UNDERSTANDING THE FUNCTION AND REGULATION OF EUKARYOTIC RELEASE FACTOR 1, ERF1

SARA ELISABETH CONARD

MICROBIOLOGY

ABSTRACT

Translation termination is an essential eukaryotic process that facilitates the end of protein synthesis and mediates the release of the nascent peptide chain. Two release factors, eRF1 and eRF3, with the help of a ribosome recycling factor assist in this process. eRF1 and eRF3 form a stable complex through conserved regions in their C-terminal domains. The eRF1/eRF3 complex, along with GTP, is absolutely vital for efficient termination in vitro and in vivo. The role of eRF1 during translation termination is to recognize the stop codon located in the ribosome aminoacyl site, stimulate GTP hydrolysis of eRF3 and enable peptide release through its GGQ motif. eRF3 aids this process in a GTP-dependent manner and through promoting conformational changes in eRF1.

Although recent advancements have elucidated how prokaryotic release factors directly recognize each stop codon (UAA, UAG and UGA), this has not aided our understanding of the mechanism of stop codon recognition utilized by eRF1, since eRF1 3-dimensional structure is significantly different. To gain a better understanding of how eRF1 is able to mediate this essential function, we extensively analyzed two motifs known to play a significant role in stop codon recognition. We found that the YCF motif most often contributed to eRF1 selective stop codon specificity while changes in the TASNIKS motif displayed non-specific stop codon selectivity.
Through our studies we also found that the abundance of eRF1 protein and *eRF1* mRNA (*SUP45* mRNA) is regulated by a post-transcriptional regulatory mechanism. The major component for this increase was contributed by an increase in *SUP45* mRNA stability. We show using *in vitro* and *in vivo* studies that limiting functional termination complexes result in increased levels of *SUP45* mRNA and eRF1 protein.

Keywords: eRF1, eRF3, translation termination, mRNA stability
DEDICATION

To my parents, Morice and Ora Conard, whose love, support and prayers have encouraged and enabled me to pursue my goals. Their dedicated love and belief that I could achieve anything carried me through the rough times. To my sister, Adell, who has been there for me throughout the years.
ACKNOWLEDGEMENTS

I truly appreciate my mentor, Dave for allowing me to perform my graduate studies in his lab. He has given me the space and freedom to build my confidence as a researcher. His guidance throughout the years has driven me to become a better scientist. I would like to thank the members of my dissertation committee, Ching-Yi Chen, James Collawn, Kim Keeling, Sunnie Thompson and Mark Walter whose helpful discussions have greatly contributed to my success in graduate school and beyond. Special thanks to Kim for being available for my endless questions, to Sunnie for career guidance and help in learning new techniques and to Mark who opened up his lab to me for a number of years.

I am grateful to the members of the Bedwell lab, past and present. Your friendship and support have made each day more pleasant.

I am forever grateful to my immediate and extended family and friends for their love and unyielding support. Your prayers, emotional and financial support have carried me through this journey.

I am eternally thankful to God for His love, grace and mercy.
TABLE OF CONTENTS

Page

ABSTRACT .................................................................................................................. ii

DEDICATION .............................................................................................................. iv

ACKNOWLEDGEMENTS ............................................................................................. v

LIST OF TABLES ........................................................................................................ vii

LIST OF FIGURES ....................................................................................................... viii

LIST OF ABBREVIATIONS .......................................................................................... x

INTRODUCTION .......................................................................................................... 1

Translation .................................................................................................................... 1
Initiation ....................................................................................................................... 1
Elongation ..................................................................................................................... 2
Termination and Ribosome recycling ....................................................................... 3
Post-transcriptional Regulatory Mechanisms ............................................................ 16
Significance .................................................................................................................. 17

IDENTIFICATION OF ERF1 RESIDUES THAT PLAY CRITICAL AND
COMPLEMENTARY ROLES IN STOP CODON RECOGNITION ......................... 18

AVAILABILITY OF FUNCTIONAL TRANSLATION TERMINATION COMPLEXES
REGULATES EUKARYOTIC RELEASE FACTOR 1 ABUNDANCE VIA MRNA
STABILITY.................................................................................................................. 54

SUMMARY ................................................................................................................... 89

GENERAL LIST OF REFERENCES ............................................................................ 95
LIST OF TABLES

Table                  Page

IDENTIFICATION OF eRF1 RESIDUES THAT PLAY CRITICAL AND
COMPLEMENTARY ROLES IN STOP CODON RECOGNITION

1  Summary of readthrough data for ciliate TASNIKS, YxCxxxF, and
   TASNIKS/YxCxxxF mutants in Saccharomyces cerevisiae eRF1 .................. 31

2  Summary of readthrough data for single amino acid changes in Saccharomyces
   cerevisiae eRF1 ...................................................................................... 41

S1 New S. cerevisiae eRF1 plasmids made for this study ......................... 53

AVAILABILITY OF FUNCTIONAL TRANSLATION TERMINATION COMPLEXES
REGULATES EUKARYOTIC RELEASE FACTOR 1 ABUNDANCE VIA mRNA
STABILITY

1  A termination defect is associated with eRF1-CΔ19 .................................. 62

2  Excess termination complexes reduce readthrough ............................... 74

S1 Strains and Plasmids used in this study .............................................. 85
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Both prokaryotic release factors, RF1 and RF2, form a compact structure but extend when bound to the 70S ribosome.</td>
</tr>
<tr>
<td>2</td>
<td>General schematic for key steps in eukaryotic translation termination</td>
</tr>
<tr>
<td>3</td>
<td>The crystal structure of human eRF1 protein</td>
</tr>
<tr>
<td>4</td>
<td>eRF1 and eRF3 interact through conserved residues in each C-terminal domain</td>
</tr>
<tr>
<td>5</td>
<td>Three cavities located in domain 1 of eRF1 are important for decoding each termination codon</td>
</tr>
</tbody>
</table>

IDENTIFICATION OF ERF1 RESIDUES THAT PLAY CRITICAL AND COMPLEMENTARY ROLES IN STOP CODON RECOGNITION

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Domain 1 of eRF1 mediates stop codon recognition</td>
</tr>
<tr>
<td>2</td>
<td>The L123I mutation from the Euplotes and Blepharisma YCF motifs reduces eRF1 recognition of the UGA codon</td>
</tr>
<tr>
<td>3</td>
<td>Introduction of the YCF motif of Paramecium tetraurelia into S. cerevisiae eRF1 results in UGA specificity</td>
</tr>
<tr>
<td>4</td>
<td>An eRF1 mutant carrying the Tetrahymena YCF motif displays UGA specificity</td>
</tr>
<tr>
<td>5</td>
<td>The Oxytricha YCF motif increases UGA recognition by S. cerevisiae eRF1</td>
</tr>
<tr>
<td>6</td>
<td>The L123F and T55K mutations enhance UGA recognition by S. cerevisiae eRF1</td>
</tr>
<tr>
<td>7</td>
<td>Introduction of the Loxodes TASNIKS and/or YCF motifs reduce UAA/UAG recognition by S. cerevisiae eRF1</td>
</tr>
<tr>
<td>8</td>
<td>Mutations in eRF1 residues near cavity three show altered stop codon recognition</td>
</tr>
</tbody>
</table>
AVAILABILITY OF FUNCTIONAL TRANSLATION TERMINATION COMPLEXES REGULATES EUKARYOTIC RELEASE FACTOR 1 ABUNDANCE VIA mRNA STABILITY

1  eRF1 levels are elevated and stabilized in the eRF1-CΔ19 strain ........................................ 64

2  Increased abundance of eRF1 is not due to removal of a cis-acting regulatory element in the mRNA .................................................................................................................. 67

3  Other mRNAs do not increase under conditions that increase SUP45 mRNA .......... 68

4  Excess eRF3 rescues the polypeptide release defect associated with eRF1-CΔ19 in vitro ........................................................ .......................................................... 71

5  The abundance of eRF3 influences eRF1 protein and SUP45 mRNA levels .......... 73

6  Other eRF1 and eRF3 mutants display elevated eRF1 levels ........................................ 76

7  Model for eRF1 regulation ..................................................................................................... 78

S1  eRF1 and eRF1-CΔ19 proteins display similar half-lives when expressed as the sole source of eRF1 .................................................................................................................. 86

S2  sup45-CΔ19 mRNA is more stable than SUP45 mRNA .................................................... 87

S1  Deletion of the cytosolic 5’→3’ exoribonuclease (xrn1Δ) increases steady-state SUP45 mRNA levels .................................................................................................................... 88

SUMMARY

1  Post-transcriptional regulation of SUP45 mRNA maintains eRF1 cellular levels depending on the efficiency of translation termination ...................................................... 93
LIST OF ABBREVIATIONS

ABCE1  ATP-binding cassette

eIF    eukaryotic initiation factor

eEF    eukaryotic elongation factor

Eo/Sc  *Euplotes octocarinatus*/ *Saccharomyces cerevisiae*

eRF1  eukaryotic release factor one

eRF3  eukaryotic release factor 3

mRNA  messenger RNA

NBD    nucleotide binding domain

NGD    no-go decay

NMD    nonsense mediated decay

ORF    open reading frame

PABP   poly-A binding protein

pre-TC  pre-termination complex

post-TC post-termination complex

PTC    peptidyl transfer center

RF     release factor

RRF    ribosome recycling factor

tRNA   transfer RNA

UTR    untranslated region
INTRODUCTION

Translation

Nascent polypeptides are synthesized from a messenger RNA (mRNA) template through a process called translation. Eukaryotic translation is composed of four main steps: initiation, elongation, termination and recycling [7]. Through this process essentially all necessary peptides and proteins are generated that are required for all activities carried out in a cell. This basic cellular function provides limitless capacity to enhance or derail normal patterns of gene regulation.

Initiation

Of the four phases of translation, initiation is the most studied. Numerous proteins are involved in this highly succinct process. Cap-dependent translation uses the eukaryotic initiation factor 4F (eIF4F) complex to recruit the 43S ribosome complex composed of a 40S subunit, eIF2-GTP-Met-tRNA ternary complex, eIF1, eIF1A, eIF3 and eIF5 to the 5’ end of the mRNA. The eIF4F complex is comprised of eIF4E which binds the cap structure, eIF4G, a scaffolding protein, and eIF4A, which aids the 43S preinitiation complex recruitment through its helicase activity [8,9]. After recruitment, the 48S initiation complex scans the 5’ untranslated region (UTR) in search of the AUG start codon. The Met-tRNA,\textsuperscript{Met} in the ribosomal peptidyl (P) site recognizes the AUG
codon in the mRNA template [10]. After the release of eIF2-GTP and eIFs, the 60S ribosome subunit joins forming an elongation competent 80S ribosome.

Cap-independent or non-canonical mechanisms of translation initiation occur through direct recruitment of the 43S complex to internal ribosome entry sites (IRES). The requirement of eIFs varies depending on the different classes of IRES with some needing multiple IFs and the others not requiring any [8,11].

**Elongation**

Initiation results in the Met-tRNA$^{\text{Met}}$ being located in the ribosome P site with the aminoacyl (A) site being empty to allow elongation of the peptide chain. Several factors are needed to carry out this process; eukaryotic elongation factor 1A (eEF1A), eEF1B, eEF2 and fungi specific eEF3 [12]. eEF1A binds to aminoacyl tRNAs along with GTP to form the ternary complex required for elongation [13]. This complex is bound to the ribosome in the A site. If cognate anticodon-codon interactions occur, the GTPase activity of eEF1A is activated by the ribosome to allow accommodation of the tRNA in the A site. The rate of accommodation is dependent on the strength of the anticodon-codon interaction. Fidelity of anticodon-codon binding is also monitored by three universally conserved bases in the 40S [12,13]. Afterwards, the amino acid located on the P site tRNA is transferred to the amino acid-tRNA in the A site. To prepare for a new aminoacyl tRNA in the A site, translocation of the P and A site tRNAs to the E and P sites are catalyzed through the activity of eEF2. The mRNA must translocate three nucleotides (1 codon) to position the next codon in the A-site for the next round of elongation. eEF1B assists in recycling the ternary complex containing eEF1A-GDP by
being a guanine nucleotide exchange factor [12]. Removal of the deacylated tRNA from the E site is facilitated by the fungi specific eEF3, which does not exist in higher eukaryotes [13].

