]. For initial selection and proofreading experiments, mRNA was transcribed *in vitro* from pGENE32-based plasmids and gel purified as described [150]. The mRNAs used for proofreading experiments have the sequence 5’- (N)\(_4\) AAG GAA AUA AAA AUG NNN GUA UAC AAA UCU (N)\(_{67}\) -3’, where NNN corresponds to UUU, CUU, UAC, or UAG. Messages for the miscoding experiments of Figure 16 and Figure 17 were synthesized by Sigma-Aldrich and have the sequence 5’-AAG GAA AUA AAA AUG NNN GUA UAC AAA UCU-3’, where NNN is the indicated A site codon. Ribosomes, translation factors, and phenylalanyl-tRNA synthetase were purified as described [81]. Tyrosyl-, lysyl-, and glutamyl-tRNA synthetases were purified with Talon Cobalt Affinity Resin (Clontech) from overexpression strains JW1629, JW2858, and JW2395, obtained from the ASKA collection (National BioResource Project - *E. coli*).
at National Institute of Genetics, Japan). Pyruvate kinase and myokinase were purchased from Sigma-Aldrich.

3.4.2 Initial selection and RF2 release assay

Initial selection and RF2 dependent peptide release experiments were carried out as described [52, 81, 135].

3.4.3 Proofreading assay

70SICs were prepared by incubating 70S ribosomes (1 µM), IF1 (2 µM), IF2 (1 µM), IF3 (2 µM), mRNA (4 µM), [35S]-fMet-tRNA\(^{\text{fMet}}\) (1 µM) and GTP (1 mM) in polymix buffer at 37°C for 1 hour. Complexes were then purified by ultracentrifugation over 1.2 mL 1.1M sucrose cushions made with polymix buffer and 15 mM MgCl\(_2\) in an SW50.2 rotor (Beckman) at 36,600 rpm for 2.5 hours. Pelleted complexes were dissolved in polymix buffer, flash frozen, and stored at -70°C. Ternary complex (TC) was made by incubating EF-Tu (10 µM), aa-tRNA (10 µM), phosphoenolpyruvate (PEP, 3 mM), pyruvate kinase (50 µg/mL), and [\(^{32}\)P]-GTP (50 µM) in polymix buffer at 37°C for 15 minutes. Following incubation, TC was passed through P30 spin columns (Bio-Rad; pre-equilibrated in polymix) to remove unbound GTP and PEP, and then diluted to 0.5 µM. 70SICs were thawed and diluted in polymix to 2 µM. Equal volumes (10 µL) of 70SIC (2 µM) and TC (0.5 µM) were mixed and incubated at 20°C for 30 s (Phe experiment) or 60 s (Tyr experiment), at which point both GTP hydrolysis and peptide bond formation had reached plateaus. Reactions were quenched with 10 µL of 40% formic acid, and
centrifuged at 20,000 × g for 15 minutes to pellet tRNA. The extent of GTP hydrolysis was determined using TLC, by spotting the supernatant on PEI-cellulose TLC plates and developing with 500 mM potassium phosphate, pH 3.5. The extent of peptide bond formation was determined using electrophoretic TLC [151], after washing the pellets with 15% formic acid, dissolving in 500 mM KOH and spotting products on cellulose TLC plates. This method enables clear separation of unreacted formyl-methionine and the various dipeptides. Each experiment was done in parallel with control ribosomes, and the ratio of GTP hydrolyzed per peptide bond formed was normalized to the cognate control reaction. Experiments were performed at least three independent times.

3.4.4 Misincorporation experiments

For the experiments of Figure 16 and Figure 17, 70SICs were prepared by incubating 70S ribosomes (0.2 µM), IF1 (0.4 µM), IF2 (0.4 µM), IF3 (0.4 µM), mRNA (1 µM), [³⁵S]-fMet-tRNA⁰⁰Met (0.2 µM), and 1X energy mix (1 mM ATP, 1 mM GTP, 10 mM PEP, 50 µg/mL pyruvate kinase, 5 µg/mL myokinase) in polymix buffer at 37˚C for 15 minutes. TC was similarly prepared in polymix by incubating EF-Tu (3 µM), EF-Ts (3 µM), tRNA (2 µM), aa-tRNA synthetase (1.5 µM), amino acid (200 µM), and 1X energy mix. Because ATP, GTP, and PEP are Mg²⁺ chelators, buffer for the 70SIC and TC preparations was supplemented with 6.5 mM additional Mg(OAc)₂ to adjust the free Mg²⁺ concentration to approximately 5 mM [137]. Equal volumes (25 µL) of 70SIC and TC were mixed and incubated at 20˚C. At various time points, 4 µL of the reaction was removed and quenched with an equal volume of 500 mM KOH. Reactions were spotted
on cellulose TLC plates subjected to electrophoretic TLC analysis. Dipeptide formation was plotted versus time, and the data were fit to a single-exponential equation to obtain apparent rates.
4.1 Effects of mutations and antibiotics on codon-independent GTP hydrolysis

4.1.1 Introduction

During decoding, interactions between the codon being translated and the aa-tRNA anticodon precede GTP hydrolysis by EF-Tu [22]. Cognate codon-anticodon interaction increases the rate of GTP hydrolysis, as do near-cognate interactions to a lesser degree [21]. It is thought that codon recognition in the A site must “signal” the GTPase active site of EF-Tu to lead to this effect, although the mechanism through which this occurs is unclear [90] [35].

