Translation termination, the last step of protein synthesis, is mediated by release factors, which recognize the stop signal in messenger RNA (mRNA) and relay the signal to the ribosome to catalyze the release of a nascent polypeptide chain. Although advanced knowledge has been obtained from the crystal structure of the ribosome and release factors, the molecular mechanisms of their function in the process are still unclear. In this study, we performed mutational analyses based on structure data and applied both biochemical and genetic approaches to further our understanding of this process in eukaryotes.

First, we utilized an established yeast genetic system that allowed us to examine the effects of ribosomal RNA (rRNA) mutations in a homogeneous background. Mutational analysis of the rRNA decoding site manifested essential roles of three phylogenetically conserved bases, A1755/A1756/G577, in translation and identified that G1645 and A1754 bases together determine eukaryotic resistance to aminoglycosides that interfere with translation fidelity. In addition, we found that mutations of these two bases had no influence on translation alone, but affected drug-induced reduction of elongation fidelity and termination efficiency in accordance with their conferred aminoglycosides susceptibility. These data, for the first time, provided in vivo functional analysis of the
eukaryotic decoding center in a homogeneous background and revealed two distinct groups of rRNA bases that affect on translation accuracy.

Second, we examined the mechanism of stop codon recognition by eRF1 using a genetic screen for viable and functional suppressors as well as site-directed mutagenesis of a *Euplotes* and *S. cerevisiae* hybrid eRF1 (Eo/Sc eRF1) that lost the ability to recognize UGA as a stop codon and could not support viability by itself. We found that S57 and N58 of the TASNIKS motif, T75 in α-helix 3 and C124 of the YxCxxxF motif are crucial for stop codon recognition. An *in vitro* peptide chain release assay of Eo/Sc eRF1 mutants also surprisingly revealed residues of eRF1 in coupling stop codon recognition, activation of GTP hydrolysis by eRF3 and polypeptide chain release.

As a result, we were able to provide new insights of ribosomes and release factors function in eukaryotic translation termination.
DEDICATION

To my parents, for their unfailing support, immeasurable encouragement and everlasting love, without which this dissertation would not have come to life.

献给我亲爱的父母，感谢他们给予我的永恒无私的爱，以及无时无刻的支持与鼓励，正是这些激励我完成这篇理学博士论文。
ACKNOWLEDGMENTS

I appreciate greatly to my mentor, Dr. David M. Bedwell, for his scientific and rigorous research attitude, open-handed and strict guidance as well as meticulous and well-organized lab managing, all of which make him an exceptional mentor and instruct me to transform from a one-sided and merely sake-seeking novice into an open-minded and dedicated researcher.

My special thanks go to dissertation committee members, Dr. Casey Morrow, Dr. Peter Burrows, Dr. James Collawn and Dr. Bradley Yoder for their unbiased criticism, fruitful suggestions and continuous encouragement.

I am also grateful to all the current and past Bedwell lab members, especially Kim Keeling, Joe Salas-Marcro and Adam Kallmeyer for their anytime available scientific discussion and magnificent friendship.

Most importantly, I thank my husband Martin Minogue being a special part of my life, whose inspiration and support has been and will always be my motivation to explore science world. Finally, I am indebted to our son, Michael Minogue, for the happiness and blessing he brings to our world.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Overview</td>
<td>1</td>
</tr>
<tr>
<td>Ribosomal RNA in Translation Termination</td>
<td>2</td>
</tr>
<tr>
<td>Ribosome and Ribosomal RNA</td>
<td>2</td>
</tr>
<tr>
<td>Decoding region of ribosomal A site</td>
<td>5</td>
</tr>
<tr>
<td>Action of Aminoglycosides on the Ribosome</td>
<td>7</td>
</tr>
<tr>
<td>Mutational Analysis of Ribosomal RNA</td>
<td>10</td>
</tr>
<tr>
<td>Measurement of Termination Efficiency</td>
<td>12</td>
</tr>
<tr>
<td>Eukaryotic Release Factors in Translation Termination</td>
<td>14</td>
</tr>
<tr>
<td>Class I and II Release Factors</td>
<td>14</td>
</tr>
<tr>
<td>Molecular Mechanism of Stop Codon Recognition</td>
<td>15</td>
</tr>
<tr>
<td>Regulation of Peptide Chain Release</td>
<td>19</td>
</tr>
<tr>
<td>Interplay between Release Factors and Ribosomes in Translation Termination</td>
<td>20</td>
</tr>
<tr>
<td>Evidence of Interaction</td>
<td>20</td>
</tr>
<tr>
<td>Significance of Understanding Translation Termination</td>
<td>21</td>
</tr>
<tr>
<td>RIBOSOMAL RNA DETERMINANTS OF EUKARYOTIC AMINOGLYCOSIDE RESISTANCE AND THEIR ROLE IN TRANSLATION FIDELITY</td>
<td>23</td>
</tr>
<tr>
<td>MUTATIONS THAT RESTORE STANDARD GENETIC CODE</td>
<td>57</td>
</tr>
<tr>
<td>RECOGNITION TO A VARIANT CODE</td>
<td></td>
</tr>
<tr>
<td>EULPLOTES HYBRID ERF1</td>
<td></td>
</tr>
<tr>
<td>DEFINE KEY DETERMINANTS OF STOP CODON RECOGNITION</td>
<td></td>
</tr>
<tr>
<td>SUMMARY</td>
<td>101</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Continued)

Page

GENERAL LIST OF REFERENCES................................................................. 106
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Standard and Variant Stop Codon Assignment</td>
</tr>
<tr>
<td><strong>IDENTIFICATION OF RIBOSOMAL RNA DETERMINANTS OF AMINOGLYCOSIDES RESISTANCE AND THEIR INDIRECT ROLE IN EUKARYOTIC TRANSLATION</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>rRNA A Site Mutations Introduced into <em>S. cerevisiae</em></td>
</tr>
<tr>
<td>2</td>
<td>Classes of Aminoglycosides Used in This Study</td>
</tr>
<tr>
<td>3</td>
<td>Aminoglycosides Sensitivity of <em>S. cerevisiae</em> Decoding Site Mutants</td>
</tr>
<tr>
<td><strong>MUTATIONS THAT RESTORE STANDARD GENETIC CODE RECOGNITION TO A VARIANT CODE EULPLOTES HYBRID ERF1 DEFINE KEY DETERMINANTS OF STOP CODON RECOGNITION</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Strains and Plasmids Used</td>
</tr>
<tr>
<td>2</td>
<td>Effect of Eo/Sc eRF1 Mutations on Colony Growth</td>
</tr>
<tr>
<td>3</td>
<td>Effect of Sc eRF1 mutations on Colony Growth</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
</tr>
</tbody>
</table>

INTRODUCTION

1 Ribosomal RNA Structure and the Decoding Region .................................................. 4
2 Conserved A Site in Helix 44 ........................................................................... 6
3 Chemical Structures of 2-deoxystreptamine (ring II)-containing Aminoglycosides .................................................. 8
4 Complete Deletion of Yeast Chromosomal rDNA Repeats ..................................... 11
5 Misincorporation Reporter and Readthrough Reporter ....................................... 13
6 Molecular Mimicry of tRNA Molecules .................................................................. 16

IDENTIFICATION OF RIBOSOMAL RNA DETERMINANTS OF AMINOGLYCOSIDES RESISTANCE AND THEIR INDIRECT ROLE IN EUKARYOTIC TRANSLATION

1 Comparison of the Decoding Sites in *E. coli* and *S. cerevisiae* ......................... 29
2 Minimum Inhibitory Concentration (MIC) of Aminoglycosides ............................... 35
3 Translational Readthrough Level of Yeast Strains with G1645 mutations ............. 39
4 Translational Readthrough Level of Yeast Strains with G1645 and A1754 mutations .......................................................... 41
5 Misincorporation Level of Yeast Strains with G1645C and G1645A/A1754G double mutations .................................................. 43
6 Structure of Eukaryotic Decoding Site (PDB 2FQN) ............................................. 47
# LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>MUTATIONS THAT RESTORE STANDARD GENETIC CODE RECOGNITION TO A VARIANT CODE EULPLOTES HYBRID ERF1 DEFINE KEY DETERMINANTS OF STOP CODON RECOGNITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mutagenesis of Eo/Sc eRF1 ........................................................................................................... 62</td>
</tr>
<tr>
<td>2</td>
<td>Efficiency of Stop Codon Recognition Mediated by Eo/Sc eRF1 Suppressor Mutants.......................... 65</td>
</tr>
<tr>
<td>3</td>
<td>Efficiency of Stop Codon Recognition Mediated by Sc eRF1 C124 and TASNIKS Mutants......................... 70</td>
</tr>
<tr>
<td>4</td>
<td>Polysome Analysis of Strains Expressing Eo/Sc eRF1 Mutants...................................................... 74</td>
</tr>
<tr>
<td>5</td>
<td>Eo/Sc eRF1 Mutants Restore Peptide Release at the UGA Stop Codon.............................................. 77</td>
</tr>
<tr>
<td>6</td>
<td>The Location of Key Determinants of Stop Codon Recognition on the Three Dimensional Structure of Human eRF1.............................................................. 80</td>
</tr>
<tr>
<td>7</td>
<td>Model for Eukaryotic Translation Termination at UAA/UAG versus UGA Stop Codons.......................... 86</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>5-FOA</td>
<td>5-fluoroorotic acid</td>
</tr>
<tr>
<td>ASL</td>
<td>anticodon stem loop</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>eRF1</td>
<td>eukaryotic release factor one</td>
</tr>
<tr>
<td>eRF3</td>
<td>eukaryotic release factor three</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>guanosine triphosphatase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAT</td>
<td>Pro-Ala-Thr</td>
</tr>
<tr>
<td>pre-TCs</td>
<td>pre-termination complexes</td>
</tr>
<tr>
<td>PTC</td>
<td>peptidyl transferase center</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal deoxyribonucleic acid</td>
</tr>
<tr>
<td>RF1</td>
<td>release factor one</td>
</tr>
<tr>
<td>RF2</td>
<td>release factor two</td>
</tr>
<tr>
<td>RF3</td>
<td>release factor three</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SMD</td>
<td>synthetic medium dextrose</td>
</tr>
<tr>
<td>SPF</td>
<td>Ser-Pro-Phe</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>YEPD</td>
<td>yeast extract peptone dextrose</td>
</tr>
</tbody>
</table>
INTRODUCTION

Overview

Protein translation, one of the fundamental cellular processes, decodes genetic information into polypeptide chains to achieve cellular function. It is performed cyclically through three highly regulated stages, initiation, elongation and termination. While translation initiation ensures a correct start and translation elongation ensures faithful deciphering, translation termination ensures a precise stop to release the correct length polypeptide chain and to allow a proper start of the next round translation. Despite the general understanding of the translation process, the molecular mechanism of its function is still obscure and termination is the least understood stage.

Termination of protein synthesis takes place on the ribosome as a response to a stop rather than a sense codon. Among all the 64 genetic codons, three different base triplets are referred to as stop codons, UAA, UAG and UGA, which are encoded in the genome and transcribed to messenger RNA (mRNA). Yet, the final release of a nascent polypeptide chain from P site transfer RNA (tRNA) requires release factors, which can recognize the stop codon at the A site of small ribosomal subunit and transmit the signal to the large ribosomal subunit to consequently trigger the hydrolysis of the peptidyl-tRNA. Therefore, the complete termination process involves interplay between the stop codon in mRNA, ribosomal components and release factors. To understand the molecular mechanism of this multistep and collaborative work, the function of each individual factor has to first be defined.
This work is mainly focused on defining the role of eukaryotic ribosomal RNA (rRNA) and release factor one (eRF1) in translation termination. Current knowledge about these two players is reviewed and preliminary evidence demonstrating their interaction in regulating the process is addressed.

**Ribosomal RNA in Translation Termination**

As the major component that contains the catalytic and regulatory centers of the ribosome, ribosomal RNA (rRNA) is involved in all aspects of protein translation. In regard to translation termination, rRNA is the main component of the ribosomal decoding region where a stop signal is recognized, and forms the peptidyl transferase center that hydrolyzes peptidyl-tRNA to release the nascent polypeptide chain in respond to the signal. Basic structures of rRNA functional centers have been known based on studies mainly in prokaryotes. However, more mutational analysis of rRNA is still needed to precisely understand how rRNA participates in eukaryotic translation termination.

**Ribosome and Ribosomal RNA**

The ribosome is a large and ancient RNA-protein biomolecule that performs protein synthesis in all types of life. The complete ribosome, 70S in prokaryotes or 80S in eukaryotes, is assembled from a large subunit and a small subunit, which are 50S and 30S in prokaryotes or 60S and 40S in eukaryotes. Each subunit has three tRNA binding sites: A (aminoacyl) site that accepts the incoming aminoacylated tRNA, P (peptidyl) site that holds the tRNA with the nascent peptide chain and E (exit) site that holds the deacylated tRNA before it leaves the ribosome (2). The A site of the small subunit is also called the
decoding center, where the anticodon of an A site tRNA pairs with a mRNA codon during elongation (Figure 1); and where a stop codon is recognized by release factors during termination. The A and P sites of the large subunit are adjacent to the peptidyl transferase center (PTC), where peptide bond formation is catalyzed between the amino acid end of an A site tRNA and the nascent peptide chain attached to the P site tRNA; and where peptide chain release is triggered by release factors. Thus, in elongation, the A site tRNA adjoin the decoding center and the PTC to relay a translational signal, while in termination, release factors help to relay the stop signal between the two subunits.

rRNAs comprise the majority of each ribosomal subunit, which make up 23S/5S and 16S in prokaryotes or 28S/5.8S/5S and 18S in eukaryotes. rRNAs also compose the major functional sites of the ribosome (3). 23S or 28S rRNA contains the catalytic moiety in the PTC that functions as a ribozyme to stimulate peptide bond formation and hydrolyze peptide chain release by a still poorly understood mechanism. 16S or 18S rRNA contributes to the main component of the decoding center and binds the codon-anticodon duplex between mRNA and tRNA during elongation to ensure the accuracy of translation (Figure 1). Whether and how rRNA is involved in the fidelity of termination still remains largely unknown. Recent crystal structures of ribosomes dramatically increased our knowledge about rRNA and provided an essential structural basis for understanding its function in translation, especially the decoding process that is also the target of many clinically important antibiotics.
Figure 1: Ribosomal RNA structure and the decoding region. The crystal structure of the prokaryotic 70S ribosome is shown on the left, with the anticodon stem loop of the A-site tRNA in the interface cavity between the 30S and 50S subunits (1). The A-site of the 30S subunit is enlarged at the bottom. The mRNA codon and cognate tRNA form Watson-Crick pairing in the decoding center, with A1492, A1493, G530 from rRNA helices 44 and 18 forming the floor and sensing the first two base pairs of the codon-anticodon double helix. On the right is the secondary structure of S. cerevisiae 18S rRNA, indicating that key nucleotides of the decoding site rRNA (in E. coli numbering) are contributed by three different rRNA helices.
Decoding Region of Ribosomal A site

Mapping of the decoding region in prokaryotic ribosomal A site has been studied using chemical protection, crosslinking and genetic analysis (4-7), all of which suggested that the anticodon of aminoacyl-tRNA and the mRNA codon were bound to highly conserved nucleotides from different helices of 16S rRNA (8). They are specifically A1492/A1493 from helix 44 and G530 from helix 18. Recent high-resolution crystal structures of ribosomes bound with mRNA and tRNA revealed that A1492, A1493 and G530 are located in the heart of the decoding site (Figure 1) (9-11). These three bases contact and form A minor interaction with the codon-anticodon duplex only when cognate codon-anticodon pairing occurs (12) and consequently induces an open to closed conformational change in the ribosome, which further stabilizes and accelerates tRNA selection (13). Therefore, rRNA is actively involved in decoding and contributes to translation fidelity, which is also consistent with previous studies in *E. coli* that found that mutations of those key nucleotides were lethal (14). Due to the lack of a high-resolution structure of termination factors bound with the ribosome, the mechanism by which rRNA is involved in the selection of release factors is still unclear.

The eukaryotic ribosome structure has not been determined and much less has been done to probe functional rRNA sites, although the three key bases in the decoding center are conserved in the same rRNA helices (Figure 1), suggesting similar mechanism maybe used to achieve translation fidelity.

In the decoding region, adjacent to those main tRNA and mRNA binding sites are also highly conserved bases from helix 44 that provide the main binding sites of aminoglycosides. This portion of helix 44 is referred to as the conserved A site in bacteria.
(Figure 2, boxed) because bases upstream or downstream of this site are variable within bacteria and their polymorphism does not affect aminoglycoside binding (15). Although the role of these bases in translation is not clear, a better understanding of drug-target interaction was achieved by the crystal structure of aminoglycosides bound to the ribosome, which revealed detailed binding sites and provided a structural basis for understanding how aminoglycosides interfere with translation fidelity.