**Termination and Ribosome Recycling**

Once the elongating ribosome and elongation factors have translated all of the sense codons on a mRNA template, it encounters a termination codon positioned in the ribosomal A-site that signals the end of the ORF [13]. Unlike sense codons, stop codons are not recognized by aminoacylated tRNAs but through the joint action of class I release factors (RFs), which recognize the stop codons, and class II RFs, which contain GTPase activity [14]. They are able to mediate the release of newly synthesized polypeptides though universal conservation of the GGQ motif, which plays an essential role in mediating a water molecule nucleophilic attack on the peptidyl-tRNA bond located in the PTC. After termination occurs, ribosomes are recycled for another round of translation [14].

**Prokaryotic termination factors.** In prokaryotes, termination codons are recognized by one of two class I release factors, RF1 and RF2. RF1 and RF2 display overlapping specificity with both being able to recognize UAA; while UAG and UGA are recognized specifically by RF1 and RF2, respectively [14]. After stop codon recognition and peptide release, the class II release factor, RF3, mediates recycling of RFs by binding and removing RFs from the ribosome [15].
RF1 and RF2 are essential prokaryotic genes and in *Escherichia coli* they are encoded by *prfA* and *prfB* [15]. Although class I RFs, they are structurally unrelated to the class I eukaryotic RF1 (eRF1). Through crystallization analysis, they were each found to contain four functional domains (Figure 1). Domain 1 contacts the ribosome GTPase center and domains 2 and 4 form a compact superdomain which is important for stop codon recognition. Moreover, domain two contains the highly conserved tripeptide motif, PxT and SPF that is important for stop codon recognition in RF1 and RF2, respectively [16-18]. Domain 3 contains the conserved glycine-glycine-glutamine (GGQ) motif that is present in all known class I release factors and is required to mediate peptide release through contacting the peptidyl-transferase center on the ribosome. Stop codon recognition by RF1 and RF2 occurs with high fidelity, with recognition of stop codons being increased at least three orders of magnitude when comparing stop to sense codons.

**Figure 1.** Both prokaryotic release factors, RF1 and RF2, form a compact structure but extend when bound to the 70S ribosome. Domains in RF1 and RF2 are colored the same with domain 1 teal, domain 2 yellow, domain 3 salmon and domain 4 blue. Conserved motifs in domain 2 and 3 are colored red. (A) Unbound RF2 comprise a compact structure (PDB:1GQE) [3]. (B) Bound RF1 leads to extension of domain 3 which contains the GGQ motif (PDB:3D5A) [6].

Also, the sequence context surrounding the stop codon, more specifically the 3’ nucleotid e after the stop codon or tetranucleotide play an important role in the ability of RFs to differentiate stop codons in prokaryotes and eukaryotes [15]. Sequence context can alter termination efficiency by more than 100-fold [18,19].

RF3 is not essential; however it is required for maximal growth efficiency. RF3 does not aid in stop codon recognition or peptidyl hydrolysis but is required in the transition of termination to recycling. GTP hydrolysis of RF3 is needed for the transition, although RF1 and RF2 do not stimulate this activity. Similarity of RF3 to eukaryotic RF3 (eRF3) is limited to their GTPase domains [14,15].

Several studies have tried to address the unanswered question of how a protein (RF1 or RF2) recognizes a codon instead of the usual tRNA. Initial studies suggested that prokaryotic RFs recognize each stop codon through a tripeptide “anticodon” located in domain 2 of each RF. The PxT and SPF motifs were thought to directly interact with the codon of the mRNA [17,20]. However, through recent crystallographic studies of RFs bound to the 70S ribosome, it was found that RFs are able to recognize stop codons by a network of H bonds between the stop codon and each specific RF [6,21,22]. Recognition of each nucleotide in the stop codon is achieved through a reading head located in domain 2 that includes the PxT and SPF motifs but extends beyond each motif. The first nucleotide is recognized by the N-terminal tip of helix α5 (Figure 1). The second nucleotide is distinguished by conserved amino acids that are part of a recognition loop that including the PxT and SPF motifs. Variation in this loop region provides the difference in specificity of RF1 and RF2. The third nucleotide is discriminated through interactions with the N- and C-terminal ends of the recognition loop [18]. Current studies
suggest that the unstructured loop between domains 3 and 4, which are located in the decoding center of the ribosome, acts as a molecular switch to relay efficient stop codon recognition to coordinate conformational changes in RFs that are required to mediate peptide release. Peptidyl release is also aided by increased affinity of RFs to the ribosome after stop codon recognition [18].

Although recent crystallographic studies have greatly advanced our knowledge on how prokaryotic RFs mediate termination, key insights into whether various conformational changes of RFs are needed in order to transition from stop codon recognition to peptide release are not fully understood. Crystallization of several eRF1 mutants that can separately allow stop codon recognition but inhibit peptide release and vice versa will provide insight into these mechanisms.

**Prokaryotic recycling factors.** After the class II release factor (RF3) has removed RF1 or RF2 from the ribosome, the ribosome recycling factor (RRF) along with EF-G and IF3 prepare ribosomes for another round of translation [13,23]. More specifically, RRF and EF-G dissociate ribosomal subunits into 50S and 30S subunits with the mRNA and tRNA being still attached to the small subunit in a process, which requires GTP hydrolysis for their release. EF-G, whose main function is translocation of the mRNA during elongation, is essential for RRF activity. IF3 binds the 30S subunit to finalize recycling through aiding in the formation of the initiation complex [14].

**Eukaryotic release factors.** Translation termination in eukaryotes is carried out by a single omnipotent class I release factor, eRF1 and a GTPase assisting class II release
factor, eRF3 [4]. Current literature suggests that once a termination codon enters the ribosomal A-site, eRF1 in complex with eRF3 is recruited to the pre-termination complex (Figure 2). First, binding of eRF1 to eRF3, allows it undergo a conformation change that is thought to facilitate the termination complex interactions with the ribosome [5]. These findings are seen through crystallography analysis of eRF1 alone and in complex with eRF3. After stop codon recognition, eRF1 and the ribosome stimulate GTPase hydrolysis of eRF3, which is thought to allow accommodation of eRF1 in the A-site and may promote another conformational change in eRF1 [24]. GTP hydrolysis of eRF3 decreases its affinity to the ribosome which mediates the departure of eRF3 from the termination complex [4,7]. Opposing views in the field state that upon accommodation, eRF1 immediately promotes peptide release while others suggest that ABCE1 (Rli1 in yeast) must bind to eRF1 to assist in peptidyl hydrolysis [7]. ATP hydrolysis by ABCE1 leads to dissociation of post-termination complexes. Further recycling is carried out by binding of eIF3/1/1A to the 40S subunit to facilitate removal of the P-site tRNA and mRNA [7,25,26].

Figure 2. General schematic for key steps in eukaryotic translation termination. Eukaryotic release factors, eRF1 and eRF3, are essential for efficient translation termination. The termination complex, which contains eRF1-eRF3-GTP, is recruited to the pre-termination complex through interactions with the poly-A binding protein. eRF1 is able to recognize each of the three stop codons, UAA, UAG, and UGA. The ribosome and eRF1 are required to stimulate GTP hydrolysis of eRF3, which facilitates its release from the post-termination complex. GTP hydrolysis of eRF3 is also vital for release of the nascent polypeptide [4].
Eukaryotic termination differs from the prokaryotic process in several ways. First, in eukaryotes both class I and class II RFs are essential for mediating efficient termination, while in prokaryotes only class I RFs are required [27,28]. Second, eRF1 is able to recognize all three stop codons, UAA, UAG and UGA [28]. The prokaryotic RF1 and RF2 only recognize two of the three stop codons and are able to mediate polypeptide release without the help of other co-factors [14]. Third, eRF3 assists in stop codon recognition and also is important for conferring various conformational changes in eRF1 (discussed below) [30]. Currently there is no known role for eRF3 in mediating recycling of class I RF or ribosomal subunits like RF3 in prokaryotes. Finally, eRF1 is assisted in peptide release through the help of ribosome recycling factor, ABCE1 [7].

Although eRF1 contains minimal sequence homology with RF1 or RF2, it is composed of similar functional domains (Figure 3). Domain 1 mediates stop codon recognition [1]. The TASNIKS and YxCxxxF motifs located in domain 1 serve as key modulators of eRF1 stop codon specificity. Domain 2 contains the universally conserved GGQ motif, which contacts the peptidyl transferase center on the ribosome to trigger hydrolysis of the ester bond of the peptidyl-tRNA molecule [31]. Domain 2 also increases eRF1 affinity for eRF3 in the presence of GTP suggesting its importance for stimulating GTP hydrolysis of eRF3 [23]. Domain 3 of eRF1 allows it to interact with several proteins, most importantly the class II release factor, eRF3, which binds to eRF1 through its conserved C-terminal domain. [32,33]. A minidomain is also present in domain 3 and extends near domain 1. Several studies imply that the minidomain aids in stop codon recognition and/or binding to the 40S subunit [34]. eRF1 has relatively low abundance
Figure 3. The crystal structure of human eRF1 protein. eRF1 contains three functional domains which are labeled according to the text. Conserved motifs are colored in red (PDB#1DT9) [1].

Note: Adapted from “The Crystal Structure of Human Eukaryotic Release Factor eRF1-Mechanism of Stop Codon Recognition and Peptidyl-tRNA Hydrolysis” by H. Song, 2000, Cell, 100. Copyright 2000 by Elsevier. Adapted with permission.
in the cell, with approximately one eRF1 molecule per twenty ribosomes [35].

eRF3 is composed of four domains (Figure 4). It contains a highly variable N-terminal domain that is responsible for its prion-forming properties. This region is also important for interactions with poly-A binding protein (PABP) and its association with the nonsense mediated decay (NMD) factor, Upf1 [4,36]. A recent study found that human eRF3 has overlapping PAM2 motifs that can bind to one or multiple PABPC1 proteins. This eRF3/ PABPC1 interaction is important for recruitment of eRFs to the pre-termination complex (pre-TC). eRF3 binding to PABPC1 also provides an opportunity for deadenylases to interact with PABPC1 and promote gradual deadenylation of the mRNA [37]. The GTPase domain of eRF3 is vital for its termination function. The affinity of eRF3 for GTP increases by two orders of magnitude in the presence of eRF1. Domains 2 and 3 are highly conserved and show strong structural similarity to other translation GTPases, eEF1α and EF-Tu. Studies also suggest that eRF3 displays functional mimicry to elongation factors eEF1α and EF-Tu in that it brings a tRNA-like molecule, eRF1, to the PTC [4,24].

The interaction of the eRFs through their C-terminal domains is absolutely essential for efficient termination (Figure 4). The last twenty-two amino acids of eRF1 C-terminus are not essential for cell viability but are needed for efficient termination while the last ten residues in eRF3 are not required [32]. Hydrophobic interactions mediate binding of eRF1 and eRF3 through highly conserved residues in each eRFs [33]. Once eRF1 is bound by eRF3 it undergoes a conformational change that increases its affinity to the ribosome [38]. After the termination complex binds the pre-TC a conformational change extends the ribosome forward two nucleotides. These findings suggest that the
Figure 4. eRF1 and eRF3 interact through conserved residues in each C-terminal domain. This interaction is essential for efficient termination. eRF1 domains are colored the same as in the eRF1 alone structure. Only eRF3 C-terminal end (after GTPase domain) was crystallized. Domain 2 is colored green and domain 3 is colored blue (PDB#3E1Y) [5].