It is known that certain antibiotics are able to induce changes in the ribosome’s structure that mimic those that occur following codon recognition. Paromomycin induces the universally conserved A1492 and A1493, which form A-minor interactions with the cognate codon-anticodon helix, to flip out of h44 in the absence of A-site tRNA [32]. Biochemical studies have shown that these two nucleotides are critical for the GTPase activation [84]. A related aminoglycoside antibiotic, streptomycin, also binds to h44 and promotes A1492 and A1493 docking with near-cognate anticodons [85]. We have recently obtained evidence that some of the mutations isolated in Chapter 2 lead to
structural changes in bridge B8 similar to ones induced by ternary complex binding, although these structures were solved in the presence of A-site tRNA anticodon-stem-loop structures [52]. So, is it possible that antibiotics or mutations can promote GTP hydrolysis in the absence of codon-anticodon recognition?

4.1.2 Results

In order to determine how ribosome mutants affect ternary complex in the absence of codon-anticodon interactions, we measured the rate of EF-Tu-dependent GTP hydrolysis stimulated by 70S ribosomes lacking mRNA, P-site tRNA or other ligands. Time courses of hydrolysis under these conditions fit to double exponential equations (Figure 18A). The slow phase, representing about 50% of the amplitude was about $5 \times 10^{-4}$ s$^{-1}$ and showed little dependence on 70S concentration, consistent with this being GTP hydrolysis by EF-Tu that is not stimulated by ribosomes. The fast phase was dependent on 70S concentration, and was inhibited by the A-site-blocking antibiotic tetracycline.
Figure 18. EF-Tu dependent GTP hydrolysis stimulated by unprogrammed ribosomes. 
(A) Representative time courses of GTP hydrolysis with control ribosomes with and without tetracycline (Tet). (B) Apparent rates for bridge B8 disrupting mutations plotted versus 70S concentration. (C) Apparent rates for A-site mutant ribosomes plotted versus 70S concentration.

Using this assay, we compared the effects of various mutant ribosomes on the fast, 70S-dependent, rate of GTP hydrolysis. For control ribosomes, secondary rate plots fit well to a Michaelis-Menten equation, with $k_{\text{cat}}$ of $5.5 \times 10^{-2}$ s$^{-1}$ and $K_M$ of 7 µM (Table 8). Compared to the same ribosomes programmed with a cognate or near-cognate message, the catalytic efficiency, $k_{\text{cat}}/K_M$, is significantly slower by ~5000- and ~150-fold, respectively [81]. Secondary rate plots for mutations G299A, G347U, Δh8, and Δh14 all fit to linear plots, consistent with large increase in both $k_{\text{cat}}$ and $K_M$ (Figure 18B). The slope of these linear plots estimates the $k_{\text{cat}}/K_M$ of the reaction (Table 8). For all these mutations this parameter was greater than the control, with relative increases of 3.6-, 1.2-, 3.5-, and 1.5-fold for G299A, G347U, Δh8, and Δh14, respectively (Table 8). Mutations in the h34, C1054A and C1200U behaved in a distinct manner. Secondary rate
plots for these mutations fit to Michaelis-Menten parameters (Figure 18C). Both displayed an increase in $k_{\text{cat}}$ and C1054A resulted in a ~3-fold increase in $K_M$ whereas for C1200U, $K_M$ was slightly lower (Table 8).

<table>
<thead>
<tr>
<th>Ribosome</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{\text{cat}}/K_M$ (s$^{-1}$×µM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>0.055</td>
<td>7.3</td>
<td>7.5×10$^{-3}$</td>
</tr>
<tr>
<td>G299A</td>
<td>-</td>
<td>-</td>
<td>27×10$^{-3}$A</td>
</tr>
<tr>
<td>G347U</td>
<td>-</td>
<td>-</td>
<td>9.0×10$^{-3}$A</td>
</tr>
<tr>
<td>Δh8</td>
<td>-</td>
<td>-</td>
<td>26×10$^{-3}$A</td>
</tr>
<tr>
<td>Δh14</td>
<td>-</td>
<td>-</td>
<td>11×10$^{-3}$A</td>
</tr>
<tr>
<td>C1054A</td>
<td>0.20</td>
<td>23.5</td>
<td>8.4×10$^{-3}$</td>
</tr>
<tr>
<td>C1200U</td>
<td>0.14</td>
<td>5.0</td>
<td>28×10$^{-3}$</td>
</tr>
<tr>
<td>C +Par</td>
<td>0.44</td>
<td>6.2</td>
<td>72×10$^{-3}$</td>
</tr>
<tr>
<td>C +Str</td>
<td>0.24</td>
<td>7.8</td>
<td>31×10$^{-3}$</td>
</tr>
<tr>
<td>C +Str +Par</td>
<td>-</td>
<td>-</td>
<td>47×10$^{-3}$A</td>
</tr>
</tbody>
</table>

A: calculated from linear fit

Table 8. Kinetics of EF-Tu dependent GTP hydrolysis with unprogrammed ribosomes

Relative to ribosome mutations, the structural and functional effects of error-promoting antibiotics have been more thoroughly studied [134, 152]. Therefore, we repeated the assay with control ribosomes in the presence of the well-studied aminoglycosides paromomycin and streptomycin (Figure 19A). Both antibiotics alone greatly increased $k_{\text{cat}}$ with little affect on $K_M$ (Table 8). However, when reactions were performed in the presence of both drugs, both $k_{\text{cat}}$ and $K_M$ increased dramatically and secondary plots could only be fit to linear lines. This synergistic effect was surprising, as previous studies of the two antibiotics with programmed ribosomes, showed that although
the two drugs can bind the ribosome simultaneously, the effects of streptomycin on GTP hydrolysis were dominant to those of paromomycin [152].

Figure 19. Effects of antibiotics streptomycin (Str, 20 µM) and paromomycin (Par 50 µM) on EF-Tu-dependent GTP hydrolysis in the presence of unprogrammed ribosomes (A) or 70SICs programmed with cognate (UUU) or near-cognate (CUU) codons.