The corresponding site in 18S rRNA shares dramatic similarity, except for two variables. Residue 1408, which forms a non-canonical Watson-Crick pair with A1493, is an adenine (A) in prokaryotes, but a guanine (G) in eukaryotes. Residue 1491, next to the universally conserved A1492, is a G in prokaryotes, but an A in eukaryotes (Figure 2). Mutational analysis of each individual base in E. coli indicated they were targets of different type of aminoglycosides (16,17) and mutations to their eukaryotic counterpart resulted in bacteria cells that exhibited similar resistance to the drug as eukaryotes (18). Therefore, it is postulated that these two residues determined the specificity of aminoglycoside action on prokaryotic ribosomes (19). However, whether these bases are really responsible for eukaryotic resistance to aminoglycosides has not been manifested.

**Figure 2:** Conserved A site in helix 44. Secondary structures of decoding stem in helix 44 of small subunit rRNA from E. coli and S. cerevisiae are shown side by side. The highly conserved ribosomal A-site is boxed. Variable nucleotides of the decoding site in E. coli are bold and *italic* bold in S. cerevisiae.
and it is quite risky to extrapolate mutational effects from prokaryotes to eukaryotes so easily.

**Action of Aminoglycosides on the Ribosome**

As a family of antibiotics, aminoglycosides are multiply charged, highly flexible compounds at physiological pH. Their positive charges are attracted to negatively charged RNA backbones and their flexible structure facilitates accommodation into binding pockets formed by loops of RNA helices or ribozyme cores to make specific contacts (20). In living cells, the actual major target of aminoglycosides is the small subunit of rRNA in both prokaryotes and eukaryotes. They bind directly to the conserved region in the decoding site of rRNA and consequently interfere with accuracy of codon decoding and inhibit fidelity at sublethal levels or kill cells at high concentration. Importantly, aminoglycosides can discriminate between prokaryotic and eukaryotic ribosomes. Prokaryotic and mitochondrial ribosomes are sensitive to many aminoglycosides; however, eukaryotic cytoplasmic ribosomes are resistant to most aminoglycosides, which is also the reason why aminoglycosides are effective antibacterial agents. Extensive structural and mutational analyses of aminoglycosides, rRNA and their complexes have been performed mainly in prokaryotes to understand the molecular mechanism of interactions between the drug and its target site.

Structurally, the majority of aminoglycosides are composed of amino sugars linked to a 2-deoxystreptamine ring (Figure 3, ring II). Ring II is disubstituted with ring I and ring III at either the 4 and 6 positions, such as kanamycin and G418, or the 4 and 5 positions, such as neomycin and paromomycin. The substitution type of aminoglycosides
Figure 3: Chemical structures of 2-deoxystreptamine (ring II)-containing aminoglycosides. The 4,5-disubstituted aminoglycosides, neomycin and paromomycin and the 4,6-disubstituted kanamycin A and G418 are shown. The rings are labeled with I-IV and the positions in the ring are labeled with Arabic numbers. 4,5 and 4,6 positions of ring II are circled. 6' of ring I having an amino group (NH$_2$) or a hydroxyl group (OH) is circled with dash line.
does not seem to affect drug binding to the ribosome, rather a hydroxyl group versus an ammonium group at position 6’ of ring I has shown distinct interactions with ribosome according to recent studies in prokaryotes (17,18,21-23). For example, certain ribosomal mutants, which showed similar reduced susceptibility to kanamycin and neomycin that have an amino group at 6’ ring I, showed less reduced susceptibility to paromomycin and geneticin (G418) that have a hydroxyl group at the 6’ position of ring I.

Mapping the aminoglycoside binding site in prokaryotic ribosomes has been greatly assisted by nuclear magnetic resonance (NMR) and crystallographic studies of aminoglycosides bound to model RNAs (16,20,24) and by mutational analysis of the decoding region of 16S rRNA, which tested the effect of specific residues on aminoglycoside susceptibility (25). Recently, the crystal structure of paromomycin bound to the ribosome revealed that upon drug binding a conformational change similar to that of cognate tRNA binding was induced, thus providing a structural basis for how the drug interferes with translation fidelity. In contrast, determinants of eukaryotic resistance to aminoglycosides have not been confirmed. Regarding the study of eukaryotic translation fidelity, though, aminoglycosides have served as suppressors to identify antisuppressors from rRNA or release factor mutants, particularly paromomycin that is known to be an efficient inducer of misreading in yeast. Apparently, more mutational analyses of the decoding region are required to further our understanding of the role of rRNA in both translation and aminoglycosides binding in eukaryotes.
Mutational Analysis of Ribosomal RNA

Mutations in rRNA have been difficult to isolate because of the high redundancy of rRNA genes (rDNA) in the genome. There are seven rRNA operons in *E. coli* and 150 to 250 tandem repeats of rDNA in eukaryotic chromosomes. To overcome this, plasmid-encoded rDNA carrying an antibiotic-resistant mutation has been used to introduce rRNA mutations into the cell. In cells transformed with the plasmid and treated with that antibiotic, the activity of genome-encoded wild type ribosomes is inhibited and only ribosomes of plasmid-encoded origin are actively translating (16,26). With this approach, quite a few mutational analyses of prokaryotic rRNA have been performed to understand drug actions on ribosomes. For example, mutations of A1492 and A1493 in the 16S decoding region have been shown to be lethal (14,27). Adjacent bases in the conserved A site of helix 44 were more tolerant of mutation and were differentially involved in antibiotics binding. However, little has been studied on their mutational effects on translation fidelity and termination (28). In eukaryotes, even fewer experiments have been carried out, mainly due to the greater complexity of their genomic rDNA. Limited experiments, though have shown that some nucleotides of 18S rRNA in the decoding region are involved in translation fidelity and aminoglycosides binding (26,29,30), which is consistent with the results from prokaryotes. However, mutational effects of some bases in the A site that confer an increase in translation accuracy are opposite to the results obtained with ribosomes of mitochondria and *E. coli*, which were found to compromise translation fidelity (29). The contrary results observed between prokaryotes and eukaryotes imply that even for the highly conserved rRNA region, we cannot easily extrapolate mutational effects from prokaryotes to eukaryotes and *vice versa*.
Meanwhile, there are drawbacks to this approach. First, inhibition of genome-encoded ribosomes by antibiotics is probably not complete, so that there may be mixed populations of wild type and mutant ribosomes. Recessive mutations could escape detection. Second, alternative unknown effects of a pre-existing antibiotic-resistant mutation on ribosomes make it risky in a mutational analysis. Therefore, a better genetic system and more mutational analyses of eukaryotic rRNA are needed for a more complete understanding of ribosome function. Recently, an ideal system for analysis of rRNA mutations in yeast was developed by the Nomura group (31). In this system, all the genome rRNA genes are completely deleted by classic homologous recombination and cell growth is supported by a multicopy plasmid encoding a single copy of rDNA (Figure 4). Thus, rRNA mutations can be introduced on a plasmid with a selective marker that is

**Figure 4:** Complete deletion of yeast chromosomal rDNA repeats. On the left is the structure of the \textit{RDN1} locus on yeast chromosome XII. The \textit{RDN1} locus contains \(\sim150\) tandem repeats of rRNA genes. A single repeat unit is expanded above \textit{RDN1}, showing the 35S rRNA coding region (long arrow) and the 5S rRNA coding region (short arrow, not to scale). Four non-rDNA repeats, A-D, are at one end of the \textit{RDN1} locus. Complete deletion was via gene replacement by a linear fragment (bottom) using \textit{HIS3} for selection. Flanking sequences L and R are homologous to each end of the \textit{RDN1} locus as indicated. Primers 1-4 were used to confirm deletions. On the right is the structure of the supporting rDNA plasmid under a Pol I promoter.
different from that of the wild type plasmid in the system. By plasmid shuffling, wild
type plasmid can be shuffled out and a homogeneous population of mutant ribosomes can
be obtained. This system is used to perform mutational analysis of yeast decoding site
rRNAs and examine their roles in translation fidelity, termination and aminoglycosides
affinity by both biochemical and functional assays.

**Measurement of Termination Efficiency**

Translation termination, as a crucial step in translational fidelity control, has an
error rate of about $10^{-4}$, which ensures that only very few abnormal products are
synthesized under normal conditions (32). A failure of translation termination happens
when a stop codon is misread as a sense codon, which is also termed stop codon
suppression or readthrough. Suppression phenotypes have long been used to identify
translation factors in genetic screens. The level of readthrough, meanwhile, has provided
a quantitative measurement of defects in translation termination. In addition to defects in
translation termination, defects in the fidelity of translation elongation can also cause
translational readthrough, where the translation elongation machinery misincorporates
amino acid residues at a stop codon in the A site by near-cognate tRNA mispairing.
Discriminating between defects of translation elongation and termination is important for
understanding how various mutations or drugs confer a readthrough phenotype and
accurately evaluating their contributions to the overall termination process. However, few
approaches are available to tell the difference, until recently when a combined application
of misincorporation and readthrough reporter systems (Figure 6) was developed (33). The
readthrough reporter measures the overall defects in translation accuracy, which has a
stop codon in between the Renilla and Firefly luciferase genes. The level of firefly luciferase activity normalized to Renilla luciferase activity at a stop versus sense codon will reflect both elongation and termination defects during translation. The misincorporation reporter has a missense mutation in a functionally important residue of the firefly luciferase gene, H245R, which results in a non-functional firefly luciferase protein during normal translation. An increased level of luciferase activity will then reflect the incidence of misincorporation during elongation. This system was applied to define the effect of rRNA mutations on translation fidelity.

**Figure 5:** Misincorporation reporter and readthrough reporter. Asterisk indicates the missense mutation, histine to arginine, at a functionally important residue 245 in the firefly gene.
**Eukaryotic Release Factors in Translation Termination**

Among all the players in translation termination, release factors that are responsible for recognizing and relaying a correct stop signal to successfully release the nascent polypeptide chain are the least conserved between prokaryotes and eukaryotes, not only in regard to structure but also function. Therefore, how stop codons are discriminated from sense codons and how the signal is transmitted from small to large subunit of the ribosome are still among the major questions regarding the mechanism of eukaryotic translation termination. More *in vivo* genetic analysis and *in vitro* biochemistry approaches to obtain a complete and detailed kinetic view of how release factors perform their function are urgently required to ultimately unravel molecular mechanism of this most mysterious process of protein synthesis.

**Class I and II Release Factors**

There are two classes of release factors mediating translation termination and they are quite different between prokaryotes and eukaryotes. Class I release factors all have three functional domains: domain 1 recognizes stop codons, domain 2 stimulates peptide chain release and domain 3 mediates the interaction with class II release factors. Prokaryotes employ two class I release factors, RF1 and RF2, recognizing UAA/UAG and UAA/UGA respectively. In contrast, eukaryotes possess one class I release factor, eRF1, recognizing all three stop codons. In addition, comparison between crystal structures of *E. coli* RF2 and human eRF1 indicate they are completely distinct proteins in terms of primary, secondary and tertiary structure (34).
The prokaryotic class II release factor, RF3, helps RF1 or RF2 dissociate from ribosomal A site after peptide chain release by inducing a large conformation change of the ribosome (35). It is dispensable and has no stable interaction with either RF1 or RF2. Eukaryotic class II release factor, eRF3, a ribosome- and eRF1-dependent GTPase, facilitates eRF1 stop codon recognition (36) and carries out GTP hydrolysis before polypeptide chain release (37). It is an essential protein and forms a stable complex with eRF1. These considerable differences between prokaryotes and eukaryotes regarding termination factors suggest they might use distinct mechanisms to recognize stop codons.

However, there is one similarity among class I release factors, the universally conserved GGQ motif in domain 2, which is required for the activation of peptidyl-tRNA hydrolysis. This may suggest vestiges of a primordial termination signal relay and peptide chain release mechanism.

**Molecular Mechanism of Stop Codon Recognition**

Domain 1 of class I release factors have been thought to be responsible for the stop codon recognition. In prokaryotes, Pro-Ala-Thr (PAT) in domain 1 of RF1 and Ser-Pro-Phe (SPF) in domain 1 of RF2 have been found to determine the specificity of stop codon recognition (38). However, such a single “peptide-anticodon” has not been identified in domain 1 of eRF1, in spite of the fact that domain 1 of eRF1 structurally mimics the anticodon loop of tRNA (Figure 5). On the contrary, eRF1 mutations affecting stop codon recognition in yeast have been found scattered between positions 51 and 132 of the polypeptide chain in domain 1, but in the 3D structure these mutations
Figure 6: Molecular mimicry of tRNA molecules. Ribbon diagram of human eRF1 and yeast tRNA$^{\text{phe}}$ structures are laid side-by-side, revealing similar shapes and over-all dimensions. The disposition of domain 1 of eRF1 matches that of the tRNA anticodon loop, with the NIKS motif mimicking anticodon bases. Domain 2 with the GGQ motif and domain 3 are indicated.
surround three putative cavities that were proposed to be the binding sites of three nucleotides of the stop codon (39). Within that region, other studies showed that there were at least two motifs involved in the specificity of stop codon recognition. The TASNIKS (Thr-Ala-Ser-Asn-Ile-Lys-Ser) motif, at the inter-loop between helix α2 and α3 of domain 1, is thought to be the most related motif because of its position mimicry of tRNA anticodon bases and high evolutionary conservation (Figure 5). Consistent with that hypothesis are data from in vitro release assays, which showed that mutations in the TASNIKS motif compromised eRF1 release activity to an extent depending on the stop codon specificity, position and nature of the substituting residues (40). The other recently found motif, YxCxxxF (Tyr-Cys---Phe) motif, is about 15 Å away from the TASNIKS motif and is postulated to affect the pattern of stop codon recognition by an interplay with the TASNIKS motif (41). Furthermore, a recent study demonstrated that eRF3 is involved in the recognition efficiency of certain stop codons by eRF1 (42). All the features of eukaryotic release factors and varieties of stop codon recognition models described above suggest that the recognition of stop codon in eukaryotes is much more regulated and complicated than in prokaryotes. Thus, an alternative approach may be necessary to identify putative peptide sequences that determine the stop codon identity of eRF1.

One possible approach to identify crucial residues involved in stop codon recognition was offered by using lower eukaryotes with variant genetic codes, where one or two out of the three stop codons are reassigned to sense codons. For instance, in _Tetrahymena_, UAA and UAG are reassigned to glutamine, leaving UGA as the only stop codon. In _Euplotes_, on the other hand, UGA encodes cysteine while UAA and UAG
remain as stop codons (Table 1). Given 50%-60% sequence identity with human eRF1, defining how ciliates eRF1 diverge from the standard genetic code might help to determine the recognition mechanism utilized by human eRF1. In particular, if domain 1 of ciliates eRF1 is sufficient to confer reassignment of stop codons in the termination process, then one may assume that a chimera with domain 1 from ciliates eRF1 and domain 2 and 3 from omnipotent eRF1 would recapitulate the variant stop codon recognition of ciliates in an omnipotent termination system. In that case, the divergent changes in domain 1 of ciliates eRF1 may count for the stop codon recoding. Actually, substantial efforts in sequencing ciliate eRF1 genes have been undertaken to find convergent changes for variant decoding. However, with ciliates eRF1 sequences expanding, convergent changes in eRF1 using the same genetic code deviation have decreased and none of the changes involved amino acids in the TASNIKS motif (43). That casts doubts on the actual role of these residue changes in stop codon recognition. Moreover, data from studying chimeric eRF1 molecules indicated that ciliates with different genetic code deviations were using actually distinct approaches (44). For example, a hybrid eRF1 of domain 1 from *Tetrahymena* and domain 2 and 3 from *S. cerevisiae* was viable as the only source of eRF1 when expressed in *S. cerevisiae* and

<table>
<thead>
<tr>
<th>Coding Species</th>
<th>Codon UAA</th>
<th>Codon UAG</th>
<th>Codon UGA</th>
<th>Genetic code</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Stop</td>
<td>Stop</td>
<td>Stop</td>
<td>universal</td>
</tr>
<tr>
<td><em>Tetrahymena</em></td>
<td>Gln</td>
<td>Gln</td>
<td>Stop</td>
<td>variant</td>
</tr>
<tr>
<td><em>Euplotes</em></td>
<td>Stop</td>
<td>Stop</td>
<td>Cys</td>
<td>variant</td>
</tr>
</tbody>
</table>
efficiently terminated at all three stop codons. A similar hybrid eRF1 with domain 1 from *Euplotes* (Eo/Sc eRF1), though, could not support cell growth alone and still lacked the capacity to terminate at UGA but maintained ability to recognize UAA and UAG as stop codons like *Euplotes* eRF1. Thus, more functional analysis of individual variant species is necessary. Characterizing molecular mechanism of stop codon reassignment by *Tetrahymena* eRF1 would help to identify other factors or domains contributing to the stop codon deviation. However, for *Euplotes* eRF1, studies can be focused on domain 1, which is sufficient to recapitulate the variant recognition of stop codons but cannot support viability in a Eo/Sc hybrid form of eRF1. Therefore, a genetic screen for gain of function mutations would provide important information about determinants of variant decoding.