Note: Adapted from “Structural insights into eRF3 and stop codon recognition by eRF1” by Z. Cheng et al., 2009, Genes & Development, 23. Copyright 2009 by Cold Spring Harbor Laboratory Press. Adapted with permission.
ribosome undergoes changes needed to mediate efficient termination, such as closing the mRNA entry and exit channel [4,34]. GTP hydrolysis by eRF3 is required for additional changes in eRF1, release of eRF3 from the ribosome and recruitment of ABCE1 [5,7]. Recent findings from our lab suggest that eRF1 must acquire a different conformation to mediate efficient release at UAA and UAG versus UGA termination codons. This was proposed after finding a hybrid eRF1 protein did not require eRF3 for efficient peptide release for UAA and UAG but eRF3 was still required for UGA [39]. ABCE1 assists in peptide release in an ATP-independent manner, and the ribosomal subunits are dissociated following ATP hydrolysis. Ribosome recycling does not require peptide release although GTP hydrolysis of eRF3 must occur for release and recycling [7,26].

*Eukaryotic recycling factors.* ABCE1 is a member of the ATP-binding cassette (ABC) protein family. It contains two nucleotide binding domains (NBD) and a cysteine-rich domain harboring two iron-sulfur clusters. Both domains are functionally important. ABCE1 was initially identified as an initiation factor and is stably associated with free 40S subunits, initiation factors and release factors [4,26,40,41]. The presence of eRF1 is required for recycling during canonical termination and ABCE1 is able to interact with eRF1 through its NBD [7]. However, ABCE1 is also important for recycling stalled ribosomal complexes [40].

Current knowledge of the eRF1 and eRF3 was aided in part by their paralogs, Dom34 (yeast)/Pelota (mammals) and Hbs1, respectively. Dom34/Hbs1 complex functions in no-go decay (NGD) by binding to the ribosomal A-site to relieve elongation complexes that are stalled by large mRNA secondary structure. This allows recycling of
the ribosomal subunits for another round of translation [25,42]. Dom34 exhibits high sequence homology to eRF1. It contains an N-terminal domain (domain 1) that is important for its function in NGD. Domains 2 and 3 are also highly related to eRF1 although Dom34 is unable to mediate peptidyl hydrolysis [43-46]. Like eRF3, Hbs1 contains a highly variable N-terminal region followed by a GTPase domain and C-terminal domain that is important for interacting with Dom34 [47,48]. Initial studies suggest that only Dom34/Hbs1 were required to recycle stalled ribosomes; however, in vitro studies found that recycling rates were increased 20-fold with the addition of the ABCE1 homologue, Rli1 [7]. Dom34 induces the GTP binding and hydrolysis activity of Hbs1. Similar to eRF1, Dom34 domain 2 contacts the GTPase domain of Hbs1. Like termination, recycling of stalled ribosomes cannot occur without GTP hydrolysis of Hbs1. However, Dom34 and Hbs1 are able to mediate subunit dissociation in a codon-independent manner [4].

To better understand the mechanistic details of translation termination, our lab uses Saccharomyces cerevisiae as a model organism. In S. cerevisiae, the eukaryotic release factors, eRF1 and eRF3, are encoded by the essential genes SUP45 and SUP35, respectively [36]. S. cerevisiae RFs are often used to study eukaryotic translation termination since they contain high similarity and identity to human RFs. More specifically, eRF1 has 68% identity and 84% similarity to human eRF1 on the protein level. This correlates with the ability of human eRF1 to support cell viability in a yeast eRF1Δ strain [35]. Although there has been progress to better understand eukaryotic stop codon recognition, the exact mechanism still remains unknown. Advances made through prokaryotic termination studies have not directly aided the field in understanding
eukaryotic termination since termination by eRFs appears to occur through distinct mechanisms. Currently, there are at least two models for eRF1 recognition of termination codons. One model suggests that residues located in the TASNIKS motif act as the anticodon of eRF1 [49]. This model stems from the initial prokaryotic studies suggesting that the PxT and SPF motifs in RF1 and RF2 acted as anticodons for the RFs. Another model proposes that three cavities or binding pockets in domain 1 recognize the stop codon (Figure 5) [2]. Each cavity would recognize either the first, second or third nucleotide of the stop codon. Data obtained from the crystallization of the eRF1/eRF3 complex found that a component of their precipitant solution, ATP, was bound to one of the proposed cavities [38]. Concrete data validating either model has yet to be published.

![Figure 5](image-url)

**Figure 5.** Three cavities located in domain 1 of eRF1 are important for decoding each termination codon. Each cavity is labeled 1, 2, or 3 with each recognizing one nucleotide of the stop codon. The residues of the TASNIKS and YCF motifs are colored in teal and yellow, respectively (PDB#1DT9) [1,2].

Note: Adapted from “The Crystal Structure of Human Eukaryotic Release Factor eRF1-Mechanism of Stop Codon Recognition and Peptidyl-tRNA Hydrolysis” by H. Song, 2000, *Cell*, 100. Copyright 2000 by Elsevier. Adapted with permission.
Most organisms conform to the ‘standard’ genetic code, which consists of 61 codons that encode amino acids and 3 stop codons. Unlike other eukaryotic eRF1 proteins, ciliate eRF1 proteins only recognize either UAA and UAG or UGA as stop codons. They have grouped reassignment of stop codons to either UAA and UAG to encode for glutamine or reassigned UGA to encode for cysteine or tryptophan [50]. Since domain 1 is thought to be responsible for stop codon recognition, alignments between universal and variant species have discovered that alterations in two conserved motifs (TASNIKS and YxCxxF) may be important for standard code organisms stop codon specificity [51]. Salas-Marcos et al. discovered that a hybrid eRF1 protein that contained domain 1 of *Euplotes octocarinatus* and domains 2 and 3 of *S. cerevisiae* (Eo/Sc eRF1) only recognized UAA and UGA as efficient termination signals and was unable to support cell growth as the sole source of eRF1. These results correlated with wild-type *E. octocarinatus* converting UGA to a cysteine codon. Fan-Minogue et al. identified the critical residues that were able to restore cell viability and UGA recognition of Eo/Sc eRF1. The hybrid eRF1 proteins, Eo/Sc C124S, Eo/Sc E57S S58N and Eo/Sc E57S S58N C124S eRF1, all decrease the readthrough at UGA comparable to wild-type *S. cerevisiae* eRF1. Interestingly, it was found through an *in vitro* peptide release assay that Eo/Sc E57S S58N eRF1 did not require eRF3 for release at UAA and UAG [52]. These findings suggest that residues E57, S58 and C124 play essential roles in UGA recognition and that E57 and S58 allow efficient termination at UAA and UAG without the need for eRF3.

Since residues in the TASNIKS and YxCxxF motifs played such an important role in altering Eo/Sc eRF1 stop codon specificity, we wanted to take a broad look at how the motifs alter specificity in wild-type *S. cerevisiae* eRF1. In chapter 2, we placed the
TASNIKS and YxCxxxF motifs, separately and together, from variant-code organisms into \textit{S. cerevisiae} eRF1 to determine how these motifs modulate eRF1 pattern of stop codon recognition. Our results suggest that the YxCxxxF motif is the main determinant in facilitating eRF1 stop codon specificity.

**Post-transcriptional Regulatory Mechanisms**

Post-transcriptional regulation is a broad term used to describe modulation of gene expression after the mRNA template is transcribed. Regulation can occur during the various stages of the mRNA life cycle (e.g. maturation, processing, localization). During this study, we will focus on post-transcriptional mechanisms that alter the stability of the mRNA. The stability or decay rate of a mRNA is controlled by \textit{cis}-acting elements located in the 5’ or 3’ UTR or in the ORF that include sequence-specific components and/or secondary structure. Interacting factors or \textit{trans}-acting factors are able to recognize these elements and alter mRNA stability by either increasing or decreasing the half-life of the message [53]. Identification of numerous RNA-binding proteins that regulate the decay of an mRNA through specific interactions with the message has become increasingly common [54]. Such proteins have the ability to inhibit or recruit the normal decay machinery which is comprised of exonucleases that either target the 5’ or 3’ end of the message [55]. In chapter 3, we seek to understand the regulation of eRF1 mRNA (\textit{SUP45} mRNA) in \textit{S. cerevisiae} and how regulation of the message affects levels of eRF1 protein and termination efficiency. We show the stability of eRF1 mRNA is modulated by the availability of functional termination complexes consisting of eRF1 and eRF3.
Significance

11% of known mutations that cause all genetic diseases are caused by a single point mutation that results in an in-frame premature stop codon (PTC)[56]. Premature termination codons cause truncation of full-length proteins and result in an increase in mRNA turnover through nonsense mediated decay (NMD). The truncated proteins are often non-functional which may lead to a broad range of genetic diseases [57]. Multiple studies have used the ability of readthrough drugs to suppress PTCs to treat multiple genetic diseases, such as cystic fibrosis, Hurler syndrome and Duchenne muscular dystrophy. However, some readthrough drugs are known to have toxic side effects. Advancements in the knowledge of translation termination will help develop possible alternative compounds that will facilitate this novel therapeutic approach. Therefore, an understanding of how a major component of translation termination, eRF1, is regulated and a mechanistic insight into its function will be a tremendous asset to this field.
IDENTIFICATION OF ERF1 RESIDUES THAT PLAY CRITICAL AND COMPLEMENTARY ROLES IN STOP CODON RECOGNITION

by

SARA E. CONARD, JESSICA BUCKLEY, MAI DANG, GREGORY J. BEDWELL, RICHARD L. CARTER, MOHAMED KHASS, AND DAVID M. BEDWELL

RNA 18 (2012) 1210-1221

Copyright 2012 by RNA Society

Used by permission

Format adapted and errata corrected for dissertation

18
ABSTRACT

The initiation and elongation stages of translation are directed by codon-anticodon interactions. In contrast, an essential and unique aspect of translation termination is stop codon recognition by a release factor protein prior to polypeptide chain release. Previous studies have identified specific regions of eukaryotic release factor one (eRF1) that are important for decoding each stop codon. The cavity model for eukaryotic stop codon recognition suggests that three binding pockets/cavities located on the surface of eRF1’s domain one are key elements in stop codon recognition. Thus, the model predicts that amino acid changes in or near these cavities should influence termination in a stop codon-dependent manner. Previous studies have suggested the TASNIKS and YCF motifs within eRF1 domain one play important roles in stop codon recognition. Furthermore, the TASNIKS and YCF motifs are highly conserved in standard code organisms that use UAA, UAG and UGA as stop codons. In contrast, these motifs are more divergent in variant code organisms that have reassigned a subset of stop codons to sense codons. In the current study, we separately introduced TASNIKS and YCF motifs from six variant code organisms into eRF1 of Saccharomyces cerevisiae to determine their effect on stop codon recognition in vivo. We also examined the consequences of additional changes at residues located between the TASNIKS and YCF motifs. Overall, our results indicate that changes near cavities two and three frequently mediated significant effects on stop codon selectivity. In particular, changes in the YCF motif, rather than the TASNIKS motif, correlated most consistently with variant code stop codon selectivity.
INTRODUCTION

In prokaryotes, translation termination occurs when a Class I release factor (RF) recognizes a stop codon located in the ribosomal A site. The two Class I release factors, RF1 and RF2, recognize UAA/UAG and UAA/UGA codons, respectively, and then facilitate polypeptide chain release [1]. The Class II release factor, RF3, is a GTPase that disassociates the Class I RF from the ribosome following polypeptide release [2]. In eukaryotes, this process differs in several ways. Polypeptide release occurs after the Class I release factor, eRF1, recognizes any one of three termination codons (UAA, UAG and UGA) [3]. The eukaryotic Class II release factor, eRF3, is a GTPase that facilitates recognition of some termination signals, and also greatly enhances the rate of polypeptide chain release mediated by eRF1 [4-6]. Thus, eRF3 plays a much more direct role in the termination process than the prokaryotic Class II factor, RF3.

eRF1 has a modular organization with three functional domains [7]. Domain one mediates stop codon recognition and contains the conserved TASNIKS and YxCxxxF (YCF) motifs [7-9]. Domain two contains the universally conserved GGQ motif that promotes polypeptide chain release [7,10]. Domain three of eRF1 interacts with eRF3 [11,12].