The effects of streptomycin on decoding have been somewhat controversial. Early studies with the drug showed that for Phe-tRNA_{Phe} ternary complexes in poly-(U) synthesis assays, streptomycin had no little effect on the rate of EF-Tu-dependent GTP hydrolysis, but slowed peptide bond formation [96]. However, later studies showed that the antibiotic stimulated GTP hydrolysis for near-cognate Leu-tRNA_{Leu} in poly-(U) synthesis, but for cognate Phe-tRNA_{Phe} it reduced the rate to one nearly identical to the near-cognate case [152]. To determine the effects of streptomycin on GTP hydrolysis in
our hands, we measured the rate of EF-Tu-dependent GTP hydrolysis for \( \text{Phe-tRNA}^\text{Phe} \) ternary complexes with 70SICs programmed with either a cognate UUU A-site codon, or a near-cognate CUU codon (Figure 19B). In this assay, the rate of GTP hydrolysis in the absence of streptomycin is very fast for cognate complexes, about 52s\(^{-1}\), and less so for near-cognate, about 0.1s\(^{-1}\). In the presence of 20 µM streptomycin, the rate of GTP hydrolysis for near-cognate complexes appears to be stimulated \( >30\)-fold to 3.4 s\(^{-1}\), but the cognate rate appears to be nearly identical to ribosomes alone, about 45s\(^{-1}\).

4.1.3 Discussion

During decoding, GTP hydrolysis is typically preceded by conformational changes in the ribosome and the EF-Tu active site that occur in response to codon-anticodon interactions [22] [35]. By measuring the rate of GTP hydrolysis in the absence of an A-site codon, we are presumably measuring the ability of the ribosome to activate EF-Tu in the absence of those important A-site interactions. Because this situation is distinct from decoding during translation, it is difficult to interpret the results mechanistically. However, several interesting insights are apparent from the data.

Similar to findings in Chapter 3, mutations that result in bridge B8 disruption and mutations in h34 display distinct behavior in stimulating GTP hydrolysis in the absences of message. Bridge B8 disrupting mutations led to large increases in both \( k_{\text{cat}} \) and \( K_M \) for the reaction relative to control ribosomes. This supports earlier observations that these mutations increase the ribosomes intrinsic ability to promote GTP hydrolysis by EF-Tu [81] [52]. For G299A ribosomes, it also indicates that the allosteric disruption
of bridge B8 can be achieved in the absence of codon-anticodon interactions [52]. Mutations in h34 increase GTP hydrolysis rates, but do not have large effects on the $K_M$ of the reaction. This is consistent with these mutations stabilizing interactions with ternary complex, likely through altering contacts with the anticodon. It also provides further insight into the results presented in Chapter 3 by indicating that changes in h34 can result in stabilizing effects on the aa-tRNA anticodon without codon-anticodon pairing in the third position (Chapter 3). It would be interesting to repeat this assay with a wide range of aa-tRNAs, as was done in Chapter 3, in order to better discern the nature of this effect.

The effects of the antibiotics streptomycin and paromomycin on this reaction are also interesting in light of previous findings. By themselves, both antibiotics appear to act in a manner similar to the two h34 mutations. However, crystal structures of both these antibiotics indicate that they primarily affect the structure of the A site closer to the 3′ end of the anticodon than to h34. The idea that these antibiotics are acting in a way similar to h34 mutations rather than the bridge B8 disrupting mutations may be significant in light of recent controversy over the mechanism of action of paromomycin. Crystal structures of the drug bound to the 30S subunit alone indicated that the antibiotic acted by inducing “domain closure” of the subunit, stabilizing interactions between EF-Tu and the ribosome's shoulder domain [35]. However, more recent structures of the antibiotic in the context of the 70S ribosome suggested that it may in fact act through inducing stabilizing contacts between the aa-tRNA and H69 [44]. Our laboratory has recently demonstrated that the important contacts for EF-Tu activation can be induced without shoulder domain
rearrangement, in contrast to the former theory [52]. The results here would suggest that the drug is acting similarly to h34 mutations, which stabilize specific contacts with the aa-tRNA, in line with the later proposal for the drug’s mechanism of action.

Crystal structures demonstrate that both of paromomycin and streptomycin can bind the 30S subunit simultaneously [134]. When we performed the assay in the presence of both drugs, the pattern of GTP hydrolysis changed from being h34-like to B8-disrupting-like (Figure 18B-C; Figure 19A). This result is surprising because previous results had indicated that in the presence of both antibiotics, the decoding process behaves the same as with streptomycin alone [152]. This contradictory finding led us to test the effects of the antibiotic in our hands. Specifically, Gromdski and Rodnina found that in the presence of streptomycin the rate of EF-Tu-dependent GTP hydrolysis is identical for both cognate and near-cognate ternary complexes, while Bilgin, et al. found the kinetics of cognate GTP hydrolysis to be unaffected by the drug. In contrast to Gromadski, and consistent with Bilgin, we found that streptomycin increased the rate of EF-Tu-dependent GTP hydrolysis for the near-cognate situation only (Figure 19B). The cause of the discrepancy is unclear, however it is worth noting that many aminoglycoside antibiotics are known to have multiple binding sites that modulate their effects [153].