**Regulation of Peptide Chain Release**

The ultimate goal of termination is to release a right length of nascent polypeptide chain, which is triggered by eRF1 after its recognition of stop codons and catalyzed by rRNA in the peptide transferase center. The efficiency of peptide chain release has been used to evaluate the ability in stop codon recognition in simple *in vitro* peptide release experiments (45-47). However, in those experiments the concentration of eRF1 was saturated and eRF3 was not included. Functional analysis *in vivo* indicated that eRF3 facilitates certain groups of termination-codon recognition by eRF1 and subsequent peptide release by a mechanism coupled to its GTPase activity (36,37). More recent studies in a defined *in vitro* translation system including all the translation factors have shown that eRF3 induces a structural rearrangement upon binding to eRF1 and carries out
GTP hydrolysis prior to polypeptide chain release (36,37), which provides strong support of the \textit{in vivo} data. These data also suggested that eRF3 may function to couple stop codon recognition to efficient peptide release. However, considering the GTPase activity of eRF3 does not affect all types of stop signals, recognition of certain stop codons may bypass eRF3 to stimulate peptide chain release. Analysis of more mutations of eRF1 stop codon recognition domain using the defined \textit{in vitro} translation system is needed to provide precise kinetic mechanism of peptide chain release regulated at different stop signals.

\textbf{Interplay Between Release Factors and Ribosomes in Translation Termination}

\textit{Evidence of Interaction}

As many other essential biological processes, translation termination is a coordinated effort of a set of related factors, including the ribosome, mRNA, release factors and other undefined proteins (48). In order to understand this complex process, we need not only to define the role of every individual factor, but also the interactions among them. Indeed, previous studies have indicated that rRNAs and release factors might have a quite close relationship. For instance, mutations in the TASNIKS motif reduced the binding of eRF1 to the ribosome and compromised termination efficiency (40,49). On the other hand, mutations in the decoding region of rRNA rescued a temperature sensitive mutation in domain 1 of eRF1, demonstrating a genetic interaction between them (29). Furthermore, a temperature sensitive termination defective mutation in the GTPase domain of eRF3 can be rescued by rRNA mutation in the decoding site, implying rRNA mutations might stabilize eRF1 on the ribosome long enough to provide
sufficient time for mutant eRF3 to hydrolyze GTP and stimulate the termination activity of eRF1 (28). Accordingly, the basic discrimination of a stop codon from a sense codon may be conducted by class-1 RFs, while the strength and stereochemistry of the interaction between stop codon and class-1 RFs may be specifically affected by rRNA sequences. More mutational analysis of rRNA and release factors and high resolution crystal structures of ribosomes and release factors complex are necessary to elucidate the molecular mechanism of translation termination of protein synthesis in eukaryotes.

**Significance of Understanding Translation Termination**

Protein translation is a fundamental biological process in the cell. The fidelity of translation, to ensure minimal deviations from the genetic coded template, is essential to preserve the function and integrity of a cell. As the last step of translation, termination plays an important role in the control of translational fidelity. Terminating at a premature stop codon would make truncated peptides, which would result in loss of protein function or even result in a dominant-negative effect on normal cellular functions. Whereas, inefficient termination, or translational readthrough of normal stop codons, would result in C-terminally extended protein versions that may dramatically alter cellular metabolism.

Indeed, nonsense and frame shift mutations cause approximately 20-40% of the individual cases of more than 200 inherited diseases, which are of high medical relevance (48). For example, about 5% of cystic fibrosis (CF) patients have premature stop mutations in the *CFTR* gene. Murine leukemia viruses express their Gag-Pol fusion protein via readthrough of a stop codon in between the two genes. The efficiency of stop
codon readthrough in this case is dependent on an interaction between reverse transcriptase and eRF1 (50). Over the past decade, research has explored pharmacological approaches designed to suppress premature stop mutations that cause diseases. Aminoglycoside antibiotics, which bind to the ribosomal decoding site and reduce translation fidelity by a conformational alteration, have been found to be promising. In this regard, identification and characterization of the factors that regulate the efficiency of translation termination will not only be important for understanding the biology of the termination process, but also for the development of therapeutics capable of treating a wide array of genetic disorders that arise as a consequence of nonsense mutations.

The aims of this study seek to increase our understanding of the molecular mechanism of translation termination by examining the role of the eukaryotic rRNA decoding site in the termination process and the mechanism of stop codon recognition by eRF1. Here, we report, for the first time, an in vivo functional analysis of the eukaryotic decoding center in a homogeneous background. The results identified determinants of eukaryotic resistance to aminoglycosides and indicated their role in translation fidelity. In a second series of experiments, using both in vivo genetic and in vitro biochemistry approaches, we identified key determinants of stop codon recognition and propose a new working model for eRF1 function.
RIBOSOMAL RNA DETERMINANTS OF EUKARYOTIC AMINOGLYCOSIDE RESISTANCE AND THEIR ROLE IN TRANSLATION FIDELITY

by

HUA FAN-MINOGUE AND DAVID M. BEDWELL

In preparation for RNA

Format adapted for dissertation
Abstract

Recent studies of prokaryotic ribosomes have dramatically increased our knowledge of ribosomal RNA (rRNA) structure, functional centers, and interactions with antibiotics. However, much less is known about how rRNA function differs between prokaryotic and eukaryotic ribosomes. In this study, we examined the effects of mutations in the decoding site of 18S rRNA of the eukaryote *S. cerevisiae*. We found that all possible mutations at G577, A1755 and A1756 (corresponding to G530, A1492 and A1493 of *E. coli* 16S rRNA) were lethal. In contrast, some mutations at the non-conserved decoding site residues G1645 and A1754 (corresponding to A1408 and G1491 of *E. coli* 16S rRNA) did not confer a significant growth defect and were shown to function as key determinants of eukaryotic resistance to different classes of aminoglycoside antibiotics. Mutations at these residues also influenced paromomycin-induced misreading of sense codons and readthrough of stop codons in accordance with their conferred sensitivity to paromomycin.

Introduction

Ribosome function has been studied by biochemical and genetic approaches for decades. These studies have shown that ribosomal RNA (rRNA), as the main component of ribosomes, plays a crucial role in all aspects of translation (Moore & Steitz, 2002). More recently, X-ray crystallographic and cryo-EM studies of prokaryotic ribosomes have provided essential structural information about various individual steps of the translation process. For example, tRNA selection is a multi-step that occurs when the anticodon of an aminoacyl tRNA base pairs with a mRNA codon located in the ribosomal
A site. Crystallographic studies have shown that three universally conserved nucleotides of *E. coli* 16S rRNA, G530 from helix 18, and A1492/A1493 from helix 44, directly contact the codon-anticodon helix to ensure the accuracy of the codon-anticodon match (Nissen et al., 2001; Ogle et al., 2001).

In other studies, a comparison of ribosome structures in the presence and absence of antibiotics has helped us understand the mechanism of action of compounds that inhibit protein synthesis, such as the aminoglycosides (Carter et al., 2000). Aminoglycosides are a family of antibiotics with a common 2-deoxystreptamine core (ring II) glycosidically linked to a glucopyranosyl (ring I) at position 4 (Hobbie et al., 2006a). Based on how additional sugars are attached to ring II, aminoglycosides can be structurally divided into 4,5- and 4,6-disubstituted classes (Table 1). Structural studies have shown that aminoglycosides target nucleotides in the rRNA decoding site, and induce a conformational change similar to the transition that occurs upon cognate tRNA binding (Ogle et al., 2001; Ogle & Ramakrishnan, 2005). As a result, aminoglycosides interfere with protein translation by facilitating amino acid misincorporation and ultimately inhibit microbial growth.

Mutational studies to confirm the proposed model of aminoglycoside action have been difficult, since rRNA genes (rDNA) are encoded in multiple copies in the bacterial genome. The *E. coli* genome contains seven rRNA operons, which, for many years, relegated functional analyses of rRNA mutations to studies of mixed populations of wild-type and mutant ribosomes (De Stasio et al., 1989; De Stasio & Dahlberg, 1990; Recht et al., 1999b). Due to the dominant feature of drug sensitivity over resistance in bacteria (Lederberg, 1951; Apirion & Schlessinger, 1968; Prammananan et al., 1999; Sander et
al., 2002), wild type ribosomes frequently mask the resistant phenotype of mutants and prevent the unambiguous determination of drug susceptibilities (Hobbie et al., 2006a). However, bacterial strains that express single rDNA operons have recently been constructed that enable the analysis of mutations that confer drug resistance in homogenous populations of mutant ribosomes (Sander P, 1996; Asai et al., 1999). Since then, several studies of the decoding site of bacterial ribosomes have been carried out (Sander P, 1996; Pfister et al., 2003a; Pfister et al., 2003b). The A1408G mutation, among all decoding site mutations, conferred the most significant resistance to aminoglycosides (Sander P, 1996; Recht et al., 1999a; Recht et al., 1999b; Pfister et al., 2003a; Gregory et al., 2005; Hobbie et al., 2005). Given a guanine at the corresponding position in eukaryotes, A1408 was concluded to be the main determinant of aminoglycosides specificity (Hobbie et al., 2006a). However, the A1408G mutation only conferred an intermediate level of resistance to aminoglycosides with a hydroxyl group at the 6´ position of ring I (Recht et al., 1999b; Gregory et al., 2005). Mutations of G1491 in the decoding site actually conferred more resistance to aminoglycosides with a 6´-hydroxyl group than the A1408G mutation (Pfister et al., 2005).

Much less is known about the nucleotides of 18S rRNA responsible for aminoglycoside resistance in eukaryotes, since most eukaryotic rRNA genes (rDNA) are much more highly repeated (Wai et al., 2000). For example, the rDNA of S. cerevisiae is encoded as ~150 copies of a 9.1kb repeat at the RDN1 locus on chromosome 12 (Raue, 1991). Each repeat unit contains the genes for 5.8S, 18S and 25S rRNAs that are transcribed by RNA polymerase I, as well as 5S rRNA gene transcribed by RNA polymerase III. Previous studies described a yeast strain in which the majority of the
chromosomal rDNA repeats were eliminated (Chernoff et al., 1994), with cell viability maintained by the expression of a rDNA repeat from a plasmid. However, the residual copies of rDNA repeats in the genome, again resulted in mixed populations of wild type and mutant ribosomes. More recently, an improved yeast system was constructed in which the chromosomal rDNA repeats are completely deleted by sequential deletion steps (rdnΔΔ). This allowed specific rRNA mutations to be expressed from a rDNA repeat on a high copy plasmid, resulting in a strain carrying a homogeneous population of mutant ribosomes.

In current study, we used this latter system to investigate nucleotides of the 18S rRNA decoding site on aminoglycosides susceptibility in yeast cells. E found that residue G1645 (corresponding to A1408 in E. coli) and A1754 (corresponding to G1491 in E. coli) both contribute to aminoglycoside resistance. Furthermore, we examined the effects of mutations that alter aminoglycosides susceptibility on translation, using stop codon readthrough and sense codon misincorporation assays that can discriminate between defects in the termination and elongation steps of translation. We found that mutations at these residues have no effect on translation accuracy, but influence both paromomycin-induced misreading of sense codons and readthrough of stop codons.

**Results**

*Mutagenesis of Nucleotides within the Yeast 18S rRNA Decoding Site*

The rRNA decoding site lays within the ribosomal A site where the anticodon of an aminoacyl-tRNA recognizes a mRNA codon. Three universally conserved nucleotides in the decoding site, G530, A1492 and A1493, directly interact with the codon-anticodon
The decoding site is also the target for aminoglycoside antibiotics, which interfere with translation fidelity (Carter et al., 2000). The nucleotides of the central portion of the decoding site in helix 44 of 16S rRNA (Figure 1A, boxed) are highly conserved among bacteria (Vicens & Westhof, 2001; Pfister et al., 2003a). Nucleotides beyond this central region are more variable within bacteria, and these polymorphisms do not appear to influence aminoglycoside binding or sensitivity (Lynch & Puglisi, 2001; Pfister et al., 2003a).

The eukaryotic decoding site in 18S rRNA shares significant similarity to its prokaryotic counterpart (Figure 1B, boxed), which implies that essential features of the decoding process may be conserved. In fact, only two nucleotides, G1645 (E. coli A1408) and A1754 (E. coli G1491) diverge within this structural element. Both mutational and structure studies have suggested these divergent residues are responsible for the enhanced sensitivity of prokaryotic ribosomes to distinct subclasses of aminoglycosides (Sander P, 1996; Recht et al., 1999a; Recht et al., 1999b; Carter et al., 2000; Vicens & Westhof, 2001; Pfister et al., 2003a; Pfister et al., 2005). However, much less has been done to investigate the role of their eukaryotic counterparts in aminoglycosides resistance, let alone their involvement in translational fidelity.

To initially investigate the role of the eukaryotic decoding site in translational fidelity, site-directed mutagenesis was first used to introduce all possible base substitutions and single base deletions of the critical nucleotides that monitor codon-anticodon pairing: G577 (corresponding to E. coli G530), A1755 (corresponding to E.
Figure 1: Comparison of the decoding sites in *E. coli* and *S. cerevisiae*. (A) Secondary structure of bacteria conserved A site in *E. coli* 16S rRNA is boxed (Pfister et al., 2003a). Bases that are protected from dimethyl sulfoxide (DMS) modification by mRNA and tRNA are marked by asterisks (Purohit & Stern, 1994). Bases that are protected from DMS modification by paromomycin are circled (Yoshizawa et al., 1998). (B) Secondary structure of corresponding conserved A site in *S. cerevisiae* 18S rRNA is boxed. The two variable bases are framed and mutations that convert them to their bacteria counterparts are indicated by arrows.
coli A1492), and A1756 (corresponding to *E. coli* A1493). Plasmids expressing these mutant rRNAs were introduced into the *rdnΔΔ* yeast strain and plasmid shuffling was used to test for the viability of strains expressing these mutations (Table 1). We found that all three possible base substitutions or the deletion of these three residues was lethal, consistent with the essential nature of these residues in the 16S rRNA of *E. coli* (Powers & HF, 1990; Yoshizawa et al., 1999). These results suggest that the essential role of these residues in translational fidelity is evolutionarily conserved in eukaryotes.

We next examined the importance of the two variable bases of the core decoding site, G1645 (A1408) and A1754 (G1491), in cell viability and aminoglycoside resistance. First, we introduced all possible base substitutions and a single base deletion of G1645 (A1408). While the G1645U and the G1645 deletion (G1645Δ) were lethal, the G1645A and G1645C mutations were viable and the mutant strains grew with normal growth rates (Table 1). The G1645A mutation restored the asymmetric internal loop found in prokaryotic species (consisting of the A1408·A1493 pair and the unpaired A1492) without any detectable growth defect (Figure 1B, Table 1), indicating this mutation does not impair ribosomal function. Consistent with this observation, the reverse mutation A1408G in the prokaryotic system also had no effect on cell growth (Pfister et al., 2003a), while the G1645C mutation has not been made. The *E. coli* residue G1491 (corresponding to the yeast residue A1754) has also been extensively mutagenized (Hobbie et al., 2005; Pfister et al., 2005; Hobbie et al., 2006b). We found that the yeast A1754G mutation also had no effect on cell growth (Table 1). Since both single substitution at these two variable bases, G1645A and A1754G, were viable, we next
Table 1: rRNA A site mutations introduced into *S. cerevisiae*

<table>
<thead>
<tr>
<th>E. coli Position</th>
<th>S. cerevisiae Mutation</th>
<th>Viability †</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>Viable (++)</td>
<td>pNOY373</td>
</tr>
<tr>
<td>G530</td>
<td>G577A</td>
<td>Lethal</td>
<td>pDB917</td>
</tr>
<tr>
<td></td>
<td>G577C</td>
<td>Lethal</td>
<td>pDB918</td>
</tr>
<tr>
<td></td>
<td>G577U</td>
<td>Lethal</td>
<td>pDB920</td>
</tr>
<tr>
<td></td>
<td>G577Δ</td>
<td>Lethal</td>
<td>pDB919</td>
</tr>
<tr>
<td>A1408</td>
<td>G1645A</td>
<td>Viable (++)</td>
<td>pDB850</td>
</tr>
<tr>
<td></td>
<td>G1645C</td>
<td>Viable (++)</td>
<td>pDB979</td>
</tr>
<tr>
<td></td>
<td>G1645U</td>
<td>Lethal</td>
<td>pDB977</td>
</tr>
<tr>
<td></td>
<td>G1645Δ</td>
<td>Lethal</td>
<td>pDB1088</td>
</tr>
<tr>
<td>G1491</td>
<td>A1754G</td>
<td>Viable (++)</td>
<td>pDB975</td>
</tr>
<tr>
<td>A1408 / G1491</td>
<td>G1645A / A1754G</td>
<td>Viable (++)</td>
<td>pDB976</td>
</tr>
<tr>
<td>A1492</td>
<td>A1755G</td>
<td>Lethal</td>
<td>pDB0007</td>
</tr>
<tr>
<td></td>
<td>A1755C</td>
<td>Lethal</td>
<td>pDB0905</td>
</tr>
<tr>
<td></td>
<td>A1755U</td>
<td>Lethal</td>
<td>pDB0908</td>
</tr>
<tr>
<td></td>
<td>A1755Δ</td>
<td>Lethal</td>
<td>pDB0906</td>
</tr>
<tr>
<td>A1403</td>
<td>A1756G</td>
<td>Lethal</td>
<td>pDB0911</td>
</tr>
<tr>
<td></td>
<td>A1756C</td>
<td>Lethal</td>
<td>pDB0909</td>
</tr>
<tr>
<td></td>
<td>A1756U</td>
<td>Lethal</td>
<td>pDB0912</td>
</tr>
<tr>
<td></td>
<td>A1756Δ</td>
<td>Lethal</td>
<td>pDB0910</td>
</tr>
<tr>
<td>U1495</td>
<td>U1758C</td>
<td>Viable (+)</td>
<td>pDB978</td>
</tr>
</tbody>
</table>

† For viable mutations, (++) and indicates normal growth and (+) indicates slow growth.
asked whether the double mutant, which recapitulated a prokaryotic decoding stem in yeast rRNA (Figure 1B), would affect the growth of yeast cells. Strikingly, the double mutation G1645A/A1754G was viable and did not exhibit any growth defect, suggesting that the combination of the two mutations does not interfere with normal ribosomal function. Furthermore, the level of 18S rRNA in each of the viable rRNA mutant strains was similar to that of the wild type strain (data not shown).