A number of organisms do not recognize all three stop codons utilized in the standard genetic code. Such “variant code” species are particularly common among the ciliated protozoa, a group of unicellular eukaryotes [13]. In general, these species either utilize UGA as a stop codon (with UAA and UAG recoded to glutamine codons) [13-15] or UAA/UAG as stop codons (with UGA recoded to cysteine or tryptophan codons) [15,16]. Past studies found that fusion of eRF1 domain one from a variant code organism
to eRF1 domains two and three from a standard code organism often conferred variant stop codon recognition [17-22]. These results demonstrated that eRF1 domain one plays a key role in stop codon recognition. The primary amino acid sequences of domain one from standard and variant code organisms have also been examined to identify more defined regions involved in stop codon recognition. Two sequence elements within domain one, the TASNIKS and YCF motifs, were found to be highly conserved in standard code organisms but more divergent in variant code species (Figure 1A) [7,13,15,23], suggesting that these sequence elements play a role in stop codon recognition. Mutagenesis studies examined the effects of changing amino acids within the TASNIKS and YCF motifs of eRF1 [8,9,18,24-26]. Those studies confirmed the general importance of both motifs in stop codon recognition. However, the relative importance of specific amino acids examined in different studies has been difficult to compare directly because of the different experimental systems and strategies used.

Several models of eukaryotic stop codon recognition have been proposed. An early model proposed that residues located in the TASNIKS motif, T55/A56/S57 (S. cerevisiae numbering) act as a linear “peptide anticodon” that interacts directly with the stop codon [27]. A second model suggested that residues in or near the TASNIKS motif serve as key determinants in decoding stop codons. These residues, located in a helical region of the protein, were proposed to assume either a relaxed or tight conformation dependent upon the stop codon recognized. Moreover, it was suggested that residues located on the helix, G54/T55 and S57/N58 (S. cerevisiae numbering), recognize the second and third nucleotide of the stop codon, respectively [28]. Further supporting a role for the TASNIKS motif in stop codon recognition, another study found that an mRNA
Figure 1. Domain 1 of eRF1 mediates stop codon recognition. A) Protein sequence alignment of eRF1 domain one from multiple species. The star symbol indicates fully conserved residues; the colon indicates strong conservation; and the period indicates weak conservation. B) Location of eRF1 residues in the context of the human eRF1 structure [7]. YCF and TASNIKS residues are in black type and bordered by dotted lines. Residues between the TASNIKS and YCF motifs are in white type. The three solid white circles indicate the cavities proposed to mediate stop codon recognition [8].
containing 4-thiouracil in the first position of the stop codon could be crosslinked to residues in (or near) the TASNIKS motif of human eRF1, particularly the K60 residue (*S. cerevisiae* numbering; underlined) [29].

Additional mutagenesis studies have shown that mutations in other regions of domain one also influence stop codon recognition. This led to the proposal of a “nonlinear” model where stop codons are recognized by positive and negative determinants [9,25]. Another group used a genetic approach to identify mutations in domain one that altered the recognition of specific stop codons *in vivo*. Those results, in conjunction with the crystal structure of human eRF1 [7], led to a model in which three cavities (or pockets) located on the surface of domain one were proposed to recognize each of the three nucleotides of the stop codon (Figure 1B) [8]. Consistent with the cavity model, a crystallization study of an eRF1/eRF3 complex found that a component of the precipitant solution, ATP, was stably bound to one of the proposed cavities [26].

In the current study, we used a previously established yeast system to determine the effects of different eRF1 mutations on stop codon selectivity [5,17,18,30,31]. In particular, we compared the relative importance of the TASNIKS and YCF motifs in stop codon recognition. To do this, we introduced amino acid changes found in six divergent TASNIKS and YCF motifs from a series of variant code species, both separately and together, into the eRF1 protein of the standard code organism *Saccharomyces cerevisiae*. We then assayed stop codon recognition of each mutant protein expressed as the sole source of eRF1 *in vivo*. We found that many of these changes altered stop codon recognition in a manner consistent with a previously proposed cavity model of stop codon recognition [8]. Additional missense mutations located between the TASNIKS
and YCF motifs near cavity three also modified stop codon discrimination in distinct ways. When taken together, our results suggest that key determinants for eRF1 stop codon recognition map to a surface of domain one that includes three cavities previously proposed to recognize the three nucleotides of stop codons. Importantly, this surface includes, but extends beyond, the TASNIKS or YCF motifs. Moreover, changes in the YCF motif correlated most consistently with variant code stop codon selectivity.

**MATERIALS AND METHODS**

*Strain used.* The *S. cerevisiae* strain used in this study was YDB447 (*MATa ura3-52 leu2-3,112 his3-D200 trp1-D901 lys2-80 suc2-D901 sup45::HIS3 [psi-]) [5].

*Plasmids.* pDB800, a centromere-based plasmid that expresses the wild-type *SUP45* gene (which encodes *S. cerevisiae* eRF1) and contains a *LEU2* selectable marker, was used as a control in all dual luciferase reporter readthrough assays. pDB967, a centromere-based plasmid that expresses wild-type eRF1 with an N-terminal HA-tag from the *GAL1* promoter with a *TRP1* selectable marker [17], was used for eRF1 deletion experiments during luciferase assays and western blot analysis. pDB1047, a centromere-based plasmid containing a *LEU2* selectable marker and expresses wild-type *S. cerevisiae* eRF1 with an N-terminal HA-tag under control of the *SUP45* promoter, served as wild-type eRF1 control for western blot analysis. All mutant forms of eRF1 were expressed from pDB1047. eRF1 mutations were made by two-stage site-directed mutagenesis of pDB1047 using the QuikChange mutagenesis kit (Stratagene). Each mutant was verified by sequencing the entire *SUP45* ORF, as well as adjacent 5′ and 3′ regions. A complete list of plasmids generated for this study is listed in Supplemental Table 1.
Viability assay. Yeast strains expressing each mutant eRF1 protein as the sole source of eRF1 were initially tested for cell viability. The viability assay was performed as previously described [17]. Briefly, LEU2 selectable plasmids expressing mutant eRF1 proteins were transformed into YDB447 (sup45Δ) containing pUKC802, which expresses wild-type eRF1 from a plasmid carrying a URA3 selectable marker. Each transformation was plated on synthetic minimal (SM) medium supplemented with 2% glucose with required amino acids (but lacking leucine and uracil). After initial growth, single colonies were streaked onto plates containing 5-fluoroorotic acid (5-FOA). Since 5-FOA inhibits growth of cells that express the URA3 gene, this led to colony formation from cells that had lost pUKC802 if the mutant eRF1 protein could support cell viability as the sole source of eRF1. If no colonies were obtained on 5-FOA plates, it was concluded that the mutant eRF1 protein was unable to support cell viability as the sole source of cellular eRF1.

eRF1 depletion experiments. To determine stop codon recognition of eRF1 mutants that were unable to support cell viability as the sole source of eRF1, relevant plasmids were transformed into YDB447/pDB967 (which expresses wild-type eRF1 under GAL1 promoter control). The resulting strains were maintained by growth on galactose-containing medium. To monitor stop codon recognition of each mutant eRF1 protein, cultures were grown in SM medium containing 2% galactose and the appropriate amino acids overnight. The next morning, cells were diluted back and allowed to grow for at least 5 hours to mid-log (defined as an A600 reading of 0.5-1.0 OD/ml). Cells were harvested, washed 3x with SM medium containing 2% glucose, and then re-suspended in SM medium with 2% glucose and amino acids and grown overnight for 6 generations.
until they again reached mid-log. Readthrough and steady-state eRF1 protein levels were tested with YDB447/pDB967 (without a mutant plasmid) as a control to confirm depletion of eRF1. YDB447 carrying a plasmid encoding wild-type eRF1 (pDB800 or pDB1047) under SUP45 promoter control was included in each experiment as a positive control.

_Dual-luciferase readthrough assays._ Readthrough assays were performed as previously described [5,17,30-32]. The dual-luciferase reporter used contained the _Renilla reniformis_ (Renilla) and _Photinus pyralis_ (firefly) luciferase genes separated by a linker region containing a readthrough cassette with either a sense or stop codon. The efficiency of termination (expressed as readthrough) of either codon was monitored by firefly luminescence activity. Firefly activity was normalized to _Renilla_ activity, which served as an internal normalization control. Readthrough levels of each mutant were determined in at least two independent experiments with each sample being assayed in quadruplicate. “Percent Readthrough” for wild-type _S. cerevisiae_ eRF1 driven by the SUP45 promoter (pDB800) or GAL1 promoter control (pDB967) was expressed as the firefly/Renilla ratio obtained from the mutant (stop codon) reporter divided by the firefly/Renilla ratio from the wild type (sense codon) reporter (x 100). The resulting values for each construct are expressed as the overall mean obtained using the same strains and conditions. For ease of comparison, the final readthrough data were expressed as the fold-change in readthrough (normalized to the wild-type control) ± standard deviation.

_Western blots._ Ten _A_600 units of cells were harvested and incubated in 10% trichloroacetic acid on ice for thirty minutes. Cells were washed with 100% acetone and
dried. After re-suspension in 120 µl of SDS lysis buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% SDS], cells were lysed by mechanical disruption using glass beads. Samples were boiled for 3 minutes and cleared by centrifugation for ten minutes in a microcentrifuge. Protein concentration was measured using the Lowery Method [33]. Total protein (20 µg) from crude extracts was resolved on an SDS-polyacrylamide gel, followed by transfer to an Immobilon-P transfer membrane (Millipore) and Western blotting. Cells from depletion experiments were collected after the galactose to glucose shift procedure as described above.

RESULTS

To examine the relative importance of residues within the TASNIKS and YCF motifs for stop codon recognition, we introduced these motifs from six variant code species into eRF1 of the standard code organism Saccharomyces cerevisiae. eRF1 mutants that supported growth in the absence of wild-type eRF1 were assayed directly to determine their pattern of stop codon recognition. Mutants unable to support cell viability as the sole source of eRF1 were transformed into a sup45Δ strain that also expressed wild-type eRF1 under GAL1 promoter control. These strains were grown in galactose medium to express both wild-type and mutant eRF1, shifted to glucose medium to halt production of wild-type eRF1, and grown for six more generations to deplete wild-type eRF1. The efficiency of translation termination at each stop codon was measured using a well-established dual luciferase readthrough reporter system [5,17,18,30,34-37].
A single amino acid change at L123 from the Euplotes and Blepharisma YCF motifs results in strong UAA/UAG specificity.

*Euplotes* species utilize UAA and UAG as stop codons, while *Blepharisma* species utilize UAA (and possibly UAG) [13,15,16]. We first examined the effect of introducing divergent amino acids from the TASNIKS and YCF motifs of *Euplotes octocarinatus* and *Blepharisma americanum* into *S. cerevisiae* eRF1. Expression of mutant eRF1 proteins containing the individual TASNIKS or YCF motifs all supported cell viability as the sole source of eRF1, while the double mutants containing both motifs from either organism were unable to sustain cell viability. The ability of the double mutants to recognize UAA, UAG and UGA stop codons was thus assayed using the shift assay described above.