While we performed our assay with identical concentrations of streptomycin as reported by Gromadski and Rodnina, differences in buffer conditions may preferentially stabilize second-site binding in one case or the other, leading to the divergent observations. Whatever the case, this observation may warrant further investigation.
4.1.4 Materials and Methods

Message-independent GTP hydrolysis assays were performed by incubating equal volumes of excess 70S ribosomes and ternary complex (0.2 µM, prepared as in Chapter 2) at 22˚C in polymix buffer. At various time points, aliquots of the reaction were removed and quenched with 1/3 volume 40% formic acid. For reactions with antibiotics, control 70S ribosomes were incubated with 100 µM paromomycin and/or 40 µM streptomycin before addition of ternary complex mixture (final concentrations: Par, 50 µM; Str, 20 µM). Programmed GTP hydrolysis assays were performed as in Chapter 2 using an RQF-3 rapid quench apparatus. Again, for indicated reactions 40 µM streptomycin was added to the 70SIC preparation prior to mixing with ternary complex. Reactions were spotted on PEI cellulose TLCs, resolved, and quantified as in Chapter 2. Data was fit and analyzed as described in text.

4.2 Effects of mRNA context on errors and accuracy

4.2.1 Introduction

Accuracy in enzymatic reactions is typically defined as the efficiency of incorporation for the cognate substrate divided by that of a competing near-cognate substrate \([\frac{(k_{\text{cat}}/K_M)^C}{(k_{\text{cat}}/K_M)^{\text{NC}}}]\) [154]. For the ribosome, this has typically been measured by analyzing non-competitive reactions comparing the kinetic properties of a cognate aa-tRNA and another aa-tRNA with a single anticodon-base mismatch to the codon [21, 27, 95, 155]. But, as it is for many reactions in vivo, the actual error rate is determined by a multitude of factors, including cellular concentration of competing
ternary complexes, and the specific rates those ternary complexes are able to react with the ribosome [13, 154, 156].

Methods to analyze errors made \textit{in vivo} are restricted by the need for a given misincorporation to lead to a measurable phenotype (see Chapter 1). Nevertheless, there is increasing evidence that translation errors are not uniform across the gene [13, 14]. For example, we have shown in Chapter 3 that different aa-tRNAs vary dramatically in their rates of misincorporation at codons containing a single basepair mismatch. Additionally, many groups have observed affects of gene context on error rates, and there is structural data suggesting that some tRNAs may be able to form unexpected basepairs with some codons [44, 46]. A comprehensive understanding of errors made during translation will require analysis of rates and errors by every aa-tRNA at every codon. While we have done nothing so ambitious, here we present several experiments that demonstrate unexpected differences in misreading events at various codons and that the structure of the mRNA outside the codon being read can influence decoding.

4.2.2 Results

To determine what aa-tRNAs are misincorporated at various codons, we designed a series of short RNA oligo messages, based on the sequence of mSM1-CUU shown in Table 9. These oligos were designed to promote ribosome binding by using the optimal Shine-Dalgarno sequence and spacing as determined by Devaraj, et al. [135]. The sequence of each oligo was identical, apart from the codon positioned in the A site. These messages assembled into 70SICs that promoted peptide bond formation at similar levels
to T7 transcribed messages (data not shown). We then made [35S]-fMet-tRNAfMet-70SICs containing one of eight different messages, and incubated these complexes (1.0 µM) with EF-Tu (20 µM), EF-Ts (1 µM), GTP (1 mM), and total tRNA extracts (5 ng/µL) charged with S100 extract and all 20 amino acids for 5 minutes at 22°C in the presence or absence of 20 µM streptomycin. The resulting dipeptide products were separated on cellulose TLCs by electrophoresis as in Chapter 3, and quantified using ImageQuant (Figure 20).

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>m291-CUU</td>
<td>5'–...AAAAAGGAAAUAAGCUUGUAUACAAA...3'</td>
</tr>
<tr>
<td>mSM1-CUU</td>
<td>5'–AAGGAGGUAAAAAUGCUUAAA–3'</td>
</tr>
<tr>
<td>mSM2-CUU</td>
<td>5'–AAAGGAAAUAAAAAUGCUUGUA–3'</td>
</tr>
<tr>
<td>mSM6-CUU</td>
<td>5'–AAAGGAAAUAAAAAUGCUUGUAUAC–3'</td>
</tr>
</tbody>
</table>

Table 9. Sequences of mRNAs used in experiments described in this subchapter. Underlined sequence is complimentary to the 3’ end of the 16S rRNA (ASD). Sequence in bold corresponds to the start codon. Sequence in red corresponds to codon positioned in the A site following initiation.

In the absence of antibiotic, the dominant spot in all cases corresponds to the expected location of product formed by the cognate aa-tRNA, although spots are visible for other dipeptide products. With the addition of 20 µM streptomycin, the relative fraction of these non-specified products increases significantly, and several additional spots appear. We have not tested the migration of every dipeptide products for this assay in our laboratory, but spots were tentatively identified based on migration patterns demonstrated by Merryman, et al. [151]. In several cases, the migration pattern did not
correspond to a product formed by any aa-tRNA containing only a single anticodon mismatch with the programmed codon.
Figure 20. Dipeptide formation for mSM1 programmed 70SICs reacted with charged total tRNA extracts as described. The large spot at the extreme left of the figure is unreacted formylmethionine, which was may not have been incorporated into active 70SICs.
We also tested these messages for their ability to promote GTP hydrolysis by ternary complexes made with five aminoacyl-tRNAs, Phe-tRNA\textsuperscript{Phe}, Val-tRNA\textsuperscript{Val}, Tyr-tRNA\textsuperscript{Tyr}, Glu-tRNA\textsuperscript{Glu}, and Lys-tRNA\textsuperscript{Lys}. Each ternary complex (0.2 µM) was incubated with 70SICs (1.0 µM) programmed with either a cognate codon or a codon containing either a first- or third-position mismatch with the anticodon, for 30 seconds at 22°C. The reactions were quenched with formic acid (40%) and the extent of GTP hydrolysis was measured as in Chapter 2 (Figure 21).
Figure 21. Extent of GTP hydrolyzed by ternary complexes with five aa-tRNAs reacted with 70SICs programmed with cognate or near-cognate codons. The programmed codon is displayed 5’-3’ from top under each bar. The “---” lane represents control reactions without message. GTP hydrolysis activity was tentatively identified as product formation above average background.