As a control to validate the rdnΔΔ strain in our hands, we also examined the affect of the U1758C mutation (corresponding to E. coli U1495C), which alters a universally conserved nucleotide in the decoding site. Mutational analysis in both prokaryotes and eukaryotes has shown that this mutation confers resistance to hygromycin B (Spangler & Blackburn, 1985; Chernoff et al., 1994; Pfister et al., 2003b). Consistent with previous studies, a strain expressing ribosomes with the U1758C was viable. As expected, the mutant strain had a slight growth defect (Table 1) and exhibited resistance to hygromycin B (data not shown).

**The Effect of rRNA Mutations on Aminoglycoside Resistance in Yeast**

The two variable bases in the core of the prokaryotic decoding site, A1408 and G1491, have been shown to be involved in aminoglycoside binding (Recht et al., 1999b; Carter et al., 2000). To determine whether their eukaryotic counterparts, G1645 and A1754, are responsible for the relative aminoglycoside resistance of yeast ribosomes, we used a minimum inhibitory concentration (MIC) assay to determine the affect of mutations at these positions on aminoglycosides susceptibility. Aminoglycosides are generally categorized into 4,5- and 4,6-di-substituted classes (Table 2). However, recent
studies have shown that the 16S rRNA of prokaryotes exhibit distinct interactions with aminoglycosides carrying a hydroxyl group versus an ammonium group at the 6´ position of ring I (Recht et al., 1999b; Pfister et al., 2003a; Hobbie et al., 2005; Pfister et al., 2005; Hobbie et al., 2006b). Because of these differences, we chose to examine the response to four aminoglycosides (Table 2): neomycin (4,5-di-substituted with an amino group at the 6´ position of ring I); kanamycin (4,6-di-substituted with an amino group at the 6´ position of ring I); paromomycin (4,5-di-substituted with a hydroxyl group at the 6´ position of ring I); and G418 (4,6-di-substituted with a hydroxyl group at the 6´ position of ring I). The wild type strain and strains with the G1645A, G1645C, A1754G and G1645A/A1754G mutations were tested to determine the relative MIC of aminoglycosides associated with each rRNA mutation (Figure 2).

Without any drug treatment, all mutant strains grew as well as the wild type strain. The wild type strain was relatively resistant to all four aminoglycosides (Figure 2 and Table 3). In contrast, the G1645A mutation increased the sensitivity to kanamycin A and neomycin (which have an amino group at the 6´ position of ring I) by 500-fold. In contrast, and the G1645A mutation increased the sensitivity to paromomycin and G418 (which have a hydroxyl group at the 6´ position of ring I) by only 7.5-fold and 3.3-fold, respectively (Table 3). These results were consistent with the reciprocal A1408G mutation in *E. coli*, which conferred a high level of resistance to aminoglycosides carrying an amino group at the 6´ position of ring I, but only moderate resistance to aminoglycosides carrying a hydroxyl group at that position (Recht et al., 1999b). In contrast, the G1645C mutation increased the sensitivity to kanamycin A and neomycin by
**Table 2:** Classes of aminoglycosides used in this study

<table>
<thead>
<tr>
<th>6′ position of ring I</th>
<th>Ring II substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>amino group (-NH₂)</td>
<td>Neomycin</td>
</tr>
<tr>
<td></td>
<td>Kanamycin A</td>
</tr>
<tr>
<td>hydroxyl group (-OH)</td>
<td>Paromomycin</td>
</tr>
<tr>
<td></td>
<td>Geneticin (G418)</td>
</tr>
</tbody>
</table>
Figure 2: Minimum inhibitory concentration (MIC) of aminoglycosides. Yeast strains with wild type or mutant rRNA were streaked on YPD plates with the indicated concentrations of aminoglycosides and incubated at 30°C for 5 days.
~5-fold, conferred the same sensitivity to paromomycin as the wild type rRNA, and actually reduced sensitivity to G418.

At the second variable nucleotide of the decoding stem in helix 44, the A1754G mutation conferred more than 60-fold greater sensitivity to paromomycin and 10-fold greater sensitivity to G418. While exhibiting only slightly more sensitivity to kanamycin A and neomycin, strains with the A1754G mutation could grow with either drug at concentrations approaching 5000 µg/ml. This was also consistent with the effect of the reciprocal G1491A mutation in bacteria, which differentially conferred greater resistance to aminoglycosides carrying a hydroxyl group at the 6´ position of ring I than to aminoglycosides carrying an amino group at that position. The most striking effect was observed with the double G1645A/A1754G mutant, which conferred the greatest sensitivity to all four aminoglycosides and could not grow in the presence of 3 µg/ml of any of the four aminoglycosides (Figure 2). Notably, this level of aminoglycoside sensitivity was similar to that observed with *E. coli* (Table 3) (Recht et al., 1999b).

**The Effect of rRNA Mutations on Paromomycin-induced Readthrough Correlates with Drug Susceptibility**

The decoding site of rRNA monitors codon-anticodon interactions during translation elongation. It also influences how the release factors (RF1 or RF2 in prokaryotes, and eRF1 in eukaryotes) recognize stop codons during translation termination. Defects in translation elongation or termination can impair translational accuracy, resulting in an increased rate of stop codon suppression (Salas-Marcos & Bedwell, 2005). A well-defined dual luciferase readthrough assay (Keeling et al., 2004)
Table 3: Aminoglycoside sensitivity of *S. cerevisiae* decoding site mutants.

<table>
<thead>
<tr>
<th>Aminoglycoside</th>
<th>MIC* for <em>S. cerevisiae</em> with indicated mutation (µg/ml)</th>
<th>MIC* for <em>E. coli†</em> (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>G1645A</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>&gt;5000</td>
<td>25</td>
</tr>
<tr>
<td>Neomycin</td>
<td>&gt;5000</td>
<td>25</td>
</tr>
<tr>
<td>Paramomycin</td>
<td>&gt;1500</td>
<td>200</td>
</tr>
<tr>
<td>G418</td>
<td>50</td>
<td>15</td>
</tr>
</tbody>
</table>

*MIC, Minimal Inhibitory Concentration
† From Recht MI et al, *EMBO J* 18, 3133; 1999
provides a quantitative measure of the efficiency of stop codon recognition (Salas-Marco & Bedwell, 2005). To examine whether decoding site mutations affect termination efficiency, we performed dual luciferase readthrough assay with strains expressing rRNA with the G1645A, G1645C, A1754G or G1645A/A1754G mutations. There were only minimal difference in the readthrough levels measured in mutant and wild type strains (Figure 3 and 4), suggesting that these mutations do not significantly impair translation accuracy or termination efficiency (consistent with their normal growth phenotype).

Paromomycin is a 4,5-di-substituted aminoglycoside that efficiently increases misincorporation and suppress nonsense mutations in yeast (Palmer et al., 1979; Singh et al., 1979; Chernoff et al., 1994; Salas-Marco & Bedwell, 2005; Davies & Rubinsztein, 2006). We next assessed the affect of these rRNA mutations on paromomycin-induced readthrough. Due to the dramatic differences in the MIC of paromomycin for the different mutations, we first grew strains expressing wild type, G1645A, or G1645C rRNAs in the presence of 25 µg/ml paromomycin. As shown in Figure 3, the wild type strain grown in the presence of 25 µg/ml paromomycin exhibited 4.5- to 8.6-fold increases in readthrough at the three stop codons. The G1645A mutation resulted in somewhat higher readthrough than wild type at the UAA and UAG codons (6.4-fold and 7.2-fold increases, respectively), but a much higher level of readthrough (36.3-fold) at the UGA stop codon. Interestingly, the G1645C mutation resulted in a much lower increase in readthrough than wild type at all three stop codons (ranging from 1.9- to 2.4-fold). The readthrough observed in strains carrying the G1645A or G1645C mutations correlated well with their relative sensitivity to paromomycin in the MIC assay.
Figure 3: Translational readthrough level of yeast strains with G1645A and G1645C mutations compared with the wild type strain at UAA, UAG and UGA stop codons. Cells were cultured in YPD medium at 30°C with or without 25µg/ml paromomycin.
Because of the higher paromomycin sensitivity associated with the A1754G and G1645A/A1754G mutations, we next grew strains expressing wild type, G1645A, A1754G or G1645A/A1754G rRNAs in the presence of 1.5 µg/ml paromomycin (Figure 4). Under these conditions, the wild type strain exhibited a 1.3- to 1.9-fold increase in readthrough. The G1645A strain was associated with a 2.2- to 2.6-fold increase in readthrough, while the A1754G strain had a 1.4- to 5.4-fold increase in readthrough. Interestingly, the G1645A/A1754G had significantly greater readthrough at all three stop codons in the presence of this low level of paromomycin, with increases ranging of 3.5-fold to 7.1-fold. The fact that A1754G and G1645A/A1754G strains exhibited higher levels of paromomycin-induced readthrough also correlates with the greater sensitivity observed with these strains.

*rRNA Mutations also Affect Paromomycin Induced Misreading*

Using the dual luciferase readthrough assay alone, it is not possible to determine whether increased readthrough is caused by a decrease in elongation fidelity or reduction in stop codon recognition. Because of this, we previously developed a luciferase misincorporation reporter system, which relies on a H245R mutation that inactivates firefly luciferase (Salas-Marco & Bedwell, 2005). The luciferase activity of this misincorporation reporter can only be restored if a histidine is improperly inserted in place of the encoded arginine residue. This system provides a measure of the level of misreading during translation elongation, and thus allows discrimination between readthrough that arises from elongation fidelity errors versus a reduced efficiency of stop codon recognition.
Figure 4: Translational readthrough level of yeast strains with G1645A, A1754G and G1645A/A1754G mutations and compared with wild type strain at UAA, UAG and UGA stop codons. Cells were cultured in YPD medium at 30°C with or without 1.5µg/ml paromomycin.
We used the misincorporation assay to examine elongation fidelity in two strains expressing rRNA mutations: the G1645C strain that decreased paromomycin-induced readthrough, and the G1645A/A1754G strain that was associated with the largest increase in paromomycin-induced readthrough (Figure 5). In the absence of paromomycin, neither mutation significantly altered the level of misreading compared to wild type, suggesting that these mutations themselves do not affect elongation fidelity (consistent with the absence of any affect on the readthrough level). However, treatment with 25 µg/ml paromomycin caused a 3.9-fold increase in misreading with the wild type strain, while the G1645C strain exhibited only a 1.7-fold increase in misreading. This suggested that G1645C mutation suppresses paromomycin-induced misincorporation. Treatment with 1.5 µg/ml paromomycin did not cause any misreading in wild type strain, while the G1645A/A1754G strain exhibited a 3.3-fold increase in misreading. These data suggest that the G1645A/A1754G mutations together facilitate paromomycin-induced misreading. The altered misincorporation in these mutant strains also correlates with their drug sensitivity.

Discussion

The rRNA Residues Involved in Monitoring Codon-anticodon Interactions are phylogenetically conserved

In our study, we examined the consequences of mutations at several positions within the yeast 18S rRNA decoding site. We found that all possible mutations of G577, A1755 and A1756 were lethal. This result is consistent with the dominant lethal effects of mutations at the corresponding positions in *E. coli* 16S rRNA (G530, A1492 and
Figure 5: Misincorporation level of yeast strains with the G1645C and G1645A/A1754G mutations compared with the wild type strain at UAA, UAG and UGA stop codons. Cells were cultured in YPD medium at 30°C with or without 25 µg/ml paromomycin to assay the wild type and G1645C mutant strains, and with or without 1.5 µg/ml paromomycin to assay between wild type and G1645A/A1754G mutant strains.
A1493, respectively) (Powers & HF, 1990; Yoshizawa et al., 1999; Abdi & Fredrick, 2005). The high level of conservation of these three nucleotides in both prokaryotes and eukaryotes is consistent with their essential role in protein synthesis. Crystallographic studies have suggested that these three nucleotides monitor translation accuracy by direct interactions with the codon-anticodon helix in the A site after an induced-fit conformational change (Ogle et al., 2002). In addition, biochemistry studies indicate that these nucleotides are required for the efficient activation of the GTPase activity of eEF1A and tRNA accommodation upon recognition of cognate tRNA species (Cochella et al., 2007). Interestingly, detrimental as mutations of these essential bases are, cells handle the aberrant rRNA differently between prokaryotes and eukaryotes. While rRNA with those deleterious mutations accumulates in the cell and causes dominant lethality in bacteria (Powers & HF, 1990), they are eliminated through “nonfunctional rRNA decay (NRD)” pathway in S. cerevisiae (LaRiviere et al., 2006), suggesting eukaryotes have tight quality control of structural and functional integrity of ribosomal RNA.

18S rRNA Residues G1645 and A1754 are Key Determinants of Aminoglycoside Resistance in Eukaryotes

The nucleotides G1645 and A1754 of the eukaryotic decoding site (corresponding to A1408 and G1491 in prokaryotes) are the only two non-conserved, core decoding site nucleotides. A previous study found that the A1408G mutation reduces bacterial sensitivity to aminoglycosides, but the reduced sensitivity was much more pronounced for aminoglycosides with an amino group at the 6’ position of ring I (Recht et al., 1999b). In the current study, the G1645A mutation conferred general sensitivity to all four types
of aminoglycosides tested, but more pronounced susceptibility to kanamycin A and neomycin that carry an amino group at the 6′ position of ring I (Figure 2, Table 3). Therefore, our data defines residue 1645 of 18S rRNA as an important determinant of aminoglycoside binding. The G1491A mutation in *E. coli* 16S rRNA confers high resistance to aminoglycosides with a hydroxyl group at the 6′ position of ring I (Pfister et al., 2005). Consistent with this observation, we found that the A1754G mutation conferred high sensitivity to both paromomycin and G418, aminoglycosides with a hydroxyl group at the 6′ position of ring I (Figure 2, Table 3). More interestingly, the double mutation G1645A/A1754G conferred high sensitivity to aminoglycosides with either moiety at the 6′ position of ring I (Table 3), effectively making yeast as susceptible to these compounds as *E. coli*. Together, these data demonstrate that the major determinants of aminoglycoside resistance in eukaryotes are nucleotides G1645 and A1754.

The crystal structure of aminoglycosides bound to the prokaryotic ribosome provides a structural basis of the drug–rRNA interaction, where A1408 hydrogen bonds with the amino or hydroxyl group of the 6′ position of ring I to form a pseudo base-pair interaction. In contrast, a guanine at residue 1408 cannot form a hydrogen bond with the amino group, but can hydrogen bond with a hydroxyl group. G1491 provides a stacking interaction with ring I to stabilize the pseudo base-pair interaction between ring I and A1408, whereas an adenine at position 1491 weakens this interaction. Therefore, A1408 and G1491 facilitate proper insertion of ring I carrying either an amino or hydroxyl group into the binding site, whereas a guanine at position 1408 and an adenine at position 1491
cause the binding pocket to become shallower and less stable, thus preventing the proper insertion of ring I (Figure 6).