Both *Euplotes octocarinatus* and *Blepharisma americanum* eRF1 proteins contain only a single divergent isoleucine at position 123 in their YCF motifs (YICDNKF) instead of the leucine residue at the corresponding position of the *S. cerevisiae* YCF motif (YLCDNKF). Introduction of the L123I mutation reduced termination efficiency at the UGA codon >11-fold when compared to wild-type (*S. cerevisiae*) eRF1 (Figure 2A; Table 1). In contrast, this mutant exhibited more efficient termination at UAA and UAG stop codons than wild-type eRF1. These results suggested that recognition of guanine (UGA) in the second position was inhibited, while adenine recognition in position 2 (UAA and UAG) was enhanced by this single amino acid substitution. The contribution of the TASNIKS motifs to the variant stop codon recognition used by these organisms was much less direct. A UAA/UAG-specific pattern of stop codon recognition was not observed in the mutant carrying the *Blepharisma KSSNIKS* motif. The *Euplotes*
TAESIKS mutant showed a partial loss of UGA recognition (as well as a slight loss of UAG recognition) (Figure 2A; Table 1). The double mutant carrying the *Euplotes* TAESIKS and YICDNKF motifs exhibited a greater loss of UGA recognition than either motif alone, but also showed defects in UAA and UAG recognition (Figure 2B). Finally, the mutant carrying both the *Blepharisma* KSSNIKS and YICDNKF motifs exhibited the same strong UAA/UAG-specific readthrough phenotype as the YCF mutant alone (Figure 2B), suggesting that *Blepharisma americanum* is a UAA/UAG specific organism. Taken together, these results indicate that the isoleucine at residue 123 plays a key role in

![Figure 2](image_url)

**Figure 2.** The L123I mutation from the *Euplotes* and *Blepharisma* YCF motifs reduces eRF1 recognition of the UGA codon. A, B) Fold change in readthrough by viable (A) and inviable (B) strains expressing the indicated mutant eRF1 proteins compared to wild-type *S. cerevisiae* eRF1. The data are expressed as mean values (relative to wild-type eRF1) ± standard deviation. The horizontal dashed lines represent wild-type *S. cerevisiae* eRF1 levels. C, D) Western blots showing steady-state eRF1 proteins levels from strains assayed in panel A and B, respectively.
excluding guanine in the second position of the stop codon in these two UAA/UAG-specific variant-code organisms, consistent with the close proximity of L123 to cavity two in the Stansfield model (see Figure 1B).

Previous findings have shown that a significant decrease in the level of eRF1 protein reduces the efficiency of translation termination at all three stop codons [5]. To exclude such non-specific effects, we routinely included two control assays. First, we measured readthrough in a strain depleted of eRF1 protein. As expected, this strain exhibited a large (15-20-fold) decrease in termination efficiency at each of the three stop codons (Figure 2B). This uniform pattern of reduced termination efficiency is distinct from the stop codon-specific results obtained with most eRF1 mutants discussed above. We also monitored the steady-state level of each mutant eRF1 protein by western blot analysis. Non-viable eRF1 mutants were assayed after a galactose to glucose shift, followed by continued growth for six generations to deplete WT eRF1 (as done for the readthrough assays). None of the *Euplotes* or *Blepharisma* substitutions resulted in a significant reduction in eRF1 abundance (Figure 2C, 2D). However, the Y1CDNKF and TAESIKS mutants displayed 2-fold and 3.6-fold increases in eRF1 abundance, respectively. Similar increases were previously noted for some eRF1 mutants [18], and are thought to reflect a regulatory mechanism that couples eRF1 abundance to the efficiency of translation termination (S. Conard, A. Kallmeyer, and D. Bedwell, unpublished). When taken together, these results indicate that the codon-specific changes in termination efficiency described above were not simply due to inadequate levels of the mutant eRF1 proteins.
Table 1. Summary of Readthrough Data for Ciliate TASNIKS, YxCxxxF and TASNIKS/YxCxxxF Mutants in *Saccharomyces cerevisiae* eRF1

<table>
<thead>
<tr>
<th>Organism (stop recognition) (^b)</th>
<th>TASNIKS/(Y_{CxxxF}) Motif</th>
<th>TASNIKS(^b)</th>
<th>YxCxxxF(^b)</th>
<th>TASNIKS/YxCxxxF (^b)</th>
<th>Viable?(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S.\ cerevisiae) (^d)</td>
<td>TASNIKS (Y_{CDNKF})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S.\ cerevisiae) (^e)</td>
<td>TASNIKS (Y_{CDNKF})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(eRF1) depleted (none) (^f)</td>
<td>----</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(B.\ americamn) (^g)</td>
<td>(K_{SNKIS}) (Y_{CDNKF})</td>
<td>0.38 ± 0.05</td>
<td>0.11 ± 0.01</td>
<td>0.97 ± 0.15</td>
<td>Yes</td>
</tr>
<tr>
<td>(E.\ octocarinatus) (^h)</td>
<td>(T_{SNKIS}) (Y_{CDNKF})</td>
<td>0.17 ± 0.05</td>
<td>0.69 ± 0.03</td>
<td>3.89 ± 0.37</td>
<td>Yes</td>
</tr>
<tr>
<td>(L.\ striatus) (^i)</td>
<td>(B_{SNKIS}) (E_{CDNT})</td>
<td>1.34 ± 0.14</td>
<td>0.78 ± 0.09</td>
<td>0.41 ± 0.04</td>
<td>Yes</td>
</tr>
<tr>
<td>(O.\ trifallax) (^j)</td>
<td>(A_{SNKIS}) (Y_{CGQKF})</td>
<td>0.32 ± 0.06</td>
<td>0.53 ± 0.04</td>
<td>0.61 ± 0.12</td>
<td>Yes</td>
</tr>
<tr>
<td>(P.\ tetraurelia) (^k)</td>
<td>(E_{SNKIS}) (Y_{CDEOF})</td>
<td>7.70 ± 0.87</td>
<td>8.05 ± 1.39</td>
<td>11.87 ± 0.99</td>
<td>No</td>
</tr>
<tr>
<td>(T.\ thermophila) (^l)</td>
<td>(K_{SNKIS}) (Y_{CDSKF})</td>
<td>0.44 ± 0.02</td>
<td>1.32 ± 0.11</td>
<td>1.23 ± 0.14</td>
<td>Yes</td>
</tr>
</tbody>
</table>

\(^a\) Stop codon usage is according to Kim et al., 2005.

\(^b\) TASNIKS, YxCxxxF, TASNIKS/YxCxxxF percent readthrough is displayed as mean ± SD.

\(^c\) Indicates whether mutant eRF1 proteins support viability as the sole source of eRF1.

\(^d\) Showing average readthrough levels for wild-type eRF1 from all viable mutant experiments.

\(^e\) Showing average readthrough levels for wild-type eRF1 from all non-viable mutant experiments.

\(^f\) Showing average readthrough levels for depleted eRF1 from all non-viable mutant experiments.
S. cerevisiae eRF1 displays UGA specificity when the Paramecium, Tetrahymena or Oxytricha YCF motifs are introduced.

Paramecium species have reassigned UAA and UAG to glutamine codons, and use only UGA as a termination codon [13,38]. To determine whether the TASNIKS and YCF motifs of Paramecium tetraurelia contain determinants for UGA specificity, we introduced the TASNIKS (EAASIKD), YCF (YFCDPQF), or both motifs from this organism into S. cerevisiae eRF1. The YCF mutant displayed strong UGA specificity, as 5- and 10-fold decreases in termination efficiency were observed at UAA and UAG codons, respectively (Figure 3A; Table 1). In contrast, termination at the UGA codon decreased only slightly (1.5-fold). These results indicate that the three amino acid changes in the YFCDPQF motif were sufficient to reduce recognition of adenine in the second position of UAA and UAG stop codons. The TASNIKS and double mutants showed strong decreases in termination efficiency at all three stop codons, suggesting that they compromised overall eRF1 protein function and/or abundance when introduced into S. cerevisiae eRF1. A large (5-fold) decrease in eRF1 abundance of the double mutant is also consistent with its general readthrough phenotype (Figure 3B). These results show that the divergent YCF motif is sufficient to provide a strong bias toward UGA stop codon specificity as observed in Paramecium species.

Like Paramecium tetraurelia, Tetrahymena species also mediate translation termination exclusively at UGA codons [13,14]. To further explore how divergent TASNIKS and YCF sequences influence stop codon selectivity, we next tested the amino acid changes found in the TASNIKS and YCF motifs of Tetrahymena thermophila. Introduction of the Tetrahymena YCF motif (YFCDSKF) into S. cerevisiae eRF1 resulted
in modest UGA specificity (Figure 4A; Table 1). However, the increases in readthrough at UAA and UAG codons (2.5-fold and 3.4-fold, respectively) were much less than was observed with the Paramecium YCF motif. UGA-specific termination was not observed with strains expressing eRF1 proteins containing the Tetrahymena TASNIKS motif (KATNIKD) or the KATNIKD / YFCDSKF double mutant. Instead, these mutants exhibited large (4-fold and 16-fold increases, respectively) in readthrough at the UAG termination codon. These results suggest that one or more of the changes in the
TASNIKS motif decrease recognition of guanine in the third position (UAG). Western blot analysis was also carried out to determine the level of mutant eRF1 proteins. The YCF mutant (YFCDSKF) resulted in a 40% reduction in eRF1 abundance, while the TASNKS (KATNIKD) mutant displayed a 60% decrease in eRF1 protein (Figure 4B). Since termination at one (or more) of the three stop codons remained at or near the normal level, we conclude that these modest decreases in eRF1 abundance were not sufficient to induce a global decrease in termination efficiency. When taken together, these results suggest that alterations within the *Tetrahymena* YCF motif reduce

---

**Figure 4.** An eRF1 mutant carrying the *Tetrahymena* YCF motif displays UGA specificity. A) Fold change in readthrough by strains expressing the indicated mutant eRF1 proteins compared to wild-type *S. cerevisiae* eRF1. The data are expressed as mean values (relative to wild-type eRF1) ± standard deviation. The horizontal dashed lines represent wild-type *S. cerevisiae* eRF1 levels. B) Western blot showing steady-state eRF1 proteins levels from strains in panel A.
recognition of adenine in position two of stop codons, while changes in the *Tetrahymena* TASNIKS motif provide a bias against guanine in position three of the stop codon. While other residues in *Tetrahymena* eRF1 probably also contribute to its UGA specificity, the TASNIKS and YCF motifs clearly provide important contributions to its overall pattern of stop codon recognition.

We next examined the effects of introducing the TASNIKS and YCF motifs from a third UGA-specific organism, *Oxytricha trifallax*, into wild-type *S. cerevisiae* eRF1. Interestingly, these changes produced distinct changes in the pattern of stop codon recognition as compared to the results obtained with the *Paramecium* or *Tetrahymena* motifs. Rather than a decrease in UAA or UAG specificity, we observed a 6-fold increase in UGA termination efficiency in the YCF mutant relative to wild-type eRF1 (Figure 5A; Table 1). This specific increase in UGA termination efficiency suggests that the *Oxytricha* YCF motif (YFCGGKF) primarily enhances recognition of guanine in the second position (UGA), without significantly reducing adenine recognition (UAA and UAG) at that position. In contrast, little or no change in the termination efficiency could be attributed to the *Oxytricha* TASNIKS motif (AAQNIKS), and termination of the TASNIKS/YCF double mutant was similar to the YCF mutant. The abundance of each mutant eRF1 protein remained similar to wild-type eRF1 with the exception of the TASNIKS mutant (Figure 5B), which was reduced to 30% of the wild-type eRF1 protein level. In spite of that decrease, the termination efficiency was largely unchanged, indicating that a sufficient amount of mutant eRF1 was present for efficient termination. These results suggest that the *Oxytricha* YCF motif enhances UGA recognition, but other
determinants in *Oxytricha* eRF1 beyond the TASNIKS and YCF motifs must act to reduce termination at UAA/UAG codons in order to confer UGA-specific termination.

![Figure 5](image)

**Figure 5.** The *Oxytricha* YCF motif increases UGA recognition by *S. cerevisiae* eRF1. A) Fold change in readthrough by strains expressing the indicated mutant eRF1 proteins compared to wild-type *S. cerevisiae* eRF1. The data are expressed as mean values (relative to wild-type eRF1) ± standard deviation. The horizontal dashed lines represent wild-type *S. cerevisiae* eRF1 levels. B) Western blot showing steady-state eRF1 proteins levels from strains in panel A.

Some amino acid changes in variant TASNIKS and YCF motifs enhance termination efficiency at one or more stop codons.