The results in Figure 21 show that these five aa-tRNAs have unique patterns of promoting GTP hydrolysis on near-cognate codons. For example, Tyr-tRNA$^{\text{Tyr}}$ showed partial activity on several near-cognate codons, while Val-tRNA$^{\text{Val}}$ was unable to promote GTP hydrolysis on any near-cognate codon tested. These results are generally
consistent with differences in near-cognate decoding demonstrated in Chapter 3, including the observation that Lys-\text{tRNA}^{\text{Lys}} \text{stimulates GTP hydrolysis for GAA codons, but Glu-\text{tRNA}^{\text{Glu}} does not for AAA codons. However, our results have a puzzling caveat that some reactions, for example Phe-\text{tRNA}^{\text{Phe}} \text{with CUU, failed to reach plateaus after the incubation period even though previous experiments had indicated the reaction should have reached completion (see Chapter 2).}

Differences between the synthetic mRNA used in these experiments and the T7 run-off transcript used in the previous chapter could account for these differences. To investigate this discrepancy further, we designed a series of synthetic messages to recreate features that differ between the mSM messages and the previously used m291 (Table 9). We tested 70SICs made with these messages in EF-Tu-dependent GTP hydrolysis and peptide bond formation with a near-cognate Phe-\text{tRNA}^{\text{Phe}} (Figure 22A). The rate of EF-Tu dependent GTP hydrolysis for the T7 transcribed m291-CUU is $0.42 \text{ s}^{-1}$. The rate of GTP hydrolysis for mSM1 is severally reduced >20-fold, to $0.02 \text{ s}^{-1}$ at least partially explaining its failure to reproduce previous experiments (Figure 22B). A message restoring SD pairing and spacing to those found in m291, mSM2-CUU slightly restored this rate to $0.07 \text{ s}^{-1}$, a 6-fold defect from m291-CUU (Figure 22B). However, a construct increasing the message length just 3 nucleotides, mSM6-CUU fully rescued the rate to $0.49 \text{ s}^{-1}$ (Figure 22B).
Consistent with these results, both m291-CUU and mSM6-CUU displayed low levels of peptide bond formation, with similar rates to GTP hydrolysis (Figure 22C). This is consistent with peptide bond formation being rate limited by GTP hydrolysis for these near-cognate messages [27]. No dipeptide formation was apparent with either mSM1 or mSM2, indicating that in these cases peptide bond formation is either much slower than GTP hydrolysis or proofreading of the near-cognate Phe-tRNA_{Phe} is more efficient.

In order to determine if the message length affected both cognate and near-cognate aa-tRNA, we measured the rates of EF-Tu dependent GTP hydrolysis for Tyr-tRNA_{Tyr} containing ternary complexes mixed with 70SICs (2 µM) programmed with synthetic oligos with the sequence 5’-A AGG AGG UAA AAA AUG NNN AAA-3’ where NNN is either UAC (cognate) or UAG (near-cognate). For the cognate case, the rate of GTP hydrolysis was fast, ~20 s⁻¹ (Table 10), comparable to rates measured for
Phe-tRNA\textsuperscript{Phe} ternary complexes under similar conditions (see Chapter 2). However, for the near-cognate case the rate was very slow, only \( \sim 0.05 \text{ s}^{-1} \) (Table 10). Here, the cognate and near-cognate rates differed by \( \sim 400 \)-fold, whereas for Phe-tRNA\textsuperscript{Phe} ternary complexes reading \textit{in vitro} transcribed messages in Chapter 2, the analogous reactions differed by \( \sim 30 \)-fold.

| A-codon | \( k_{\text{GTP apparent (s}^{-1})} \) |  \\
|---------|-----------------|---------|
| UAC     | 20.8 \( \pm \) 1.2 |  \\
| UAG     | 0.052 \( \pm \) 0.024 |  \\

A: error represents fitting error

Table 10. Rates of GTP hydrolysis for Tyr-tRNA ternary complexes reading short, synthetic mRNAs.

4.2.3 Discussion

Here, we present data that speaks to the complicated interactions that determine translation errors. In Figure 16, we show that in the presence of the error-promoting antibiotic streptomycin, a number of aa-tRNAs are misincorporated in programmed 70SICs. Several of the dipeptide products formed seem to correspond to misincorporation of aa-tRNAs that would not typically be thought of as near-cognate. For example, at GUU and GUC codons, we see evidence of a dipeptide product that most closely resembles fMet-Ser [151]. Serine has four isoacceptor tRNAs with anticodons UGA, CGA, GCU, and GGA, none of which form more than one Watson-Crick base pair with the GUU and GUC codons. Additionally, a product formed at CUU and CUC appears to
correspond with fMet-His, which would need to form a typically highly unfavorable 2\textsuperscript{nd} position mismatch in order to be incorporated. More thorough characterization of these products, perhaps by mass spec or HPLC will be needed to positively identify these products. In line with this evidence of idiosyncratic misincorporation events, our single-time point survey of EF-Tu-dependent GTP hydrolysis with a variety of aa-tRNA and codons demonstrates that tRNAs have significant differences in which codons they respond to as near-cognate (Figure 21).