We found that a strain expressing the G1645C mutation was also viable. However, this mutation resulted in a dramatically different response to aminoglycosides. First, it caused a much smaller decrease in resistance to kanamycin A or neomycin than the G1645A mutation. It did not significantly alter paromomycin resistance, and conferred even greater resistance to G418 than the wild type (G1645) residue. Although there has not been a corresponding mutational analysis or structural study in bacteria, our data suggests that the G1645C mutation is less favorable for the insertion of aminoglycosides with a ring I that contains a 6´ hydroxyl group than G1645. In addition, the G1645C mutation facilitates the insertion of aminoglycosides with a ring I that contains a 6´ amino group to a much lesser extent than the G1645A mutation. The other two non-viable mutations at residue 1645 (corresponding to the bacterial residue A1408), were G1645U and the deletion of G1645 (G1645Δ). These mutations were presumably lethal because G1645U has the potential to form a Watson-Crick base pair with A1756 (corresponding to bacterial 1493), while the G1645Δ disrupts the local internal loop that may inhibit the extension of A1756 (bacterial A1493) and/or A1755 (bacterial 1492) into the minor groove to monitor the codon/anticodon interaction that is required for accurate tRNA selection in the ribosomal A site.
Figure 6. Structure of the eukaryotic decoding site (PDB 2FQN).
**Effect of Mutations at the Non-conserved Residues of the Decoding Site on Translation Fidelity and Termination Efficiency**

Our results indicate that the two non-conserved nucleotides of the eukaryotic decoding site do not participate directly in tRNA selection or stop codon recognition, since mutations at these residues do not have significant effects on these processes. However, they greatly stimulate the misreading and readthrough induced by aminoglycosides, in accordance with their conferred drug susceptibility. Together, these results suggest that the growth inhibition of these compounds correlates with their affinity for binding the decoding site, and consequently their effects on translation accuracy. This is consistent with the observation that aminoglycosides with high affinity to bacterial rRNA can kill these organisms, while the same compounds cause much more subtle effects on misreading and readthrough in eukaryotes. Interestingly, mutations at these non-conserved residues can also influence the phenotype associated with mutations in the release factors. For example, the rdn15 (A1491G) mutation was isolated as a suppressor of the conditional lethality associated with a yeast eRF1 mutation \( (sup^{45-R2^t}) \) (Velichutina et al., 2001). While these non-conserved nucleotides do not directly contribute to confirmation changes required for normal decoding, they can influence translation in the presence of mutant release factors. Further genetic studies with this novel rdnΔΔ strain should all allow hypotheses from structural and evolutionary studies to be tested.
Materials and Methods

Strains, Plasmids and Genetic Methods

The *S. cerevisiae* strain NOY891 (MATa ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 rdnΔΔ::HIS3) (Wai et al., 2000) was used to generate homogeneous rRNA population, in which chromosomal ribosomal DNA (rDNA) repeats are completely deleted and cell growth is supported by a high copy plasmid pNOY353 (*TRP1* selectable marker) carrying a single rDNA repeat under *GAL7* promoter control for transcription by RNA polymerase II (Wai et al., 2000). The expression of rDNA was maintained by growth on synthetic minimal (SM) galactose (tryptophan dropout) plates. High copy plasmid pNOY373 (*LEU2* selectable marker), carrying a single rDNA repeat under its native promoter control for transcription by RNA polymerase I, was used as template for mutagenesis. Since the whole rDNA repeat in pNOY373 is 9.1kb and to avoid unexpected mutations, the ~3.9kb *PstI*-*MluI* fragment containing 18S rRNA gene was subcloned into pBluescript II KS+ (*HindIII::MluI*) (pDB840) vector for introducing mutations. All rDNA mutations are site-directly introduced using a QuikChange site-directed mutagenesis kit (Stratagene). After confirming the mutation by sequencing the whole *PstI*-*MluI* fragment, it was cloned back to replace the corresponding regions in pNOY373. Wild type or mutated pNOY373 (*LEU2* selectable marker) were introduced into NOY891 using a plasmid shuffle approach where transformants were plated on SM glucose medium containing leucine, but not tryptophan. The viable strains that were confirmed carrying homogeneous rRNA population were then plated or cultured with YEPD (1% Bacto-yeast extract, 2% peptone, 2% dextrose) rich medium to obtain best growth.
Antibiotics and Determination of MIC

Antibiotics kanamycin (K-1377), neomycin (N-6386), paromomycin (P-5057) and hygromycin B (H-7772) are obtained from sigma. G418 (geneticin) (345810) is obtained from Calbiochem.

The minimum inhibitory concentration (MIC) tests were performed first in YEPD (1% Bacto-yeast extract, 2% peptone, 2% dextrose) medium to obtain a rough range of MIC. Cultures were started from single colonies and grown in YEPD medium at 30°C overnight. 1:500 dilution of the overnight culture was inoculated for 24hrs in 5ml YEPD with a 5-fold series of dilutions of one of the following aminoglycosides: kanamycin, neomycin, paromomycin and G418. For each drug a range from 5–1000 µg/ml was tested. The MIC for liquid medium was defined as the drug concentration at which the growth of the cultures was completely inhibited after 24h incubation at 30°C. To fine tune the MIC, cultures were grown to mid log-phase in YEPD medium at 30°C, and equal cell numbers (determined by A_{600} units) were streaked at 30°C on YEPD plates with different MIC of aminoglycosides determined in liquid medium. For strains grown out of MIC, further increased concentration was tested. For strains with no growth at MIC, a bit lower concentration was tested to confirm the MIC. The MIC for plate culture, also the reported MIC, was determined as the drug concentration at which the growth of the culture is completely inhibited after 5 days incubation at 30°C.

Dual Luciferase Readthrough Assays

Dual luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promege) as previously described (Keeling et al., 2004). Briefly, yeast strains
were transformed with the indicated readthrough reporter plasmids. Approximately 10^4 cells from each strain were assayed for luminescence with a Berthold Lumat LB9507 luminometer. Assays were done in quadruplicate and the percent readthrough in each strain is expressed as the Firefly / Renilla luciferase activity (nonsense) divided by the Firefly / Renilla luciferase activity (sense) multiplied by 100. The measurement of readthrough efficiency under paromomycin treatment was performed the same except strains with readthrough reporter plasmids were cultured with indicated drug concentration through the assay.

**Misincorporation Assays**

The misincorporation assay was performed using a misincorporation reporter system (Salas-Marco & Bedwell, 2005). The misincorporation reporter constructs also contain dual luciferase genes with an in-frame linker between upstream Renilla gene and downstream firefly gene carrying or not carrying a His245Arg (CGC) mutation. After transforming yeast strains with the misincorporation reporters, the assay were performed the same as readthrough assay. The percentage misreading was expressed as Firefly (mutant) / Renilla luciferase activity divided by the Firefly (wild type) / Renilla luciferase activity multiplied by 100. The measurement of misreading under paromomycin treatment was performed the same except strains with misincorporation reporter plasmids were cultured with indicated drug concentration through the assay.
Acknowledgements

We thank Masayasu Nomura for providing the \textit{rdn\Delta\Delta} yeast strain and plasmids. This work was supported by NIH grant RO1 GM 68854 (to DMB).

References


MUTATIONS THAT RESTORE STANDARD GENETIC CODE RECOGNITION TO A VARIANT CODE EUPLOTES HYBRID ERF1 DEFINE KEY DETERMINANTS OF STOP CODON RECOGNITION

by

HUA FAN-MINOQUE, MING DU, ADAM KALLMEYER, JOE SALAS-MARCO, SUNNIE THOMPSON, ANDREY V. PISAREV, TATYANA V. PESTOVA AND DAVID M. BEDWELL

In preparation for Molecular Cell

Format adapted for dissertation
Abstract

The eukaryotic polypeptide release factor eRF1 recognizes UAA, UAG, and UGA stop codons in organisms that utilize the standard genetic code, while the eRF1 in *Euplotes octocarinatus*, a variant code organism, recognizes only UAA and UAG stop codons. We previously showed that a hybrid eRF1 carrying the *Euplotes* domain 1 fused to *S. cerevisiae* domains 2 and 3 (Eo/Sc eRF1) recognized UAA and UAG, but not UGA, as stop codons and could not support cell viability. We now report the identification of mutations in Eo/Sc eRF1 that restore UGA recognition and cell viability. The location of these mutations supports the cavity model for stop codon recognition. Furthermore, an in vitro peptide release assay revealed that mutants with changes in the TASNIKS motif no longer requires eRF3 for release at UAA and UAG codons. These results lead us to propose an integrated model for eRF1 and eRF3 function during translation termination.

Introduction

Translation termination occurs when one of three stop codons, UAA, UAG or UGA, enters the ribosomal A site. Two classes of release factors mediate this process in both prokaryotes and eukaryotes, although the mechanisms are quite distinct. Prokaryotes have two class I release factors, RF1 and RF2. RF1 recognizes UAA and UAG stop codons through the action of the linear tri-peptide anticodon Pro-Ala-Thr (PAT), while RF2 recognizes UAA and UGA codons using the tri-peptide anticodon Ser-Pro-Phe (SPF) (Ito et al., 2000; Nakamura and Ito, 2002). In these critical stop codon recognition sequences, the first and third amino acids are thought to discriminate the second and third purine bases, respectively. The prokaryotic class II release factor, RF3, uses GTP
hydrolysis to recycle the class I factors after polypeptide chain release (Zavialov et al., 2001). Eukaryotes only have one class I release factor, eRF1, which recognizes all three stop codons (Kisselev et al., 2003). The mechanism used by eRF1 to recognize the three stop codons is still unknown. The eukaryotic class II release factor, eRF3, facilitates eRF1 stop codon recognition and carries out GTP hydrolysis prior to polypeptide chain release (Alkalaeva et al., 2006; Salas-Marco and Bedwell, 2004).

eRF1 proteins from eukaryotic species share significant homology at the primary amino acid sequence level and are composed of three distinct functional domains (Song et al., 2000). Domain 1 is thought to recognize stop codons in the ribosomal A site, and contains the highly conserved TASNIKS and YxCxxxF motifs (Bertram et al., 2000; Frolova et al., 2002; Inagaki et al., 2002; Song et al., 2000). Domain 2, which contains the universally conserved GGQ motif, interacts with the peptidyl transferase center of the ribosome to trigger peptidyl-tRNA hydrolysis (Frolova et al., 1999; Seit-Nebi et al., 2001; Song et al., 2000). Domain 3 of eRF1 mediates eRF3 binding (Eurwilaichitr et al., 1999; Ito et al., 1998).

Organisms that deviate from the standard genetic code are called variant code organisms. Among these organisms, many ciliate species have reassigned one or more stop codons. For example, *Tetrahymena* species reassigned both UAA and UAG to glutamine codons and retain only UGA as a stop codon, while *Euplotes* species reassigned UGA to a cysteine codon and retain UAA and UAG as stop codons. Ciliate eRF1 proteins retain substantial overall amino acid sequence homology with eRF1s from standard code organisms. It has been suggested that highly conserved amino acid motifs in standard code organisms that are more degenerate in variant code organisms may
represent key residues that mediate stop codon recognition (Kim et al., 2005; Knight et al., 2000; Lozupone et al., 2001; Song et al., 2000). The TASNIKS and YxCxxxF motifs (amino acids 55-61 and 122-128 of \textit{S. cerevisiae} eRF1, respectively) have been implicated in stop codon recognition by this approach. Subsequent in vitro studies have shown that various mutations in these elements alter the efficiency of polypeptide chain release (Frolova et al., 2002; Seit-Nebi et al., 2002). However, definitive evidence that these sequence elements are responsible for stop codon recognition has remained elusive.

In a recent study, domain 1 from \textit{Euplotes octocarinatus} eRF1 was fused to domains 2 and 3 of \textit{S. cerevisiae} eRF1 and the resulting hybrid protein (referred to hereafter as Eo/Sc eRF1) was tested for the ability to complement a knockout of the essential \textit{SUP45} gene that encodes eRF1 (Salas-Marco et al., 2006). This Eo/Sc eRF1 could not support viability as the sole source of eRF1 in yeast cells, and was subsequently shown to recognize UAA and UAG stop codons, but not the UGA stop codon. These results demonstrated that domain 1 of \textit{Euplotes} eRF1 was sufficient to recapitulate variant stop codon recognition. In the current study, we use this Eo/Sc eRF1 to show that mutations at residues S57 and N58 of the TASNIKS motif, T75 in \(\alpha\)-helix 3, and C124 of the YxCxxxF motif (all \textit{S. cerevisiae} eRF1 numbering) completely restore UGA recognition by this hybrid eRF1 protein.
Result

Identification of Intragenic Suppressor Mutations in a Hybrid Eo/Sc eRF1 that Restore Growth

We previously found that a hybrid Eo/Sc eRF1 was unable to support growth when provided as the only source of eRF1 in yeast cells (Figure 1) (Salas-Maro et al., 2006). Dual luciferase readthrough assays (Keeling et al., 2004; Salas-Maro and Bedwell, 2005) revealed that Eo/Sc eRF1 efficiently recognized UAA and UAG as stop codons, but not the UGA codon. These findings led us to hypothesize that intragenic suppressor mutations that restore UGA recognition should also restore the growth of cells expressing Eo/Sc eRF1, thus identifying functional determinants of UGA recognition.

A centromeric plasmid carrying the Eo/Sc hybrid eRF1 gene was subjected to random mutagenesis and a pooled mutant library was transformed into a sup45Δ strain that also carried a plasmid encoding the wild type SUP45 under GAL1 promoter control (Figure 1A). Transformants were selected at 30°C on plates containing glucose to shut off expression of the wild type SUP45 gene. Three independent suppressors that restored growth carried the same Cys to Ser substitution at codon 124 (C124S; S. cerevisiae eRF1 numbering) in the highly conserved YxCxxxxF motif.

Mutations at C124 of the YxCxxxxF Motif Restore UGA Recognition by Eo/Sc Hybrid eRF1

The Eo/Sc C124S eRF1 mutant restored cell viability, but was associated with a slow growth and cold-sensitive phenotype (Figure 1B and Table 2, Supplemental Materials). To determine the efficiency of stop codon recognition by Eo/Sc C124S eRF1,
**Figure 1:** Mutagenesis of Eo/Sc eRF1. (A) The Eo/Sc eRF1 plasmid used for random mutagenesis and the Sc eRF1 plasmid under GAL1 promoter control used to support growth of the sup45Δ strain while screening for suppressors of the hybrid Eo/Sc eRF1. (B) Plasmid shuffling to test viability of the Eo/Sc C124S eRF1. 5-FOA plates were used to shuffle out wild type eRF1. Plates were incubated at 22°C, 30°C and 35°C for 5 days. (C) Readthrough levels of wild type Sc eRF1, Eo/Sc eRF1 and Eo/Sc eRF1C124S at 35°C. Readthrough in strains carrying the Eo/Sc eRF1 that cannot support cell growth as the sole source of eRF1 was carried out using a galactose to glucose shift protocol as described in the Materials and Methods.
a dual luciferase-based readthrough assay was carried out (Grentzmann et al., 1998; Howard et al., 2000; Keeling et al., 2004; Salas-Marco and Bedwell, 2004, 2005; Salas-Marco et al., 2006). This assay monitors the efficiency of stop codon recognition, since efficient release factor binding should preclude suppression of the stop codon by a near-cognate tRNA. As controls, stop codon recognition was also measured in strains expressing either wild type Sc eRF1 or the original Eo/Sc hybrid eRF1 (Figure 1C). Stop codon recognition was efficient (<0.3% readthrough) at all three stop codons in the strain expressing wild type Sc eRF1. Since the Eo/Sc eRF1 (expressed under SUP45 promoter control) was unable to support cell viability, growth of the strain expressing this construct was maintained by a plasmid expressing wild type Sc eRF1 under GAL1 promoter control. This strain was grown in SM galactose medium, and then shifted to SM glucose to shut off wild type eRF1 expression. After growth for 6 more generations, the residual level of Sc eRF1 was ~10% of normal (Salas-Marco et al., 2006), which allowed us to estimate the stop codon recognition of Eo/Sc eRF1. We found that Eo/Sc eRF1 had low readthrough (<0.7%) at the UAA and UAG stop codons, while >10% readthrough was observed at the UGA codon. These results confirmed our previous finding that Eo/Sc eRF1 efficiently recognizes UAA and UAG codons, but is severely compromised in UGA recognition.

We found that the strain expressing Eo/Sc C124S eRF1 exhibited <0.4% readthrough at UAA and UAG stop codons, indicating efficient recognition was retained these stop codons (Figure 1C). Remarkably, only ~0.15% readthrough was measured at the UGA stop codon, which was less than the readthrough observed with wild type Sc eRF1. These results indicate that the C124S mutation successfully restored efficient
recognition of the UGA stop codon without significantly compromising recognition of the UAA or UAG stop codons.