The results described above show that divergent residues in the *Paramecium*, *Tetrahymena* and *Oxytricha* YCF motifs confer UGA specificity by reducing UAA/UAG termination or enhancing UGA recognition. These three motifs, YFCDPQF, YFCDSKF,
and YFCGGKF, respectively, share the L123F alteration. We also showed that the sole amino acid change in the Euplotes and Blepharisma YCF motifs, L123I, conferred a strong bias against UGA termination. When taken together, these results suggest that the identity of the amino acid at residue 123 has a strong effect on the efficiency of UGA recognition. To explore this further, we introduced L123F alone into S. cerevisiae eRF1 and examined its effects on stop codon recognition (Figure 6A; Table 2). The L123F mutation resulted in a ~3-fold enhancement of termination efficiency at the UGA codon, while termination at UAA/UAG remained similar to wild-type eRF1 levels. Little or no change was observed in the steady-state level of the L123F eRF1 protein relative to wild-type eRF1 (Figure 6B). These results demonstrate that the L123F alteration acts to enhance UGA recognition, rather than reduce UAA/UAG recognition.

Our earlier results showed that the presence of the Tetrahymena TASNIKS motif (KATNIKD) resulted in a bias against efficient termination at the UAG codon when placed in the context of S. cerevisiae eRF1, suggesting that these changes together prevent guanine binding in position three (Figure 4A). Of the three changes in this motif, T55 is closest to cavity three of the Bertram model. Accordingly, we also examined the consequences of introducing a T55K mutation into S. cerevisiae eRF1 (Figure 6C; Table 2). Surprisingly, this mutant protein recognized each of the three stop codons more efficiently than WT eRF1 (ranging from 20% better at UAA to 70% better at UGA). The steady-state level of the T55K eRF1 protein was only slightly reduced relative to wild-type eRF1 (Figure 6D). These results suggest that the T55K change in the first position of the TASNIKS motif may act to counterbalance other negative changes that contribute to variant stop codon selection in order to optimize UGA specificity in Tetrahymena.
species. When taken together, these results suggest that the L123F and T55K mutations enhance recognition of specific stop codons in variant-code species, presumably to fine-tune the overall termination efficiency.

**Figure 6.** The L123F and T55K mutations enhance UGA recognition by *S. cerevisiae* eRF1. A, C) Fold change in readthrough by strains expressing the indicated mutant eRF1 proteins compared to wild-type *S. cerevisiae* eRF1. The data are expressed as mean values (relative to wild-type eRF1) ± standard deviation. The horizontal dashed lines represent wild-type *S. cerevisiae* eRF1 levels. B, D) Western blot showing steady-state eRF1 proteins levels from strains in panel A.

The *Loxodes* TASNIKS and YCF motifs both contribute to UGA-specific stop codon recognition.

The results above suggest that the YCF motif generally plays a more prominent role than the TASNIKS motif in determining stop codon specificity for the five variant-code organisms examined above. To test this further, we introduced the TASNIKS, YCF or TASNIKS/YCF motifs of the UGA-specific species *Loxodes striatus* into *S. cerevisiae*
eRF1 (Figure 7). Unlike the other UGA-specific ciliates above that carry the L123F change in their YCF motifs, this organism contains the standard code leucine at position 123 [13]. We found that the *Loxodes* TASNIKS mutant (RAQNIKS) reduced UAA/UAG recognition by two to four-fold, while the YCF mutant (FLCENTF) reduced UAA/UAG recognition by five to ten-fold (Figure 7A, 7B; Table 1). Thus, the introduction of either motif into *S. cerevisiae* eRF1 resulted in varying degrees of UGA specificity. The double mutant (RAQNIKS/FLCENTF) reduced termination at UAA and UAG codons (11-fold and 15-fold, respectively), significantly more than either the RAQNIKS or FLCENTF mutants alone. These results suggest that the *Loxodes* TASNIKS and YCF motifs act together to suppress termination at UAA and UAG codons and optimize UGA-specific termination. Once again, western blot analysis indicated that the steady-state level of each eRF1 mutant protein remained similar to (or above) wild-type eRF1 levels (Figure 7C, 7D).

*Mutations between the TASNIKS and YCF motifs have distinct effects on stop codon recognition.*

In their model for stop codon recognition, Bertram et al. proposed that eRF1 cavity one binds the conserved uracil in position one of stop codons, while cavities two and three bind the purines in positions two and three, respectively [8]. The YCF motif occupies most of the space between the three proposed cavities on the surface of eRF1, while the first residues of the TASNIKS motif are distal to cavity three (see Figure 1B). The results described above showed that the introduction of several variant code TASNIKS and YCF elements into *S. cerevisiae* eRF1 altered stop codon recognition in a manner
that is generally consistent with this cavity model. This led us to hypothesize that changes in other amino acids between the TASNIKS and YCF motifs may also influence the pattern of stop codon recognition.

Figure 7. Introduction of the Loxodes TASNIKS and/or YCF motifs reduce UAA/UAG recognition by S. cerevisiae eRF1. A, B) Fold change in readthrough by strains expressing the indicated mutant eRF1 proteins compared to wild-type S. cerevisiae eRF1. The data are expressed as mean values (relative to wild-type eRF1) ± standard deviation. The horizontal dashed lines represent wild-type S. cerevisiae eRF1 levels. C, D) Western blot showing steady-state eRF1 proteins levels from strains in panel A.

Seven additional eRF1 mutants carrying single amino acid changes were generated. Introduction of T29A, I32A or I72A mutations individually into S. cerevisiae eRF1 caused severe readthrough at all stop codons, indicating that these mutations each caused general defects in termination (Table 2). These results show that these residues
play important but poorly defined role(s) in the structure or function of eRF1. The A71V mutation showed a 2 to 3-fold defect in termination at all three stop codons, and thus did not influence stop codon selectivity. Other mutations showed more specific roles on stop codon recognition. The V68A mutation exhibited a 4-fold reduction in termination efficiency at UAA and a more severe (14-fold) reduction in termination at UGA, but no change in termination at the UAG codon. These results suggest that the V68A mutation caused a strong bias against stop codons with an adenine in position three, consistent with the location of this residue near cavity three.

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Percent Readthrougha</th>
<th>Viable?b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UAA</td>
<td>UAG</td>
</tr>
<tr>
<td>WTc</td>
<td>0.36 ± 0.11</td>
<td>0.37 ± 0.13</td>
</tr>
<tr>
<td>WTd</td>
<td>0.51 ± 0.16</td>
<td>0.41 ± 0.11</td>
</tr>
<tr>
<td>depleted eRF1</td>
<td>7.29 ± 1.71</td>
<td>6.46 ± 1.52</td>
</tr>
<tr>
<td>T29A</td>
<td>6.46 ± 0.83</td>
<td>4.17 ± 0.66</td>
</tr>
<tr>
<td>I32A</td>
<td>8.47 ± 0.68</td>
<td>4.81 ± 1.13</td>
</tr>
<tr>
<td>E52A</td>
<td>0.57 ± 0.09</td>
<td>5.83 ± 0.70</td>
</tr>
<tr>
<td>T55K</td>
<td>0.27 ± 0.03</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>V68A</td>
<td>1.81 ± 0.28</td>
<td>0.62 ± 0.19</td>
</tr>
<tr>
<td>A71V</td>
<td>0.75 ± 0.05</td>
<td>0.63 ± 0.07</td>
</tr>
<tr>
<td>I72A</td>
<td>5.71 ± 0.38</td>
<td>6.88 ± 0.85</td>
</tr>
<tr>
<td>Y122A</td>
<td>7.06 ± 0.67</td>
<td>9.09 ± 0.88</td>
</tr>
<tr>
<td>Y122F</td>
<td>0.53 ± 0.10</td>
<td>5.29 ± 0.79</td>
</tr>
<tr>
<td>L123F</td>
<td>0.45 ± 0.07</td>
<td>0.38 ± 0.07</td>
</tr>
</tbody>
</table>

a Readthrough values are represented as mean ± SD.
b Indicates whether mutant eRF1 proteins support viability as the sole source of eRF1.
c Showing average readthrough levels for wild-type eRF1 from all viable mutant experiments.
d Showing average readthrough levels for wild-type eRF1 from all non-viable mutant experiments.

Based on structural studies, the Y122 residue in the YCF motif is adjacent to cavity one and is predicted to form a hydrogen bond with E52, which borders cavity three.
To examine the importance of this interaction, we next mutated each of these residues in *S. cerevisiae* eRF1. The E52A mutation significantly reduced termination at UAG and UGA codons (14 and 11-fold, respectively), but not at the UAA codon, suggesting that this mutation reduced termination at codons with guanine in positions 2 or 3. The Y122A mutant exhibited high levels of readthrough at all three stop codons, indicating that this mutation resulted in a non-functional protein in vivo. To overcome this problem, we introduced a more conservative change, Y122F (which differs only by the loss of the hydroxyl group thought to participate in the hydrogen bond with E52). The Y122F mutant displayed efficient termination at UAA and UGA codons, but 13-fold less efficient termination at the UAG codon (Figure 8A; Table 2). This suggested that recognition of guanine in the third position of the stop codon was inhibited by the Y122F mutation. The Y122A, Y122F and V68A mutants each displayed two to three-fold reductions in the abundance of eRF1 protein (Figure 8B). Since the Y122F and V68A mutants each retained efficient termination at one or more stop codons, we conclude that these eRF1 levels were sufficient to facilitate efficient termination in vivo.
Figure 8. Mutations in eRF1 residues near cavity three show altered stop codon recognition. A) Fold change in readthrough by strains expressing the indicated mutant eRF1 proteins compared to wild-type *S. cerevisiae* eRF1. The data are expressed as mean values (relative to wild-type eRF1) ± standard deviation. The horizontal dashed lines represent wild-type *S. cerevisiae* eRF1 levels. B) Western blot showing steady-state eRF1 proteins levels from strains in panel A.
DISCUSSION

The variant genetic codes of most ciliated protozoa appear to use either UAA/UAG or UGA as stop codons [13], and a number of studies have implicated amino acid changes within the TASNIKS and YCF motifs as important mediators of those two outcomes [7-9,13,15,18,23-26]. In this study, we examined the relative importance of these motifs from six variant-code species in the context of *S. cerevisiae* eRF1. We found that the single amino acid change L123I found in both the *Euplotes* and *Blepharisma* YCF motifs reduced UGA-specific termination by 12-fold, resulting in the conversion of *S. cerevisiae* eRF1 to primarily a UAA/UAG pattern of stop codon recognition. Consistent with our results, Stansfield and colleagues found that an L123V mutation resulted in a 5-fold decrease in UGA-specific termination [8]. We previously demonstrated that a C124S mutation in a *Euplotes octocarinatus / S. cerevisiae* hybrid eRF1 protein converted it from a UAA/UAG pattern of recognition to omnipotent recognition (UGA recognition was restored while UAA/UAG recognition was maintained) [18]. These results demonstrate that single amino acid changes at these two adjacent residues near cavity two either enhance (C124S) or inhibit (L123I) recognition of guanine in the second position of the stop codon (UGA) [8,18], and are entirely consistent with their location directly adjacent to cavity two of the Bertram model.

This study provides new insights into possible mechanisms of stop codon reassignment. It is generally assumed that recoding requires the acquisition of amino acid changes within eRF1 that reduce its ability to recognize some, but not all, stop codons. However, our results show that the acquisition of variant stop codon usage involves multiple amino acid changes with both negative and positive affects on stop
codon recognition that may act cooperatively to optimize variant code utilization. For example, we found that incorporating the two or three amino acid changes from the *Paramecium* or *Tetrahymena* YCF motifs into *S. cerevisiae* eRF1 significantly reduced termination at UAA/UAG codons, while maintaining efficient UGA termination (see Figures 3A and 4A). However, incorporation of the *Oxytricha* YCF or a single divergent residue shared by all three organisms (L123F) did not reduce UAA or UAG termination. Instead, it enhanced termination at the UGA codon by two to five-fold (Figure 5A). Similarly, we found that the T55K mutation from the *Tetrahymena* TASNIKS motif enhanced termination efficiency at all three stop codons by up to four-fold. These results demonstrate that some amino acid changes reduce the recognition of one or more stop codons, while others buffer that effect by enhancing the recognition of other stop codons. The net effect is to optimize recognition of one (or a subset) of stop codons.