More surprising is the observation that variation in length and non-coding sequence can have significant effects on decoding. The initial mSM1 oligo tested differed from the m291 mRNA in several ways, most notably in overall length (22 nt versus 138), Shine-Dalgarono strength (AGGAGG versus AGGAAA), and spacing between the Shine-Dalgarono and the start codon (6 nt versus 5). Short spacing between the Shine-Dalgarono sequence and the start codon can promote +1 frameshifting and destabilize the P-tRNA, although both the 5 and 6 nt spacing are in the optimal range for tRNA stability [135]. There is also some evidence that Shine-Dalgarono interactions can affect decoding accuracy [157]. The small differences we see between mSM1 and mSM2 constructs are consistent with this, but more thorough experiments are needed to confirm this effect. Strikingly, the 3' length of the mRNA seems to have the greatest effect on near-cognate decoding, although to our knowledge this effect has not previously been observed. It is difficult to rationalize a physiological role for this increased stringency on short mRNA, as typical transcripts contain a stop codon well before the end of the message. This behavior may be an artifact of the \textit{in vitro} system, however it could play a role in non-
stop mRNA recognition or mRNA cleavage during starvation. Notably, a recent
observation of unexpected error-prone decoding following misincorporation of an aa-
tRNA led to the discovery of a post-decoding quality control mechanism that may play a
significant role in bacteria [158, 159].

4.2.4 Materials and Methods

RNA oligonucleotides described in Table X were ordered from Sigma-Aldrich as
in Chapter 3. Total tRNA extracts were purchased from Roche.

Total tRNA extracts were charged as described, except using S100 in place of
purified synthetase and with all 20 amino acids present at 100 μM concentration [149].
Dipeptide formation was performed as follows. 70SICs were made as in Chapter 2,
except with [35S]-fMet-tRNAfMet and the indicated mSM1 oligo. Ternary complexes
formed by incubating EF-Tu (20 μM), EF-Ts (1 μM), pyruvate kinase (50 μg/mL), PEP
(2 mM), GTP (1 mM), and charged total tRNA (5 ng/μL) for 15 minutes at 37°C.
Reactions were performed by mixing 5 μL 70SICs (1 μM ± 40 μM streptomycin) with 5
μL ternary complex mix (20 μM EF-Tu) and incubating at room temperature (~22°C) for
5 minutes before addition of 5 μL 500 mM KOH. Quenched reactions were spotted on
PEI cellulose TLCs, run and analyzed as described [151].

Single time-point GTP hydrolysis assays were done similarly to multiple time-
point assays described in Chapter 2, except that reactions were mixed by manual
pipetting rather than a rapid quench device. Equal volumes (5 μL) of ternary complex
mix (0.2 μM) and 70SIC (1 μM) were mixed and incubated for 30 seconds at room
temperate (~22°C) before addition of 5 μL 40% formic acid. The extent of GTP hydrolysis was measured as in Chapter 2.

GTP hydrolysis and dipeptide formation time courses were performed in simultaneous reactions similar to proofreading assays performed in Chapter 3. EF-Tu•[γ32P]-GTP•Phe-tRNA^Phe was mixed with [35S]-fMet-tRNAfMet containing 70SICs on a RQF-3 rapid quench device and quenched with the addition of 35% formic acid. Quenched reactions were centrifuged at 15,000 RPM to pellet tRNA, and supernatant was removed to analyze the extent of GTP hydrolysis by PEI cellulose TLC as in Chapters 2 and 3. Pellets were washed with 15% formic acid and resuspended in 500 mM KOH. Dipeptide formation was analyzed by electrophoretic TLC as in Chapter 3.
Chapter 5: Conclusions and Perspectives

As reviewed in Chapter 1, ribosomal accuracy mutations have been important tools for studying the decoding mechanism. The work presented here built upon years of dedicated research by other scientists who have constructed the contemporary model of aa-tRNA selection. In the shadow of that prior work, any novel insights I may have contributed are minor, but nevertheless may further a complete understanding of the process.

5.1 Locations of ribosomal ambiguity mutations mark accuracy centers of the 30S

The combination of the specialized ribosome system with the missense and nonsense suppression reporter lacZ constructs proved immensely successful in identifying ribosomal ambiguity mutations. The method was sensitive enough that <2-fold changes in β-galactosidase expression could be reliably be distinguished on X-gal plates, and thorough enough to allow the identification of >130 independent isolates and multiple isolations of 16/34 residue alterations (Table 3). To our knowledge, only six of these mutations had been previously shown to influence the fidelity of elongation,
demonstrating the effectiveness of this method against other techniques to isolate rRNA mutations (reviewed in Chapter 1).

Over 80% of our mutations mapped to three distinct locations in the 30S subunit: 1) the A site, particularly h34, 2) h12, and 3) h8/h14. The locations of many r-am mutations in r-proteins overlap with these sites, especially S4/S5 mutations that flank h12, and mutations in L19, which along with L14 and h8/h14, forms intersubunit bridge B8 (Figure 11). Mutations in all three of these sites resulted in >10-fold increases in missense errors, whereas mutations at other sites led to no more than 4-fold increases in errors (Figure 9 and Figure 10). Therefore, it seems reasonable to conclude that these three sites are the primary locations of possible r-am mutations in the 30S subunit.