The cysteine residue of the YxCxxxF motif in domain 1 appears to be universally conserved among eukaryotic eRF1 proteins (including variant code ciliate species) (Kim et al., 2005; Kisselev et al., 2003). To determine whether other amino acid substitutions at this position also restore UGA recognition by Eo/Sc eRF1, we tested each of the other 18 amino acids at this position. We found that only C124N could support cell viability, although it was associated with a cold-sensitive phenotype like the C124S mutation (Table 2 in the Supplemental Materials). Readthrough assays carried out with strains expressing Eo/Sc C124N eRF1 revealed very low readthrough (~0.1%) at the UGA stop codon, and 0.2 to 0.4% readthrough at the UAA and UAG codons (Figure 2B). These results indicate that the C124N mutation, like C124S, is able to efficiently restore UGA stop codon recognition without compromising UAA and UAG stop codon recognition.

While characterizing the C124N mutation, we found a fast growing colony that had spontaneously acquired a second mutation, A75S (S. cerevisiae eRF1 numbering; corresponds to T75 in Sc eRF1). Interestingly, this mutation is located directly adjacent to C124 in the three-dimensional structure of eRF1. Strains expressing Eo/Sc eRF1 proteins with a combination of either the A75S/C124N or A75S/C124S mutations grew better at 30°C and 35°C than a strain expressing an Eo/Sc eRF1 with either C124 mutation alone (Table 2 in the Supplemental Materials). Furthermore, the Eo/Sc A75S eRF1 supported cell growth as the sole source of eRF1. Readthrough assays carried out on strains expressing Eo/Sc A75S eRF1 showed very low readthrough (0.2%) at UAA and UAG codons, but 0.8% readthrough at UGA (Figure 2B). Thus, the A75S mutation
Figure 2: Efficiency of stop codon recognition mediated by Eo/Sc eRF1 suppressor mutants. (A) Alignment of TASNIKS and YxCxxxF motifs from Saccharomyces eRF1 and Euplotes eRF1. Numbering from Saccharomyces eRF1 was used. (B) Readthrough of stop codons measured in strains expressing wild type Sc eRF1 or Eo/Sc C124S, C124N, A75S or C124N/A75S eRF1. (C) Western blot quantitation of eRF1 levels from strains in (B). (D) Readthrough of stop codons measured in strains expressing wild type Sc eRF1 or Eo/Sc C124S, E57S/S58N, or E57S/S58N/C124S eRF1. (E) Western blot quantitation of eRF1 levels from strains in (D). All strains were grown in SM glucose medium at 35°C.
restored UGA recognition less efficiently than the C124S or C124N mutations. However, the combination of the A75S and C124N mutations resulted in less than 0.2% readthrough at the UGA codon, and about 0.2% and 0.3% readthrough at the UAA and UAG codons, respectively. Given that growth is better when A75S is combined with either C124N or C124S, it appears that combining these mutations in eRF1 optimizes an aspect of eRF1 function not reflected by the readthrough assay.

Since changes in the steady-state level of the Eo/Sc eRF1 suppressors might influence the overall termination efficiency, we next examined the relative abundance of each Eo/Sc eRF1 by western blot analysis. We found that the steady-state level of eRF1 was not reduced by any of the suppressor mutations. In fact, we found that the steady-state level of the hybrid eRF1 was elevated 2 to 3-fold in the strains that had regained UGA recognition (Figure 2C). These results are consistent with our recent finding that a regulatory mechanism increases steady-state eRF1 mRNA (and protein) levels when translation termination is compromised in *Saccharomyces cerevisiae* (Kallmeyer and Bedwell, manuscript in preparation).

**TASNIKS Mutations also Restore UGA Recognition and Function Cooperatively with C124S in the Eo/Sc Hybrid eRF1**

Previous studies have implicated the NIKS motif (residues 58 to 61 of *S. cerevisiae* eRF1) in stop codon recognition (Frolova et al., 2002; Seit-Nebi et al., 2002). This motif is highly conserved in eRF1 proteins from standard code organisms, and crosslinking studies suggest that it is in close proximity to stop codons in the A site (Chavatte et al., 2002). Furthermore, this motif is much more divergent in variant code
organisms, suggesting that divergence from the consensus TASNIKS sequence may contribute to changes in stop codon recognition in variant code species (Inagaki and Doolittle, 2001; Knight et al., 2000). In *Euplotes octocarinatus* eRF1a (used in this study), this motif has the sequence TAESIKS, which differs from the consensus TASNIKS by the presence of a glutamic acid (E) at residue 57 and serine (S) at residue 58 (Figure 2A). To determine the importance of these variations on stop codon recognition, we introduced single amino acid changes in the Eo/Sc hybrid eRF1 (E57S or S58N) or a double mutant (E57S/S58N) that restored the standard TASNIKS motif. Viability assays indicated that the single mutations did not allow growth in cells expressing these mutants as the sole source of eRF1. In contrast, the Eo/Sc eRF1 containing both mutations (Eo/Sc E57S/S58N eRF1) restored viability, but growth was again cold sensitive (Table 2, Supplemental Materials).

We next asked whether combining mutations in the TASNIKS and YxCxxxF motifs could further improve growth of the strain expressing Eo/Sc eRF1. We found that strains expressing Eo/Sc eRF1 proteins containing either single TASNIKS mutation, E57S or S58N, in conjunction with the YxCxxxF mutation C124S mutation were viable and had better growth than a strain expressing the Eo/Sc C124S eRF1 alone (Table 2, Supplemental Materials). The triple mutant, Eo/Sc E57S/S58N/C124S eRF1, exhibited the best growth. These results indicated that changes in the TASNIKS motif and C124 are cooperative with respect to cell growth. We also examined the affects of combining the E57S/S58N mutations with A75S in Eo/Sc eRF1. Viability assays showed that this combination of mutations also improved growth as compared to the two TASNIKS or the
A75S mutation alone. The E57S/S58N/A75S mutant grew less well than the E57S/S58N/C124S mutant, but better than the A75S/C124S mutant.

Readthrough assays carried out with a strain expressing Eo/Sc E57S/S58N eRF1 showed 0.2% readthrough at UAA and UAG, but 0.7% readthrough at UGA (Figure 2D). These results indicated that the E57S/S58N mutations were less efficient in restoring UGA recognition than the C124S mutation alone. However, the E57S/S58N/C124S triple mutant exhibited less than 0.2% readthrough at all three stop codons. Readthrough associated with an Eo/Sc eRF1 carrying all three mutations was improved over Eo/Sc eRF1 proteins carrying the two TASNIKS mutations or the C124S mutation separately, again suggesting that the C124S mutation acts cooperatively with the E57S/S58N mutations to fine-tune overall eRF1 function. Consistent with this premise, western blot analysis of the steady-state levels of these eRF1 proteins revealed a 2.3-fold excess of Eo/Sc C124S eRF1 and a 4-fold excess of Eo/Sc E57S/S58N eRF1, while the Eo/Sc E57S/S58N/C124S eRF1 level was reduced to a level only 1.3-fold above normal (Figure 2E). Since increased eRF1 levels correlate with a defect in eRF1 function, these results suggest that overall eRF1 function was better when all three mutations were present.

**TASNIS and C124 Mutations Reduce Stop Codon Recognition by S. cerevisiae eRF1**

Our results indicate that both the TASNIKS motif and C124 are important for UGA recognition by Eo/Sc eRF1. To determine the role of the corresponding residues in Sc eRF1, we introduced the C124S mutation and altered the TASNIKS motif to the TAESIKS element found in *Euplotes* eRF1 by introducing single (S57E or N58S) or double mutations (S57E/N58S). Viability assays showed that Sc eRF1 with the single
mutations, C124S, S57E or N58S, did not eliminate cell viability, but all reduced the
growth rate relative to a strain expressing wild type Sc eRF1 (Table 3 in the
Supplemental Materials). The double TASNIKS mutant (S57E/N58S) resulted in a more
severe growth defect. Readthrough assays carried out with a strain expressing Sc C124S
eRF1 showed 0.35% and 0.2% readthrough at UAA and UAG respectively, similar to
wild type Sc eRF1 (Figure 3A). Readthrough at the UGA codon was less than 0.2%,
which was about two fold less than the readthrough observed with wild type Sc eRF1.
These results indicate that the C124S mutation makes UGA recognition more efficient in
a standard code eRF1, as it does in the variant code Eo/Sc eRF1.

Readthrough assays of Sc eRF1 proteins carrying the S57E, N58S and
S57E/N58S mutations showed a low level of readthrough (0.2 to 0.35%) at the UAA and
UAG stop codons. However, 0.8% to 1.0% readthrough was observed at the UGA stop
codon in strains expressing these mutant proteins, a ≥2-fold increase in readthrough
compared to wild type Sc eRF1 (Figure 3A). This suggests that these changes in the
TASNIKS motif of Sc eRF1 diminished the efficiency of UGA recognition, consistent
with the observation that mutations that improve the TASNIKS homology in Eo/Sc eRF1
increased the efficiency of UGA recognition. We found that the level of the Sc S57E,
N58S or C124S eRF1 proteins was normal (Figure 3B). In contrast, the S57E/N58S
double mutant showed a slight (1.4-fold) increase in eRF1 abundance, suggesting a
partial defect in eRF1 function.

When we combined the S57E or N58S TASNIKS mutations with C124S in Sc
eRF1, we found that none of the strains expressing pairwise combinations or the triple
mutant were viable (Table 3 in the Supplemental Materials). To determine the effect of
Figure 3: Efficiency of stop codon recognition mediated by Sc eRF1 C124 and TASNIKS mutants. (A) Readthrough of stop codons measured in strains expressing Sc eRF1 with C124S or viable TASNIKS mutations. (B) Western blot quantitation of eRF1 levels from strains in (A). (C) Readthrough in strains carrying the wild type Sc eRF1, depleted of eRF1, or combinations of Sc eRF1 mutations that result in an inability to support growth as the sole source of eRF1. For mutant eRF1 proteins unable to support growth, readthrough was measured using a galactose to glucose shift protocol as described in the Materials and Methods. All strains were grown in SM glucose medium at 35°C.
the mutations on stop codon recognition, we carried out readthrough assays on strains expressing these mutant Sc eRF1 proteins in yeast cells after wild type Sc eRF1 had been depleted by growth in the absence of wild type Sc eRF1 for six generations. A strain constitutively expressing wild type Sc eRF1 was used as a positive control, while a strain depleted of eRF1 was used as a negative control (Figure 3C). The wild type strain exhibited 0.5% readthrough at the UAA stop codon, while 5.3% readthrough was observed in the eRF1-depleted strain. The strain expressing Sc S57E/C124S eRF1 exhibited a wild type level of readthrough (0.6%) at the UAA codon. In contrast, the strains expressing Sc N58S/C124S eRF1 and S57E/N58S/C124S eRF1 resulted in 5.1% and 4.0% readthrough, respectively, at the UAA stop codon (8 to 10-fold higher than wild type eRF1). This was similar to the readthrough observed following complete eRF1 depletion.

At the UAG stop codon, readthrough in the strain expressing wild type eRF1 was 0.2%, while readthrough in the eRF1-depleted strain was 4.7%. Readthrough in the strain expressing Sc S57E/C124S eRF1 was 1.1% (5 fold higher than wild type eRF1). Readthrough in strains expressing Sc N58S/C124S eRF1 or Sc S57E/N58S/C124S eRF1 was 4.8% and 3.9% respectively, again near the level observed following eRF1 depletion. At the UGA stop codon, 0.8% readthrough was observed in the strain expressing wild type Sc eRF1, while 18.4% readthrough was measured following eRF1 depletion. Readthrough at the UGA codon in strains expressing Sc eRF1 carrying a single TASNIKS mutation and C124S (S57E/C124S or N58S/C124S) was normal (0.6%), while readthrough in a strain expressing both TASNIKS mutations with C124S
(S57E/N58S/C124S) was 2.6%, or 4.3-fold higher than the strain expressing wild type Sc eRF1.

These results show that the S57E/C124S eRF1 exhibited a partial defect in UAG recognition. The Sc N58S/C124S eRF1 and Sc S57E/N58S/C124S eRF1 both had severe defects in UAA and UAG recognition, while the latter mutant also exhibited a partial defect in UGA recognition. These results suggest that the S57 and N58 residues in the TASNIKS motif function in UAA, UAG, and UGA recognition in conjunction with the C124 position. In particular, N58 of the TASNIKS motif clearly plays a critical role in UAA and UAG recognition. In contrast, the C124S mutation exerted a negative effect of UAA and UAG stop codon recognition when combined with the N58S TASNIKS mutation, suggesting that C124 plays a key role in maintaining stop codon recognition together with this TASNIKS residue. Only when all three mutations are combined does UGA recognition deteriorate. When taken together, these results suggest that the TASNIKS and YxCxxxF motifs function together to maintain efficient recognition of all three stop codons.

**Compromised eRF1 Function Leads to General Translation Defects.**

Although the Eo/Sc eRF1 suppressors restored UGA stop codon recognition and cell viability, the level of growth was poor compared to cells expressing wild type Sc eRF1. Moreover, differences in growth frequently did not correlate with the efficiency of UGA stop codon recognition, suggesting that defects in eRF1 function other than stop codon recognition may be involved. Consistent with this premise, we observed reduced rates of $[^{35}S]$-methionine/cysteine incorporation and increased paromomycin sensitivity
in strains carrying the hybrid eRF1 and suppressors (Supplemental Figure 1). To further determine whether the slow-growth phenotype associated with the Eo/Sc eRF1 suppressors resulted from translation defects, we analyzed polyribosome profiles in strains expressing wild type Sc eRF1 or the Eo/Sc C124S, E57S/S58N or E57S/S58N/C124S eRF1 proteins. Polysomes were prepared with or without cycloheximide treatment. Cycloheximide is a general translation elongation inhibitor that prevents the dissociation of ribosomes from the mRNA during cell lysis and fractionation. With this drug, defects in translation initiation can be detected by the reduction of the polysome/monosome (P/M) ratio (Choi et al., 1998). In the absence of this drug, most ribosomes in a wild type strain will run off the mRNA during processing. However, strains with defects in elongation (Anand et al., 2003) or termination should show a persistence of ribosomes bound to mRNA that would be manifested by a decrease in the fraction of free subunits in the cell.

In polysome profiles from each of the strains treated with cycloheximide, we observed a reduction in the P/M ratio compared to that of wild type Sc eRF1 (Figures 4A and B). The strain expressing the Eo/Sc C124S eRF1 showed the most severe P/M reduction (55%), while strains expressing the Eo/Sc E57S/S58N and E57S/S58N/C124S eRF1s exhibited smaller reductions of 20% and 28%, respectively. These results suggest that these mutations result in a reduced frequency of translation initiation, consistent with the reduced growth and protein synthesis rates observed (see Supplemental Results). We also observed “halfmers” in polysome profiles from the strain expressing Eo/Sc C124S eRF1 (Figure 4A, arrows). Halfmers are generally indicative of 48S initiation complexes.
**Figure 4:** Polysome analysis of strains expressing Eo/Sc eRF1 mutants. (A) Polysome profiles. The location of 40S subunits, 60S subunits, 80S monosomes and polysomes are indicated. Arrows indicate the location of halfmers. (B) Quantitation of polysomes versus monosomes in polysome profiles shown in (A). (C) Quantitation of free subunits versus total ribosomes and ribosomal subunits in polysome profiles shown in (A). Each polysome profile and quantitation shown was repeated at least twice with similar results.
that accumulate on the mRNA due to an inability to efficiently form functional 80S ribosomes (Helser et al., 1981; Nielsen et al., 2004; Sydorsky et al., 2003). This finding suggests that the Eo/Sc C124S eRF1 causes not only termination defects, but also initiation defects. The ratio of free/total subunits from cycloheximide-treated cells did not change appreciably in strains expressing any of the mutant eRF1s (Figure 4C).

In polysome profiles prepared without cycloheximide treatment, we found that free 40S and 60S subunits relative to total subunits was consistently reduced roughly one-third in strains expressing the Eo/Sc C124S eRF1, Eo/Sc E57S/S58N eRF1 and Eo/Sc E57S/S58N/C124S eRF1 compared to polysome profiles prepared from a strain expressing wild type Sc eRF1 (Figures 4A and 4C). These data suggest that the Eo/Sc eRF1 suppressors exhibit reduced rates of translation termination and/or recycling, resulting in slower ribosome release from cellular mRNAs in the absence of cycloheximide.