To test the contribution of residues beyond the TASNIKS and YCF motifs, we altered a series of amino acids between the TASNIKS and YCF motifs into *S. cerevisiae* eRF1 to determine whether other patterns of stop codon recognition could be obtained (Figure 8). Among these, mutant eRF1 proteins carrying the Y122F, E52A or V68A mutations exhibited intriguing patterns of stop codon recognition. Y122F is thought to hydrogen bond to E52 [24], which borders cavity three in the Bertram cavity model. The Y122F mutant displayed a 13-fold reduction in termination efficiency at UAG while termination at UAA and UGA codons was largely unaffected. This indicates that the Y122F mutation strongly and specifically inhibited recognition of guanine at the third position (as in UAG). These findings are in general agreement with a previous study that showed a 3-fold decrease in termination efficiency at UAG using an *in vitro* release assay.
using human eRF1 [24]. In contrast to Y122F, we found that the E52A mutant exhibited efficient termination at UAA, but a strong defect in termination at UAG and UGA (reduced 14-fold and 11-fold, respectively). This indicates that E52A shows a strong bias against guanine residues at the second or third positions (UAG and UGA), a distinct phenotype from the Y122F mutant. The different nature of these phenotypes indicates that the potential hydrogen bonding of E52 and Y122 is not their only contribution to standard code stop codon recognition.

Residue V68 is also directly adjacent to cavity three. eRF1 carrying the V68A mutation mediated efficient termination at UAG, while termination at both UAA and UGA was decreased (4-fold and 14-fold, respectively). Thus, the V68A mutant inhibited recognition of adenine at the third position (as in UAA/UGA), a result that is consistent with cavity three serving to bind the third nucleotide of the stop codon. A recent crystallization study of an eRF1/eRF3 complex found ATP, a component of the precipitant solution, bound to this same pocket [26]. Importantly, V68 was identified as one of the hydrophobic residues that bound the adenine base, implicating the potential importance of this residue in decoding stop codons [26]. However, those authors concluded that the bound ATP was located in a cavity that bound the second, rather than the third, nucleotide of the stop codon. Our results demonstrate that the Y122F, E52A and V68A mutations generally exhibit strong effects on selection of the third nucleotide of the stop codon. Furthermore, given the importance of L123 and C124 in recognition of the second nucleotide of the stop codon, we believe that the sum of our results are most consistent with the cavity model proposed by Bertram and co-workers [8].
Unfortunately, there is a significant body of conflicting data in the literature regarding several of these key eRF1 mutations. For example, Bertram and colleagues analyzed a V68A mutant and reported that termination remained similar to wild-type eRF1 in *S. cerevisiae* cells [8]. The reason for this discrepancy with our results is not known. Two other studies examined the E52A mutation (E55A in human eRF1) [24,26]. In our study, we found that this mutation allows termination at UAA, but largely excludes UAG or UGA codons. In contrast, Cheng et al. [26] reported that an E52A mutant exhibited only subtle (1.5 to 2-fold) defects at all three stop codons in an *S. cerevisiae* eRF1 depletion strain expressing mutant derivatives of human eRF1. Another study characterized the E52A mutation in human eRF1 using an fmet release assay (a highly purified system) [24]. They reported that the E52A mutant eRF1 showed a strong defect at the UAG codon, and more subtle (2-fold) defects at UAA and UGA codons. Again, the reasons for these different results are not clear. However, the use of such diverse assay systems, eRF1 species, and experimental conditions has previously made it difficult to compare results from different studies in the context of a unified model for stop codon recognition.

There are several advantages to the experimental system used in the current study. First, results were obtained using a homologous in vivo *S. cerevisiae* system in which mutant eRF1 derivatives were maintained in the absence of selective pressure for suppressor mutations until the assays were carried out, thus minimizing the potential for second-site mutations that could influence the results obtained. Second, a relatively small number of amino acids were changed in the context of *S. cerevisiae* eRF1 in each experiment, and swaps of entire domains to produce hybrid eRF1 proteins that could
complicate the interpretation of results was avoided. Third, control strains expressing either wild-type *S. cerevisiae* eRF1 or a strain completely depleted of eRF1 were included. These controls demonstrated that this system provides a broad dynamic range for both decreased termination efficiency (ranging from 17 to 26-fold; see Table 1) and increased termination efficiency (up to five-fold). Together, these results show that our system allows quantitation of termination efficiency over a 130-fold range. Finally, many other proteins have been shown to influence the efficiency of translation termination in vivo [39]. Consequently, the use of in vivo termination measurements ensures that all data were obtained in a physiologically relevant environment. When taken together, our results provide a unified picture of the functional consequences of these mutations in the context of *S. cerevisiae* eRF1 in its natural in vivo environment.

Our current data reinforces previous findings that the TASNIKS and YCF motifs play important roles in stop codon recognition. However, it also clearly shows that changes in the YCF consistently result in stronger and more specific effects on the expected variant patterns of stop codon selection. It is informative to consider these results in the context of our recent study that examined a hybrid eRF1 containing domain one of *Euplotes octocarinatus* fused to domains two and three of *Saccharomyces cerevisiae* eRF1 (referred to as Eo/Sc eRF1) [18]. We found that Eo/Sc eRF1 showed a UAA/UAG pattern of stop codon recognition, and did not efficiently recognize the UGA codon. When the *Euplotes* variant TAESIKS motif in Eo/Sc eRF1 was changed to the standard TASNIKS sequence (Eo/Sc eRF1 E57S/S58N) we found that omnipotent stop codon recognition was restored. Importantly, we also showed that termination at UAA/UAG codons no longer required eRF3 (either in vitro or in vivo), while UGA
termination retained the usual eRF3 requirement. Those results led us to propose a conformational model in which eRF3 induced eRF1 to acquire a final position/conformation on the pre-termination complex after stop codon recognition that was dependent on the bound stop codon (UAA/UAG vs. UGA). We reasoned that the Eo/Sc eRF1 E57S/S58N mutant maintained the UAA/UAG position/conformation constitutively, thus relieving the requirement for eRF3 to enhance termination at those stop codons. However, this mutant continued to require eRF3 in order to attain the alternate position/conformation required for termination at UGA. The implications of this model is that the primary role of the TASNIKS motif is not direct stop codon recognition; instead, it acts to couple stop codon recognition and GTP hydrolysis to the acquisition of alternate positions/conformations of eRF1 on the pre-termination complex prior to polypeptide release. The data presented in the current study are generally consistent with the TASNIKS motif mediating such a coupling function, since the introduction of TASNIKS motifs from most variant code organisms showed only a limited ability to determine stop codon specificity. In contrast, the YCF motifs from those organisms were generally much more efficient at conferring the stop codon specificity of each organism, consistent with a role in direct molecular contacts with the residues of the stop codon as predicted by the cavity model [8]. Ultimately, structural studies on these distinct termination complexes will be required to confirm the validity of this model.
ACKNOWLEDGEMENTS

We thank Heflin Center Genomics Core Facility for DNA sequencing of eRF1 mutant alleles. This work was supported by NIH grant GM068854 (to DMB).

REFERENCES


<table>
<thead>
<tr>
<th>Designation</th>
<th>Description</th>
<th>Organism Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDB1037</td>
<td>pDB1047 carrying Sc eRF1 TAESIKS</td>
<td>Euploites octocarinatus</td>
</tr>
<tr>
<td>pDB1196</td>
<td>pDB1047 carrying Sc eRF1 YICDNKF</td>
<td>E. octocarinatus/ B. americanum</td>
</tr>
<tr>
<td>pDB1195</td>
<td>pDB1047 carrying Sc eRF1 TAESIKS/YICDNKF</td>
<td>Euploites octocarinatus</td>
</tr>
<tr>
<td>pDB1124</td>
<td>pDB1047 carrying Sc eRF1 KSSNIKS</td>
<td>Blepharisma americanum</td>
</tr>
<tr>
<td>pDB1125</td>
<td>pDB1047 carrying Sc eRF1 KSSNIKS/YICDNKF</td>
<td>Blepharisma americanum</td>
</tr>
<tr>
<td>pDB1112</td>
<td>pDB1047 carrying Sc eRF1 EAASIKD</td>
<td>Paramecium tetraurelia</td>
</tr>
<tr>
<td>pDB1113</td>
<td>pDB1047 carrying Sc eRF1 YFCDPQF</td>
<td>Paramecium tetraurelia</td>
</tr>
<tr>
<td>pDB1111</td>
<td>pDB1047 carrying Sc eRF1 EAASIKD/YFCDPQF</td>
<td>Paramecium tetraurelia</td>
</tr>
<tr>
<td>pDB1198</td>
<td>pDB1047 carrying Sc eRF1 KATNIKD</td>
<td>Tetrahymena thermophila</td>
</tr>
<tr>
<td>pDB1199</td>
<td>pDB1047 carrying Sc eRF1 YFCDSKF</td>
<td>Tetrahymena thermophila</td>
</tr>
<tr>
<td>pDB1197</td>
<td>pDB1047 carrying Sc eRF1 KATNIKD/YFCDSKF</td>
<td>Tetrahymena thermophila</td>
</tr>
<tr>
<td>pDB1110</td>
<td>pDB1047 carrying Sc eRF1 AAQNIKS</td>
<td>Oxytricha trifallax</td>
</tr>
<tr>
<td>pDB1114</td>
<td>pDB1047 carrying Sc eRF1 YFCGGKF</td>
<td>Oxytricha trifallax</td>
</tr>
<tr>
<td>pDB1109</td>
<td>pDB1047 carrying Sc eRF1 AAQNIKS/YFCGGKF</td>
<td>Oxytricha trifallax</td>
</tr>
<tr>
<td>pDB1152</td>
<td>pDB1047 carrying Sc eRF1 RAQNIKS</td>
<td>Loxodes striatus</td>
</tr>
<tr>
<td>pDB1154</td>
<td>pDB1047 carrying Sc eRF1 FLCENTF</td>
<td>Loxodes striatus</td>
</tr>
<tr>
<td>pDB1158</td>
<td>pDB1047 carrying Sc eRF1 RAQNIKS/FLCENTF</td>
<td>Loxodes striatus</td>
</tr>
<tr>
<td>pDB1104</td>
<td>pDB1047 carrying Sc eRF1 T29A</td>
<td></td>
</tr>
<tr>
<td>pDB1103</td>
<td>pDB1047 carrying Sc eRF1 I32A</td>
<td></td>
</tr>
<tr>
<td>pDB1101</td>
<td>pDB1047 carrying Sc eRF1 E52A</td>
<td></td>
</tr>
<tr>
<td>pDB1200</td>
<td>pDB1047 carrying Sc eRF1 T55K</td>
<td></td>
</tr>
<tr>
<td>pDB1105</td>
<td>pDB1047 carrying Sc eRF1 V68A</td>
<td></td>
</tr>
<tr>
<td>pDB1102</td>
<td>pDB1047 carrying Sc eRF1 A71V</td>
<td></td>
</tr>
<tr>
<td>pDB1108</td>
<td>pDB1047 carrying Sc eRF1 I72A</td>
<td></td>
</tr>
<tr>
<td>pDB1106</td>
<td>pDB1047 carrying Sc eRF1 Y122A</td>
<td></td>
</tr>
<tr>
<td>pDB1107</td>
<td>pDB1047 carrying Sc eRF1 Y122F</td>
<td></td>
</tr>
<tr>
<td>pDB1181</td>
<td>pDB1047 carrying Sc eRF1 L123F</td>
<td></td>
</tr>
</tbody>
</table>
AVAILABILITY OF FUNCTIONAL TRANSLATION TERMINATION COMPLEXES REGULATES EUKARYOTIC RELEASE FACTOR 1 ABUNDANCE VIA MRNA STABILITY

by

SARA E. CONARD, ADAM K. KALLMEYER, KIM M. KEELING, ANDREY V. PISAREV, MING DU, TATYANA V. PESTOVA AND DAVID M. BEDWELL

In preparation for RNA

Format adapted for dissertation

54
ABSTRACT

Eukaryotic translation requires a dedicated set of factors for each of the four steps of protein synthesis: initiation, elongation, termination, and recycling. The two eukaryotic release factors, eRF1 and eRF3, must form a functional complex to facilitate efficient translation termination. In this study, we describe a novel post-transcriptional regulatory mechanism in *Saccharomyces cerevisiae* that controls the abundance of eRF1 protein by modulating the stability of its mRNA. We show that cells expressing a mutant eRF1 lacking the last 19 amino acids (eRF1-CΔ19) exhibit a six-fold increase in the steady-state level of eRF1 protein and mRNA. This increase is mediated (at least in part) by an increase in the stability of *SUP45* mRNA (which encodes eRF1). Strains expressing other mutant forms of eRF1 and eRF3 also display smaller increases in eRF1 protein (and mRNA) abundance. Previous studies have shown that eRF1-CΔ19 is unable to form stable termination complexes with eRF3. Consistent with this defect, we found that the addition of excess eRF3 to an *in vitro* peptide release assay rescues the termination defect associated with eRF1-CΔ19. Furthermore, overproduction of eRF3 in a strain expressing eRF1-CΔ19 to drive termination complex formation attenuates the up-regulation of eRF1 abundance, while depletion of eRF3 in a wild-type strain increases eRF1 levels. Taken together, these results suggest that eRF1 levels are controlled by a regulatory mechanism that modulates the stability of *SUP45* mRNA as a function of the availability of functional termination complexes.
INTRODUCTION