If this is so, why then did we fail to isolate mutations at other sites known to be critically important for decoding, specifically h18 (Table 3. [160] [161] [162])? One possible explanation is that mutations in this critical helix are too deleterious, so that even if they increase error frequency, the loss of total translation activity would prevent their effects from being apparent. However, an alternative explanation is that mutations at this and other functionally important decoding sites are more likely to result in restrictive phenotypes rather than r-am. Consistent with this idea, the h18 mutations previously studied generally have more dramatic effects on restricting errors, and none have been directly shown to increase missense suppression or near-cognate aa-tRNA incorporation (Table 3. [139] [161]). More detailed characterization of mutations in h18 will help reveal the functional role of this highly conserved region.
5.2 Disruption of Bridge B8 contributes to both phases of decoding

The largest cluster of mutations isolated by our genetic screen, in h8 and h14, was also arguably the least expected. Prior to carrying out this work, only one study had previously implicated mutations in the bridge B8 region as having consequences for decoding [99]. Shortly after we had analyzed the results of the screen, but before publication of our work, a cryo-EM study of the ternary complex stalled on the ribosome suggested that h14 forms a direct contact with the switch 1 loop of EF-Tu, and through that contact activates the factor for GTP hydrolysis [49]. To test this hypothesis, we constructed truncations of both h8 and h14 and showed that these mutations, as well as G347U in h14, led to increased rates of GTP hydrolysis for both cognate and near-cognate ternary complexes, the opposite of the effect predicted from the structure (Figure 12). Later, crystal structures of ribosome containing the G347U mutation demonstrated that disruption of bridge B8 was an unappreciated structural rearrangement leading to activation of EF-Tu for GTP hydrolysis (Figure 8B. [52]). This study also showed that the G299A mutation in h12 leads to a similar disruption of B8, indicating that these two sites are allosterically linked, a novel mechanism of ram mutation function (Figure 8C).

An important unanswered question about the role of bridge B8 in initial selection is: how does B8 disruption lead activation of EF-Tu for GTP hydrolysis? We postulate in Chapter 2 that a structural connection to h5 might play a role. Crystal structures of ternary complex bound to the ribosome indicate that h5 is the site of several contacts with EF-Tu, that induce a structural rearrangement of the factor unique to its ribosome bound state [50]. Fagan, et al. showed that B8 disruption leads to a conformation of h5 that is
similar to that seen in the presence of bound ternary complex [52]. Recently, analysis of a A55U mutation in h5 demonstrated that this mutation is dominant negative for growth, but does not inhibit peptide bond formation [163]. However, recent work from another student in our laboratory indicates that other mutations in h5 lead to severe defects in EF-Tu-dependent GTP hydrolysis (Dedonato, E. Undergraduate Honors Thesis). Continued analysis of these mutations should provide clues to the GTPase activation mechanism.

In Chapter 3, we present evidence that mutations disrupting bridge B8 (G299A, Δh8, Δh14, and G347U) decrease fidelity of the second decoding phase, proofreading (Table 7). The importance of B8 on both decoding phases is particularly interesting in light of recent findings by Zaher and Green showing that the mutations rpsD12 decreases fidelity of initial selection, but has no effect on proofreading [95]. The rpsD12 mutation results in a truncation of S4, in the same region as G299. Other mutations in S4 have previously been noted for having contradictory ram and restrictive phenotypes [86]. Elucidating how these mutations lead to their distinct phenotypes will be especially enlightening.

Less is understood about the second phase of decoding relative to the initial selection phase. Kinetic studies show that aa-tRNAs with a proflavin moiety at positions 16/17 undergo a conformational change following GTP hydrolysis and before peptidyl transfer, indicating that movement from the A/T to the A/A state is rate limiting for peptide bond formation [164]. Single molecule studies show that both cognate and near-cognate aa-tRNAs undergo reversible fluctuations from the mid-FRET GTPase activation state to the high-FRET accommodated state, before either attaining a stable high-FRET
state or dissociating [165] [28]. However, there is some debate as to whether accommodation of the aa-tRNA or peptidyl transfer is rate limiting for the overall decoding reaction [155]. Additionally, it is still unclear what the mechanism of rejection is for near-cognate aa-tRNA [44].

How bridge B8 effects proofreading is unclear. Our assay here only demonstrates that the ratio of accommodation to rejection is higher for near-cognate aa-tRNAs with these mutant ribosomes, but does not indicate whether it is the rate of accommodation or rejection that is affected. All atom simulations of the accommodation process indicate that the aa-tRNA may contact the bridge B8 area during its journey to the peptidyl transfer center, but it does not appear that this is a critical barrier to movement [59]. The most obvious path of dissociation for the aa-tRNA is through the factor-binding site, so it is possible that B8 disruption might stabilize EF-Tu and prevent dissociation. However, kinetic studies do not indicate that EF-Tu dissociation normally happens at a faster rate than near-cognate rejection, indicating removal of EF-Tu may not be so necessary [21]. Another possibility is that B8 disruption indirectly stabilizes the near-cognate aa-tRNA in the A site, reducing the probability of rejection. Further dissection of the effects of these mutations on accommodation and stability of various ligands should provide insight into the proofreading mechanism.

5.3 Mutations in h34 increase misincorporation for specific near-cognate codons

Studies of aa-tRNA selection in vivo have interchangeably used reporters for both missense and nonsense suppression. These two situations are subtly different, missense
suppression requires misincorporation of a near-cognate aa-tRNA in favor of a cognate aa-tRNA, while nonsense suppression requires incorporation of an aa-tRNA in favor of a release factor that normally decodes the stop codon. However, results from both of these reporters are generally consistent with decoding defects, and in this Chapter 2, screens done in parallel for nonsense and missense suppression largely isolated mutations at identical 16S residues (Table 3). There are two notable exceptions to this overlap; mutations C1054U and G1491A were isolated repeatedly in the nonsense suppression screen, while not at all in the screen for missense suppression. When these mutations were tested for activity in the missense reporter strain, G1491A resulted in a mild (~2-fold) increase in error rate, despite a >8-fold effect on nonsense suppression, and C1054U proved to be hyperaccurate (Figure 9 and Figure 10). Mutations at other A-site residues (C1054G and C1200U) also had dramatically different effects on nonsense suppression and missense suppression.