*The Eo/Sc eRF1 Suppressor Mutations Restore Polypeptide Chain Release at UGA Codons*

We next examined the relative efficiency of the Eo/Sc eRF1 suppressors in mediating polypeptide release at different stop codons using a recently described *in vitro* peptide release assay (Alkalaeva et al., 2006). In this assay, mammalian pre-termination complexes (pre-TCs) containing peptidyl-tRNA in the P site and a stop codon in the A site were assembled on Met-Val-His-Cys (MVHC-STOP) mRNAs encoding a MVHC tetrapeptide followed by either a UAA, UAG or UGA stop codon using 40S and 60S ribosomal subunits, purified initiation and elongation factors, and aminoacylated tRNAs.
Pre-TCs were purified by sucrose gradient centrifugation, and peptide release was initiated by the addition of eRFs. Release of M-V-H-[\textsuperscript{35}S]C tetrapeptide as a function of time was monitored by scintillation counting of supernatants after TCA precipitation of reaction mixtures.

We first verified the ability of yeast eRFs (Sc eRF1 and Sc eRF3) to promote peptide release on mammalian pre-TCs. Incubation of pre-TCs for 20 minutes with saturating amounts of Sc eRF1 alone or in combination with Sc eRF3 in the presence of GTP resulted in nearly complete peptide release, just as with human (Hs) eRFs (Figure 5A). Release mediated by the combination of Sc eRF1 and Sc eRF3 was inhibited by the non-hydrolyzable GTP analog GMPPNP, again as with Hs eRFs (Alkalaeva et al., 2006).

To investigate the kinetics of peptide release, the concentrations of Sc eRFs were reduced, but release factors continued to be in excess over pre-TCs so that their recycling was not required. Like Hs eRF1, Sc eRF1 alone promoted slow peptide release, which was strongly increased by Sc eRF3 in the presence of GTP (Figure 5B, closed and open circles). These results indicated that the behavior of yeast and human eRFs was identical in this \textit{in vitro} system, which justified the use of mammalian pre-TCs to study the activities of yeast release factors.

We next compared the rate of peptide release induced by Sc eRF1, Eo/Sc eRF1, Eo/Sc E57S/S58N eRF1, and Eo/Sc C124S eRF1 in the presence or absence of Sc eRF3. As in the case of Sc eRF1, slow peptide release at UAA and UAG stop codons promoted by the original Eo/Sc eRF1 hybrid alone was strongly stimulated by Sc eRF3•GTP (Figures 5C and 5D, closed and open squares). However, peptide release promoted by Eo/Sc eRF1 and Sc eRF3•GTP was nevertheless slower than peptide release promoted by
Figure 5: Eo/Sc eRF1 mutants restore peptide release at the UGA stop codon. A) Endpoint peptide release assay with excess Sc eRF1 and Sc eRF3. B) Kinetics of peptide release with Sc eRF1 and Sc eRF3. C) Kinetics of peptide release with Eo/Sc eRF1 and mutant derivatives at UAA stop codon. D) Kinetics of peptide release with Eo/Sc eRF1 and mutant derivatives at UAG stop codon. E) Kinetics of peptide release with Eo/Sc eRF1 and mutant derivatives at UGA stop codon. Representative data for each experiment is shown. Reactions with eRF1s are denoted by closed symbols, while reactions with eRF1s and Sc eRF3 are denoted by open symbols.
Sc eRF1 and Sc eRF3•GTP, which was most likely due to the heterogeneity of the system: even though mammalian pre-TCs could tolerate yeast release factors well, the use of the Eo/Sc eRF1 hybrid introduced an additional challenge. Consistent with the in vivo data, peptide release induced by Eo/Sc eRF1 on the UGA codon was slow even in the presence of Sc eRF3•GTP (Figure 5E, closed and open squares). Again, reflecting the in vivo situation, the E57S/S58N and C124S mutations in Eo/Sc eRF1 restored its activity at the UGA stop codon: the rates of peptide release on the UGA codon by Eo/Sc E57S/S58N eRF1 and Eo/Sc C124S eRF1 in the absence and in the presence of Sc eRF3/GTP (Figure 5E, closed and open triangles and diamonds, respectively) were similar to the rates of peptide release by Eo/Sc eRF1 on UAA and UAG codons (Figure 5C and 5D, closed and open squares). The C124S mutation did not change the activity of Eo/Sc eRF1 at UAA and UAG codons, and as in the case of the original Eo/Sc eRF1 hybrid, the low activity of Eo/Sc C124S eRF1 alone was also strongly enhanced by Sc eRF3•GTP (Figures 5C and 5D, closed and open diamonds). Surprisingly, the rate of peptide release on UAA and UAG codons by Eo/Sc E57S/S58N eRF1 alone was as high as the rates of peptide release on these codons by Eo/Sc eRF1 and Eo/Sc C124S eRF1 in the presence of Sc eRF3•GTP, and was not stimulated further by Sc eRF3•GTP (Figure 5C, open and closed triangles). Taken together, these data indicate that the E57S/S58N and C124S mutations restored the peptide release activity of Eo/Sc eRF1 at the UGA stop codon. In addition, the E57S/S58N mutations enabled Eo/Sc eRF1 to promote efficient peptide release on UAA and UAG codons independently of eRF3.
Discussion

Various models have been proposed to explain how eRF1 mediates stop codon recognition. First, phylogenetic comparisons of eRF1 proteins from diverse species found that the NIKS motif is highly conserved among standard code organisms, but frequently diverges in variant code organisms (Kim et al., 2005; Knight et al., 2000). By analogy to features of the peptide anticodon found in prokaryotes, Nakamura noted that residues T55/A56/S57 (the “TAS” of the TASNIKS motif; \textit{S. cerevisiae} numbering) shares key features of the bacterial PAT and SPF motifs, and suggested that it may function as a linear peptide anticodon in eRF1 (Nakamura et al., 2000). In another model, it was proposed that the region at the end of $\alpha$-helix 3 (which includes the TASNIKS motif) forms a flexible element that can assume a tight or relaxed conformation as a function of the stop codon recognized (Muramatsu et al., 2001). Specifically, it was suggested that amino acids, G54 and T55 (\textit{S. cerevisiae} numbering) interact with the second nucleotide of the stop codon, while S57 and N58 recognize the third nucleotide.

Subsequently, a genetic screen in yeast identified several mutations throughout domain 1 that increased readthrough of UAG or UGA stop codons (Bertram et al., 2000). Based on these results, a model was proposed in which the three nucleotides of the stop codon bind to three pockets (or cavities) located near many of the identified mutations (Figure 6A). In this model, cavities 1, 2, and 3 bound the first, second, and third nucleotides of the stop codon, respectively. Later, a study that considered these data in conjunction with evolutionary rates of amino acid changes in domain 1 agreed in principle with the cavity-binding model, but proposed that the orientation of the stop
Figure 6: The location of key determinants of stop codon recognition on the three-dimensional structure of human eRF1. Only the relevant portion of domain 1 is shown. A) Residues identified by genetic screens in this study or by Stansfield and co-workers (Bertram et al., 2000) are indicated in yellow. B) Key residues located near cavity 3 and the TASNIKS motif are shown in yellow. Residues identified by genetic screens in this study (black type), by Stansfield and co-workers (Bertram et al., 2000) (purple type), or other TASNIKS residues (red type) are labeled. Black circles indicate cavities 1, 2, and 3, while the white circle indicates the TASNIKS motif. The residue designations and numbering corresponds to human eRF1 (+3 relative to Sc eRF1).
codon binding to the 3 pockets be reversed (Inagaki et al., 2002). Finally, Kisselev and colleagues probed the effect of mutations in residues of domain 1 that are conserved in standard code organisms, but have diverged in many variant-code organisms. They found that both the NIKS residues within the TASNIKS motif (S. cerevisiae residues 55-61) and the YxCxxxF motif (S. cerevisiae residues 122-128) influence polypeptide chain release, leading them to propose a non-linear model in which stop codon recognition is modulated by positive and negative determinants (Frolova et al., 2002; Seit-Nebi et al., 2002).

In the current study, we identified mutations that restored UGA recognition to a yeast strain expressing a hybrid Eo/Sc eRF1 that initially recognized only UAA and UAG stop codons. This stringent selection demanded that UAA and UAG recognition be retained while gain-of-function mutations restored UGA recognition. We identified the C124S mutation in three independent suppressors, suggesting that this mutation was one of the strongest suppressors that could be obtained. The identification of mutations at C124 was unexpected, since this residue is conserved in all known standard and variant code eukaryotes. In the model of Inagaki et al., it was proposed that C124 is involved in the recognition of the first base of the stop codon (Inagaki et al., 2002). This cannot be correct, since we found that the Eo/Sc eRF1 recognizes only UAA and UAG codons, while the Eo/Sc C124S eRF1 recognizes all three stop codons. In contrast, C124 (C127 in human eRF1) is positioned directly between cavities 2 and 3 in the model of Stansfield and co-workers (Bertram et al., 2000) (Figure 6A), which would put it in an ideal position to discriminate between different stop codons. Furthermore, the A75S mutation (which corresponds to V78 in human eRF1), which also restored UGA recognition, is located in
α-helix 3 directly adjacent to C124 (C127 in human eRF1) in the floor of cavity 3. These results highlight the importance of cavities 2 and 3 in discriminating U-purine-purine codons, and suggest that cavity 3 plays an essential role in distinguishing the UGA and UGG (Trp) codons.

We found that the E57S/S58N mutations (S60/N61 in human eRF1) that converted the TAESIKS sequence found in domain 1 of Euplotes eRF1a to the consensus TASNIKS motif also restored UGA recognition. While this observation could be taken to suggest that these residues play a key role in recognizing the second and/or third positions of stop codons, they are located somewhat distant to cavity 3 (Figure 6B). Based on the growing body of data supporting the cavity model, two possibilities could explain this result. First, many studies have shown that a tetranucleotide termination signal (the stop codon and the following nucleotide) is important for recognition by eRF1 (Bonetti et al., 1995; Brown et al., 1993; Manuvakhova et al., 2000; Martin, 1994). Given these results, it is possible that S60 and N61 (human eRF1 numbering) of the TASNIKS motif influence stop codon recognition by interacting with the fourth nucleotide of the stop signal. We also found that the Eo/Sc eRF1 E57S/S58N no longer requires eRF3 for peptide release at UAA and UAG codons. This suggests that the TASNIKS motif may also function as a conformational switch that couples stop codon recognition, GTP hydrolysis, and peptide release. This functional coupling must require a high degree of communication between the three domains of eRF1, since stop codon recognition by eRF1 domain 1 and GTP hydrolysis by eRF3 (mediated by eRF1 domain 3) must precede polypeptide chain release by eRF1 domain 2 (Alkalaeva et al., 2006; Salas-Marcos and Bedwell, 2004; Song et al., 2000).
eRF1 performs three distinct functions during translation termination: it recognizes a stop codon, activates the GTPase activity of eRF3 and triggers peptide release. Peptide release is strongly stimulated by eRF3 in a GTP-dependent manner, but the mechanism of stimulation is unknown. eRF3 could stimulate peptide release by enhancing eRF1’s association with pre-TCs, by increasing the catalytic rate of peptidyl-tRNA hydrolysis, or both.

There is some evidence that eRF3 stimulates the initial binding of eRF1 to pre-TCs. Binding of the eRF1 (GGQ→AGQ) mutant (which is defective in peptide release) to pre-TCs in the absence of eRF3•GTP results in a 2 nucleotide forward shift of a corresponding toe-print (Alkalaeva et al., 2006). However, this shift appears at much lower concentrations of eRF1 if eRF3•GTP is present (Pestova, unpublished results). If the solution structure of eRF1 is similar to its crystal structure (Song et al., 2000), then eRF1 must adopt a more closed conformation to fit into the ribosomal binding pocket since the distance between decoding and peptidyl transferase centers is smaller than the distance between the TASNIKS and GGQ motifs of crystallized eRF1. Binding to eRF3•GTP might theoretically close eRF1’s structure to meet the ribosomal constraints, thereby enhancing eRF1’s association with pre-TCs. However, pre-TCs that contain eRF1/eRF3•GTP remain inactive in peptide release because the GGQ loop of eRF1 is not properly positioned at the PTC, and eRF1 must induce ribosome-dependent hydrolysis of GTP by eRF3 in order to adopt the conformation and/or position required to trigger peptide release (Alkalaeva et al., 2006). Importantly, mutations in eRF3 that reduce the efficiency of GTP hydrolysis increased readthrough in a codon-dependent manner (Salas-Marco and Bedwell, 2004). Since readthrough is a measure of the efficiency of eRF1
binding at a stop codon, this suggests that a reduced rate of GTP hydrolysis leads to less efficient recognition of those stop codons at which readthrough increased. Thus, in addition to the conformational changes required to activate eRF1 to induce peptide release on all stop codons, GTP hydrolysis also leads to changes that particularly facilitate recognition of a subset of stop codons. This suggests that stop codon recognition might therefore occur in two stages: initial recognition prior to GTP hydrolysis, and tightening of the interaction after GTP is hydrolyzed.

The second stage might be particularly important for recognition of a subset of termination codons, which includes all four UGA-N stop signals (Salas-Marco and Bedwell, 2004). The initial recognition of a stop codon might in turn influence the rate of activation of GTP hydrolysis, making it codon-dependent. Efficient recognition of a UAA or UAG stop codon might activate rapid GTP hydrolysis by eRF3 and promote efficient peptide release, whereas eRF1 binding of a UGA codon might activate GTP hydrolysis less efficiently (possibly to allow discrimination between UGA and UGG codons). Peptide release would occur at the UGA codon as long as GTP hydrolysis took place before dissociation of the termination complex. This might partially account for the higher basal readthrough observed at the UGA stop codon (Bonetti et al., 1995).

eRF1 alone promotes slow peptide release. It is likely that to achieve a higher rate of peptide release, eRF1 must undergo similar conformational/positional rearrangements as when it is bound by eRF3 and GTP hydrolysis occurs. However, in the absence of eRF3 such changes are slow. Remarkably, we found that Eo/Sc E57S/S58N eRF1 did not require eRF3 for peptide release at UAA and UAG codons, but required eRF3 for release at the UGA codon. This suggests that Eo/Sc E57S/S58N eRF1 must be capable of
efficiently and spontaneously achieving the conformation and the position required for efficient peptide release on pre-TCs with UAA and UAG stop codons, but not with a UGA stop codon.

Based on the preceding considerations, we propose the following model for the codon-dependent stimulation of eRF1-induced peptide release by eRF3 (Figure 7). According to this model, eRF1 binding (state 1) to eRF3•GTP yields an eRF1/eRF3•GTP complex that has a higher affinity for pre-TCs, in part probably due to conformational changes in eRF1 (state 2). eRF1/eRF3•GTP cannot facilitate peptide release until GTP is hydrolyzed. Upon binding to pre-TCs and stop codon recognition, eRF1 induces GTP hydrolysis by eRF3, which could be faster at UAA and UAG codons and slower at the UGA codon. GTP hydrolysis induces rearrangement of termination complexes, which includes tightening of the interaction of eRF1 with the stop codon, particularly in the case of the UGA codon, and proper positioning of the GGQ loop in the PTC. After GTP hydrolysis, the final positions/conformations of eRF1 on pre-TCs with UAA/UAG (state 3) and UGA (state 4) codons are probably somewhat different. Both states 3 and 4 can facilitate efficient polypeptide release, although the higher basal readthrough observed at UGA codons (Bonetti et al., 1995) suggests that peptide release in state 3 might be somewhat more efficient. These two conformations of active eRF1 may be necessary to allow eRF1 to readily recognize the UAA and UAG in state 3, while allowing it to not only recognize UGA, but also discriminate against the UGG tryptophan codon in state 4. Our data suggest that Eo/Sc E57S/S58N eRF1 gained the ability to reach state 3, but not state 4, in the absence of eRF3.
Figure 7: Model for eukaryotic translation termination at UAA/UAG versus UGA stop codons.
Among species that use a variant genetic code, two types of stop codon reassignment are found. Some variant code species use only UAA and UAG as stop codons (e.g., *Euplotes* and *Blepharisma* species), while others use only UGA as a stop codon (e.g., *Tetrahymena*, *Stylonychia* and *Loxodes* species) (Inagaki et al., 2002; Kim et al., 2005). The proposed model could readily explain why these two specific patterns of stop codon usage have repeatedly arisen during the evolution of variant code species (Lozupone et al., 2001; Tourancheau et al., 1995). Since it is implicit in this model that peptide release at UAA and UAG stop codons requires a different eRF1 conformation than release at UGA codons, the observed stop codon specificity could arise simply by eRF1 losing the ability to acquire either state 3 or 4 (thus eliminating peptide release at UAA/UAG or UGA codons, respectively). Further studies are needed to define the complex mechanism of eRF1 function and particularly of its stimulation by eRF3.

**Materials and Methods**

**Strains and Plasmids**

The *S. cerevisiae* strain and plasmids used in this study are described in Table 1 of the Supplemental Materials.

**Random Mutagenesis**

Random mutagenesis of plasmid DNA Encoding Eo/Sc eRF1 was carried out using *E. coli* XL1-red cells (Stratagene). Mutant libraries were transformed into YDB447/pDB967 (with Sc eRF1 under *GAL1* promoter control) and plated on SM glucose plates to terminate expression of Sc eRF1 and select plasmids expressing Eo/Sc
eRF1 mutants that had regained UGA recognition. Site-directed mutations of eRF1 were introduced using a QuikChange site-directed mutagenesis kit (Stratagene).

**Viability Assays and Drug Sensitivity**

The ability of plasmids expressing mutant eRF1 was assessed by a plasmid shuffle technique on SM glucose plates containing 5-fluoroorotic acid (5-FOA), which selects for colonies that had lost pUKC802 that expresses wild type Sc eRF1. For viable strains, loss of pUKC802 was confirmed by a lack of growth on SMD URA dropout and plasmids were re-extracted and sequenced to confirm that the mutation was still present. To assay for cold sensitivity, cultures were streaked at 22°C, 30°C and 35°C on 1% Bacto-yeast extract, 2% peptone, 2% dextrose (YEPD) plates. To assess the paromomycin sensitivity, 0.5 A$_{600}$ units of cells and a series of 10-fold serial dilutions were spotted on YEPD plate with different paromomycin concentration at 35°C.

**Dual Luciferase Readthrough Assays**

Dual luciferase readthrough assays of viable eRF1 mutants were performed as described (Salas-Marco et al., 2006). Readthrough assays in inviable mutants were measured following a galactose to glucose shift procedure. First, a sup45Δ strain (YDB447) that co-expressed both wild type Sc eRF1 under GAL1 promoter (pDB967) and the indicated Eo/Sc hybrid eRF1 under SUP45 promoter control was grown in SM galactose medium for several generations. During mid-log growth the cells were harvested and re-suspended in SM glucose medium to a cell density of 0.01 A$_{600}$ units/ml.
After six generations, cells were harvested and assayed for readthrough levels using the dual luciferase assay.

**Yeast Polysome Profile Analysis**

Cultures were grown in YEPD medium at 35°C to a cell density of 0.6 A₆₀₀ units/ml. Cycloheximide was added (when indicated) to a final concentration of 0.1 mg/ml and the culture was immediately chilled on ice for 5 min. Cells were harvested, washed twice in 2.5 ml of ice-cold lysis buffer (20 mM Tris-HCl [pH 8.0], 140 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 1% Triton-X-100, 0.1 mg/ml cycloheximide (when indicated), and 1.0 mg/ml heparin). Cell pellets were re-suspended in 0.7 ml lysis buffer and lysed by vortexing with glass beads. Following centrifugation, 50 A₂₅₄ units of each supernatant was layered on top of an 11 ml linear 20-50% sucrose gradient and centrifuged in an SW41Ti rotor at 3,5000 rpm for 160 min at 4°C. Each experiment was repeated at least two times with similar results.

**Peptide Release Assay**

Peptide release was performed as described (Alkalaeva et al., 2006). Pre-termination complexes (pre-TCs) were formed on MVHC-STOP mRNA (a derivative of the previously described MVHL-STOP mRNA) with the mammalian initiation and elongation factors, ribosomal subunits and aminoacyl tRNAs that included [³⁵S]Cys-tRNA⁵⁰, and purified by centrifugation through a 10-30% linear sucrose gradient for 95 min in an SW55 rotor at 4°C. For the initial characterization of peptide release mediated by Sc eRF1 and Sc eRF3 (Figure 5A), 0.02 pmol pre-TCs were incubated in 40 ml
reaction mixtures with 10 pmol Sc eRF1, 20 pmol Sc eRF3, and 1 mM GTP (or 1 mM GMPPNP as indicated) for 20 minutes at 37°C. To determine the kinetics of peptide release, 0.5 pmol Sc eRF1, Eo/Sc eRF1, or mutant derivatives of Eo/Sc eRF1 and 1 pmol Sc eRF3 were used, while the amount of pre-TCs remained at 0.02 pmol. Release of M-V-H-[\(^{35}\)S]C tetrapeptide was monitored by scintillation counting of supernatants after TCA precipitation of reaction mixtures.

**Supplemental Data**

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, one figure and three tables.
Supplemental Figure 1: Translation defects associated with Sc eRF1 and Eo/Sc eRF1 mutants. A) $[^35S]$ methionine/cysteine incorporation. B) Paromomycin sensitivity. Overnight cultures of strains expressing wild type Sc eRF1, Sc S57E/N58S eRF1, and Eo/Sc C124S, E57S/S58N and E57S/S58N/C124S eRF1s were grown at 35°C and spotted with 1:1, 1:10, 1:100 and 1:1000 dilutions on YPD plates with no paromomycin, 75 µg/ml paromomycin, and 200 µg/ml paromomycin.
Table 1: Strains and plasmids used.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YDB447</td>
<td>MAT $\alpha$ ura3-52 leu2-3,112 ade1-14 lys2− trp1− his3− $\sup45::HIS3$ [psi−]</td>
<td>{Salas-Marco, 2006 #2935}</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sc eRF1 derivatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDB967</td>
<td>YCplac22 carrying GAL1 HA-Sc eRF1, CEN4, TRP1, $amp^R$</td>
<td>{Salas-Marco, 2006 #2935}</td>
</tr>
<tr>
<td>pUKC802</td>
<td>YEplac24 carrying SUP45 Sc eRF1, 2μ, URA3, $amp^R$</td>
<td>{Salas-Marco, 2006 #2935}</td>
</tr>
<tr>
<td>pDB950</td>
<td>YCplac33 carrying SUP45 HA-Sc eRF1, CEN4, URA3, $amp^R$</td>
<td>{Salas-Marco, 2006 #2935}</td>
</tr>
<tr>
<td>pDB1047</td>
<td>YEplac111 carrying SUP45 HA-Sc eRF1, CEN4, LEU2, $amp^R$</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1040</td>
<td>pDB1047 carrying Sc eRF1 C124S</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1045</td>
<td>pDB1047 carrying Sc eRF1 S57E</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1046</td>
<td>pDB1047 carrying Sc eRF1 S57E, C124S</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1041</td>
<td>pDB1047 carrying Sc eRF1 N58S</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1042</td>
<td>pDB1047 carrying Sc eRF1 N58S, C124S</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1044</td>
<td>pDB1047 carrying Sc eRF1 S57E, N58S</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1043</td>
<td>pDB1047 carrying Sc eRF1 S57E, N58S and C124S</td>
<td>this study</td>
</tr>
</tbody>
</table>
Table 1: Strains and plasmids used (continued).

**Eo/Sc eRF1 derivatives**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDB948</td>
<td>YCplac111 carrying <em>SUP45</em> Eo/Sc eRF1, <em>CEN4</em>, <em>LEU2</em>, {Salas-Marco, 2006 #2935}</td>
<td></td>
</tr>
<tr>
<td>pDB974</td>
<td>pDB948 carrying Eo/Sc eRF1 C124S</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1033</td>
<td>pDB948 carrying Eo/Sc eRF1 C124N</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1032</td>
<td>pDB948 carrying Eo/Sc eRF1 A75S</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1030</td>
<td>pDB948 carrying Eo/Sc eRF1 A75S, C124S</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1029</td>
<td>pDB948 carrying Eo/Sc eRF1 A75S, C124N</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1031</td>
<td>pDB948 carrying Eo/Sc eRF1 E57S, A75S, S58N</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1038</td>
<td>pDB948 carrying Eo/Sc eRF1 E57S</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1035</td>
<td>pDB948 carrying Eo/Sc eRF1 E57S, C124S</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1039</td>
<td>pDB948 carrying Eo/Sc eRF1 S58N</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1036</td>
<td>pDB948 carrying Eo/Sc eRF1 S58N, C124S</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1037</td>
<td>pDB948 carrying Eo/Sc eRF1 E57S, S58N</td>
<td>this study</td>
</tr>
<tr>
<td>pDB1034</td>
<td>pDB948 carrying Eo/Sc eRF1 E57S, S58N, C124S</td>
<td>this study</td>
</tr>
<tr>
<td>Mutation</td>
<td>TAESIKS</td>
<td>YxCxxxF</td>
</tr>
<tr>
<td>----------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A75</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A75S</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A75S</td>
<td>—</td>
<td>C124S</td>
</tr>
<tr>
<td>A75S</td>
<td>—</td>
<td>C124N</td>
</tr>
<tr>
<td>—</td>
<td>E57S</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>S58N</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>E57S/S58N</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>E57S</td>
<td>C124S</td>
</tr>
<tr>
<td>—</td>
<td>S58N</td>
<td>C124S</td>
</tr>
<tr>
<td>—</td>
<td>E57S/S58N</td>
<td>C124S</td>
</tr>
<tr>
<td>A75S</td>
<td>E57S/S58N</td>
<td>—</td>
</tr>
</tbody>
</table>

Symbols used to denote growth on YPD plates: (–) no growth; (+/–) very weak growth; (+) weak growth; (++) moderate growth; (+++) good growth. For comparison, growth of wild type Sc eRF1 is (++++).
Table 3: Effect of Sc eRF1 mutations on colony growth

<table>
<thead>
<tr>
<th>Mutation</th>
<th>TASNIKS</th>
<th>YxCxxxF</th>
<th>22°C</th>
<th>30°C</th>
<th>35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>—</td>
<td>C124S</td>
<td></td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>S57E</td>
<td></td>
<td>—</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>N58S</td>
<td></td>
<td>—</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>S57E/N58S</td>
<td></td>
<td>—</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S57E</td>
<td>C124S</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>N58S</td>
<td>C124S</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S57E/N58S</td>
<td>C124S</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Symbols used to denote growth on YPD plates: (−) no growth; (+/−) very weak growth; (+) weak growth; (++) moderate growth; (++++) good growth; wild type growth (+++++).
Acknowledgements

The authors thank Kim Keeling and Mark Walter for helpful discussions and critically reading the manuscript. This work was supported by NIH grants RO1 GM 68854 (DMB) and RO1 GM 80623 (TVP).

References


Ito, K., Ebihara, K., and Nakamura, Y. (1998). The stretch of C-terminal acidic amino acids of translational release factor eRF1 is a primary binding site for eRF3 of fission yeast. RNA 4, 958-972.


Kim, O.T., Yura, K., Go, N., and Harumoto, T. (2005). Newly sequenced eRF1s from ciliates: the diversity of stop codon usage and the molecular surfaces that are important for stop codon interactions. Gene 346, 277-286.


SUMMARY

In this project, we carried out studies on two different components of eukaryotic translation termination, rRNA and eRF1, with the aim to further our understanding of the molecular mechanism of this process. With regard to the role of rRNA in translation termination, we utilized an established yeast system in which all genomic rDNA was completely deleted and for the first time performed \textit{in vivo} functional analysis of eukaryotic decoding center in a homogeneous background. Our data first identified that G1645 and A1754, two bases in the decoding site, together determine eukaryotic resistance to aminoglycoside antibiotics. G1645 and A1754 are the only polymorphic bases in the conserved A site and the corresponding sites in bacteria are A1408 and G1491 respectively. In prokaryotes, for drug development purposes, each base has been extensively studied individually since they were shown to be the main targets of aminoglycosides by both crosslinking and structural data. These data also indicated that the two bases had distinct interactions with different sub-types of aminoglycosides, however, the combination of mutations at these two bases had never been studied in either prokaryotes or any eukaryote. Our study is the first mutational analysis of these bases in eukaryotic rRNA and revealed that both sites had to be mutated to their prokaryotic counterparts to make yeast as sensitive to aminoglycosides as \textit{E. coli}, suggesting that two residues make a combined contribution to aminoglycoside resistance in eukaryotes. Then, we found that mutations at these residues, although they had no direct effect on translation fidelity, influenced both paromomycin-induced misreading of
sense codons and readthrough of stop codons. On the other hand, mutations of the three nucleotides involved in sensing codon-anticodon were all lethal. Although this finding suggested their essential role in translation, we were not able to examine their functional mechanism. Future study will be needed to select and purify tagged lethal mutant ribosomes and perform *in vitro* biochemistry assays to define their functional effect on translation termination.

With regard to eRF1, we utilized a previous characterized hybrid eRF1 with domain 1 from *Euplotes* eRF1, which was not able to support cell growth as the only source of eRF1, to screen for viable suppressors. The non-viable hybrid eRF1 has been shown to lack UGA recognition, thus restoration of UGA recognition would be required to promote viability. Surprisingly, a specific serine mutation at the universally conserved C124 in the YxCxxxF motif was found to support viability and restore recognition of UGA as a stop codon. More interestingly, mutations to all the other amino acids except to asparagine were not viable. This result implied that C124 is in a specific position that plays a crucial role in eRF1 stop codon recognition. In the 3D structure of eRF1, C124 is located right between two cavities that were proposed to be the binding sites for the second and the third nucleotides of the stop codon. However, its universal conservation made it unlikely that C124 was the only factor determining stop codon specificity. Other motifs and residues that are variable must be involved. The TASNIKS motif, conserved in standard code species but variable in variant code species, is an ideal candidate. Mutations that restored TASNIKS motif in Eo/Sc eRF1 were found to support cell growth and restore UGA recognition as a stop codon.
The readthrough assay mainly measures the efficiency of the stop codon recognition step rather than the whole termination process. Readthrough assay of our mutants at three different stop codons revealed that C124 has a dominant role in UGA recognition and the specific mutation C124S is a guarantee of efficient UGA recognition. With C124 in the YxCxxxF motif, the TASNIKS motif is essential for efficient UGA recognition, since changing either S57 or N58 of Sc eRF1 to that in *Euplotes* eRF1 decreases the efficiency of UGA recognition and reconstructing TASNIKS motif in *Euplotes* eRF1 restores UGA recognition. However, with the C124S mutation, retaining the TASNIKS motif is not necessary to recognize UGA but appears to be more related to the recognition of the other two stop codons. Since altering either C124 or the TASNIKS motif alone does not affect UAA and UAG recognition, this implies that improper cooperativity between C124 and TASNIKS motif could alter eRF1’s conformational positioning toward UAA and UAG recognition. This twist may also impede the binding of eRF3 and then the structural rearrangement required for proper positioning of pre-termination complex followed by peptide release (6). The lack of proper positioning in the A site and delay of release can also explain the greater sensitivity to paromomycin and overall translation reduction. Moreover, the cold sensitive phenotype may result from the twisted and unstable structure that is stabilized at higher temperature (32). Taken together, our data suggested a specific role of C124 of YxCxxxF motif and N57/S58 of TASNIKS motif in UGA recognition.

On the other hand, an *in vitro* peptide release assay of our Eo/Sc hybrid eRF1 mutants identified a type of peptide release independent of eRF3 at UAA and UAG but not UGA stop codon. The mutation that had the effect was E57S/S58N, which restored
the TASNIKS motif and UGA recognition in Eo/Sc eRF1. This surprising result suggested that the TASNIKS motif not only affects stop codon recognition but also the coupling between recognition and release. These findings also imply that UAA and UAG recognitions are different from UGA recognition, which may be achieved by eRF1 applying different conformations. Consistent with this hypothesis, GTPase mutant of eRF3 only affected efficiency of a subgroups not all of the stop codons. All the above data led us to propose a different conformation model for eRF1 function. In this model, eRF1 itself binds to the stop codon in the A site and promotes peptide release inefficiently, which is referred to inactive state. Upon binding to eRF3 and GTP, eRF1 obtains higher binding affinity to the stop codon via a possible conformation change and switch to active state. Our E57S/S58N mutation, which can facilitate release independent of eRF3 at UAA and UAG stop codons, implies that eRF1 and eRF3-GTP complex applies different conformations at UAA and UAG from UGA. The necessity for the different conformational states may be due to the added difficulty of discriminating between UGA and UGG codons. However, further stimulation of peptide release requires GTP hydrolysis by eRF3. Hydrolysis of GTP would further induce conformation change of eRF1 to fit the binding pockets in both small and large subunits of ribosome, which consequently promotes efficient polypeptide release.

This different conformation model also corresponds to the type of variant decoding. Among all the species using variant code, there are actually two types of stop codon reassignment: UAR (R: A or G) = stop (Euplotes and Blepharisma) and UGA = stop (Tetrahymena, Stylonychia and Oxytricha). With both our biochemical and genetic data, we hypothesize that some species evolved eRF1 to only a UAR recognizing state...
(Euplotes) or a UGA recognizing state (Tetrahymena). Therefore, evolution may provide strong support for our hypothesis of different conformation states. Future work will focus on the molecular determinants of these states and how they couple and uncouple stop codon recognition and peptide release.

The results for my studies highlight the multi-step complex of translation termination. In future work, a system with a double knockout of rRNA gene and eRF1 will allow us to identify residues or motifs that are involved in the signal relay during translation termination to understand the interplay between ribosomes and release factors,
GENERAL LIST OF REFERENCES