We thought that these effects could be due to an effect of these mutations on RF2, which is typically responsible for reading the UGA stop codon in the nonsense suppression construct. Another student, Aishwarya Devaraj, assayed these mutations for RF2-dependent peptide release, but found no effects large enough to explain the \textit{in vivo} results (Table 6). However, when we measured the effects of mutations C1054U and C1054A on proofreading, we found they had no defect for Phe-tRNA\textsubscript{Phe} reading a near-cognate CUU codon, but were were significantly defective in proofreading a Tyr-tRNA\textsubscript{Tyr} reading a near-cognate UAG codon (Figure 15; Table 7). This indicated that the
in vivo results might be the result of these mutations only affecting decoding for specific codon contexts.

In order to test this hypothesis, we carried out the experiments shown in Figure 17, measuring the rates of incorporation for four different aa-tRNAs on near-cognate codons containing mismatches at either the 1\textsuperscript{st} or 3\textsuperscript{rd} position. Ribosomes containing mutations that result in bridge B8 disruption increased misincorporation in every context tested. In contrast, mutations C1054A and C1200U increase misincorporation only for specific codon-anticodon combinations, primarily those containing 3\textsuperscript{rd} position mismatches. These mutations seem to have the greatest effects for specific 3\textsuperscript{rd} position mismatches, but the pattern of which mismatch is most favored is different for C1054A than C1200U.

As described in Chapter 3, C1054 protrudes from h34 and interacts with the 5’ position of the tRNA anticodon in the A site. Residue C1200 interacts with the phosphate backbone 3’ to C1054, so mutations to C1200 may affect the tRNA through C1054. We propose that mutations at these residues create spurious favorable contacts with the aa-tRNA that stabilize certain codon-anticodon mismatches, particularly those at the 3\textsuperscript{rd} position.
In Chapter 3, we describe data from 30S crystal structures with bound tRNA\textsuperscript{Arg}\textsubscript{ICG} that creates a purine-purine wobble pair with a CGA codon. In contrast with the structure of this tRNA bound to a CGC codon, the purine-purine pair induces C1054 to swing underneath the anticodon and stack with the insosine base (Figure 23A and B). This is not the only indication that the C1054 interaction is sensitive to the different ligands in the A site. From the forty-five crystal structures of ribosomes containing an A-site tRNA or tRNA mimic available in the PDB (http://www.rcsb.org/pdb/), C1054 appears to adopt at least four unique interactions: 1) the indirect packing with the 5’ anticodon base ribose as seen in Figure 23A, 2) the stacking interaction shown in Figure 23B, 3) an interaction

![Figure 23. Interactions of C1054 with tRNAs in the A site. (A) Interaction with tRNA\textsuperscript{Arg}\textsubscript{CGI} from PDB:1XNR. (B) Interaction with tRNA\textsuperscript{Arg}\textsubscript{CGI} from PDB:1XNQ. (C) Interaction with tRNA\textsuperscript{Phe}\textsubscript{AAG} from PDB:1IBL. (D) Interaction with tRNA\textsuperscript{Met}\textsubscript{UAf5C} from PDB:4GKJ.](image-url)
where the Watson-Crick face of C1054 is directly facing the 5′ anticodon base (Figure 23C), and 4) an interaction seen only in structures with an anticodon-stem-loop (ASL) mimic of human Lys$^3$-mcm$^5$s$^2$U(34)UU or human mitochondrial Met- $^5$CAU where C1054 slides up the anticodon stem to interact with either the ribose or the tRNA phosphate backbone (Figure 23D). While it is unclear what features of each of these complexes result in the idiosyncratic positioning of C1054, it is clear that the residue can form unique interactions with several tRNAs [41] [40]. Our data in Chapter 3 suggests that these or other interactions may play a significant role in stabilizing certain codon-anticodon pairings. Further analysis will be needed to establish what role these interactions play decoding throughout the genetic code.

### 5.4 Future studies of ribosome mutations and decoding

The collection of 16S _ram_ mutations isolated in Chapter 2 has been useful for investigating the role of distinct regions of the ribosome in the decoding mechanism, as demonstrated by the results presented in Chapters 2-4. This collection could additionally help address several remaining questions regarding the role of the ribosome in decoding. Importantly, accommodation, and other events occurring subsequent to GTP hydrolysis by EF-Tu, is poorly described in current models. We have demonstrated in Chapter 3 that several 16S mutations, in bridge B8, h12, and the ribosomal A site, affect the ribosome’s ability to proofread near-cognate aa-tRNA. Investigating the effects of these mutations on
the kinetic steps following GTP hydrolysis may help elucidate what events contribute to accuracy during proofreading.

One reason why the proofreading has been the less-studied phase of decoding is that EF-Tu-dependent GTP hydrolysis is rate limiting for near-cognate reactions in high fidelity conditions [27]. Unlike bulk systems, single molecule experiments offer the opportunity to observe accommodation even for near-cognate complexes [28, 165]. It is possible that comparing these mutant ribosomes using the smFRET methods would reveal how they affect the second decoding phase.

Another potential area of investigation stems from our observations in Chapter 3 that mutations in the A site have distinct effects on specific mismatched tRNAs. The data presented in Figure 17 represent only a small number of possible mismatch contexts that could be affected by the mutations. Expanding this experiment to include other codons or tRNAs could determine the pattern of interactions that are stabilized or promoted by various A-site mutations. Additionally, may be some utility in identifying mutant ribosomes that facilitate the incorporation of non-canonical amino acids. These amino acids are typically incorporated by charging onto suppressor tRNAs, which the ribosome may use less efficiently than standard tRNAs [166]. Modulating the A site could provide a path for efficient incorporation the suppressor species.

Our results in Chapter 3 and 4, along with other recent research, suggest that even for wild-type ribosomes, error frequencies are not uniform at all codons. Several assays developed in Chapter 3 and 4 may be useful in investigating errors made at various codons during translation.
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