The four stages of eukaryotic translation are initiation, elongation, termination and recycling. The molecular details of some stages, such as initiation of protein synthesis, have been studied extensively [1]. However, other steps in this process have only begun to be studied at the mechanistic level. Changes in the abundance of ribosomes or various translation factors can be associated with a range of diseases, including cancer [2]. However, regulatory circuits that normally modulate the levels of translation factors to fine-tune specific steps of the translation process are largely unknown.

Eukaryotic release factors 1 (eRF1) and 3 (eRF3) are the two proteins required for translation termination. eRF1 is an essential factor that facilitates both stop codon recognition and polypeptide release [3,4]. Unlike the prokaryotic functional homologues RF1 and RF2, which separately recognize UAA and UAG, or UAA and UGA respectively, eRF1 recognizes all three termination codons [5-7]. eRF1 and eRF3 form a stable complex that is required for efficient translation termination. They interact through their C-terminal domains, and this complex formation is required for efficient termination (and cell viability) in vivo [8-10]. Recent studies have described the importance of a series of conformational changes within the eRF1/eRF3 complex that are required for termination to occur [11,12]. These changes are promoted upon eRF1 binding to eRF3•GDP, nucleotide exchange from eRF1/eRF3•GDP → eRF1/eRF3•GTP, and GTP hydrolysis by eRF3 [13,14]. These changes aid in the initial interaction of the termination complex with the ribosome, accommodation of the termination complex into the ribosomal A site, stop codon recognition, and finally, release of the nascent polypeptide chain [15,16]. Previous studies have reported that eRF3 is present in a six-
fold molar excess relative to eRF1 in the yeast *Saccharomyces cerevisiae* [17,18]. The importance for this imbalanced stoichiometry for translation termination is unclear. However, the excess of cellular eRF3 could be associated with other important interactions, such as its association with yeast poly(A) binding protein Pab1p or its interaction with Upf1p at nonsense mutations during the early stages of nonsense-mediated mRNA decay (NMD) [19,20].

In the current study, we found that expression of a C-terminally truncated form of eRF1 (eRF1-CΔ19) as the sole source of eRF1 in *Saccharomyces cerevisiae* results in elevated steady-state levels of both eRF1 protein and its encoding *SUP45-CΔ19* mRNA [21]. Interestingly, the increase in eRF1 expression does not appear to reflect increased *SUP45* promoter activity. Instead, we found that the stability of *sup45-CΔ19* mRNA is two- to four-fold greater than wild-type *SUP45* mRNA. We show that overproduction of eRF3, thought to drive termination complex formation with eRF1-CΔ19, enhances termination efficiency and reduces eRF1-CΔ19 protein and mRNA levels. We propose a model in which the stability of *SUP45* mRNA is increased when functional eRF1/eRF3 translation termination complexes are limiting, which leads to corresponding increases in the abundance of *SUP45* mRNA and eRF1 protein. This regulatory mechanism appears to tightly control the abundance of the limiting release factor (eRF1) to fine-tune the overall efficiency of translation termination.
MATERIALS AND METHODS

Yeast strains and growth conditions. The *Saccharomyces cerevisiae* strains used in this study are listed in Table S1. YDB638 (*MAT* *ura3*-52 *leu2*-3,112 *lys2*-80 *his3*-Δ200 *trp1*-Δ901 *suc2*-Δ901 *sup45*-CΔ19::*LEU2* [psi-]) was generated by integrating a linearized plasmid, pDB973, containing the *sup45*-CΔ19 allele into the *sup45Δ* strain YDB447 using standard yeast genetic techniques. Briefly, pDB973 was linearized by digestion with BplI and transformed into YDB447/pUKC802 (which expresses wild-type eRF1). pUKC802 was then evicted using 5-FOA selection. The presence of the *sup45*-CΔ19 allele was confirmed by western blot analysis. YDB668 (*MAT* *ura3*-52 *leu2*-3,112 *lys2*-80 *his3*-Δ200 *trp1*-Δ901 *suc2*-Δ901 *sup45*-1stop::*LEU2* [psi-]) was generated by integrating the *sup45*-1stop allele using the linearized plasmid pDB1243 (*sup45*-1stop/YIp lac128). YDB669 (*MAT* *ura3*-52 *leu2*-3,112 *his3*-Δ200 *trp1*-Δ901 *lys2*-80 *suc2*-Δ901 *GAL* + *mel upf1::kanMX* [psi-]) and YDB670 (*MAT* *ura3*-52 *leu2*-3,112 *lys2*-80 *his3*-Δ200 *trp1*-Δ901 *suc2*-Δ901 *sup45*-CΔ19::*LEU2* *upf1::kanMX* [psi-]) were generated by transforming a DNA cassette containing the *kanMX* open reading frame (ORF) with *UPF1* flanking sequences into YDB340 and YDB638, respectively. Each knockout was confirmed using PCR and measurement of *CYH2* pre-mRNA levels. YDB630 (*MAT* *leu2*-3,112 *his3*-11,15 *trp1*-1 *ura3*-1 *ade1*-14 *xrn1::C. albicans HIS3* [psi-]) was generated by transforming into YJW614 a knockout cassette containing the *C. albicans HIS3* gene with XRN1 flanking sequences generated using primers DB2125 and DB2126. YDB632 (*MAT* *leu2*-3,112 *his3*-11,15 *trp1*-1 *ura3*-1 *ade1*-14 *ski2::C. albicans TRP1*) was generated in the same manner from YJW614 using primers DB1173 and DB1174 and the *C. albicans TRP1* gene. Synthetic medium contained either 2% glucose
(dextrose; SD) or 2% galactose (SGal) and other required nutritional supplements. Yeast peptone dextrose (YPD) medium is a rich medium containing 2% glucose [22].

*Immunoprecipitation, western blots and protein half-life analysis.* Metabolic labeling was carried out in methionine-free SD medium by the addition of 200 µCi/mL EXPRESS [³⁵S] protein labeling mix (Perkin Elmer) to 2.5 OD₆00 of cells in 0.5 ml fresh SD medium for 10 minutes [23]. Cells were added to trichloroacetic acid (TCA; 7% final concentration) and pelleted. Cold 100% acetone was used to wash pellets twice and pellets were then dried. Yeast were subject to mechanical lysis with glass beads in 50 µL of lysis buffer (50 mM Tris pH 7.5, 1% SDS, 1 mM EDTA). 800 µL of IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA) was added, and the cell debris was removed by centrifugation. 600 µL of the supernatant was removed, combined with 4µL of eRF1 polyclonal antibody and incubated overnight at 4°C. Protein-antibody complexes were recovered by incubating with Protein A Sepharose beads at 4°C for an hour. The beads were washed twice with IP buffer followed by a wash with 1% 2-mercaptoethanol. The complexes were then boiled in SDS loading buffer and proteins recovered were resolved on an 8% SDS-PAGE gel. The gel was fixed, dried and developed using a PhosphorImager (GE Healthcare).

eRF1 protein half-life measurements were carried out by placing *SUP45* wild-type (pDB967) or sup45-CΔ19 (pDB1245) under *GAL1* promoter control. Cultures were initially grown in SGal medium and allowed to reach an optical density of 0.5 A₆00 units/ml. Cells were harvested by centrifugation and re-suspended in synthetic medium lacking a carbon source. After collection of the first time point, glucose was added to a 2% final concentration to specifically inhibit transcription of the *SUP45* gene. Aliquots
were collected at designated times for the remaining time points. Depletion of eRF3 was achieved by placing the SUP35 gene under GAL1 promoter control. After initial growth in SGal medium, cultures were grown overnight in medium containing 2% glucose for 6 to 8 generations. Equal amounts of total protein were measured using the Lowery method and subjected to Western blotting [24].

Dual luciferase assays. Dual luciferase assays were performed as previously described [21,25-27]. The dual luciferase reporter contained in-frame Renilla and firefly luciferase genes separated by a linker region containing a readthrough cassette with either a sense or stop codon. Readthrough of a stop codon in the linker region was determined as downstream firefly luciferase activity relative to upstream Renilla luciferase activity. Reporters containing a sense codon instead of a stop codon served as 100% readthrough control. “Percent Readthrough” values expressed as mean of n=4 ± standard deviation.

Northern blots and mRNA half-life analysis. To monitor mRNA half-life, transcription was inhibited either by the addition of 3 µg/mL thiolutin or by transcriptional shut-off of the GAL1 promoter using a galactose to glucose shift [28]. Aliquots were harvested at the designated times after transcriptional inhibition. A strain expressing SUP35 mRNA under GAL1 promoter control in the plasmid pDB1244 was used for eRF3 depletion after a shift to SD medium. For Northern blots, total RNA was isolated for each sample and equal amounts of total RNA were loaded onto a formaldehyde-agarose gel. To control for differences in loading, mRNA levels were normalized to rRNA or actin mRNA (as indicated). mRNA half-lives were determine by first-order kinetics using the equation t_{1/2}=0.693/k. Individual data points were obtained in at least two independent experiments (and usually three or more experiments). Steady-state levels are expressed
as fold change relative to wild-type (mean ± standard deviation). Radiolabeled $^{32}\text{P}\text{-DNA}$ probes to specific mRNAs were made by PCR amplifying and gel purifying DNA fragments labeled using Ready-to-Go Labeling Beads (GE Healthcare). Unincorporated dCTP was removed using a Bio-Spin Column (Bio-Rad).

**Polypeptide release assay.** The termination assay was performed as previously described with the following modifications for utilization of the yeast release factors [11,29]. Mammalian initiation and elongation factors and ribosomes were used to form pre-termination complexes on an mRNA template. Yeast release factors containing N-terminal 6X His-tags were purified from *E. coli*. The addition of release factors triggered peptide release, which was monitored by measuring the release of [$^{35}\text{S}$]-labeled nascent chains by TCA precipitation. The amount of yeast release factors used (unless otherwise specified) was 0.5 pmol eRF1 and 0.2 pmol eRF3. The amount of pre-TCs used for all reactions was 0.02 pmol.

**RESULTS**

*Increased abundance of eRF1 in a yeast strain that has a defect in translation termination.*

We previously constructed a *Saccharomyces cerevisiae* strain that expressed a *sup45* allele containing a precise deletion of the last 19 codons of the *SUP45* gene (*sup45-CΔ19*) as the sole source of eRF1. This construct was previously used to identify residues of eRF1 subject to CK2 protein kinase-mediated phosphorylation [21]. The absence of the last 19 amino acids of eRF1 severely impairs the ability of eRF1-CΔ19 to associate with eRF3 in a stable termination complex [21,30]. In addition, expression of
eRF1-CΔ19 as the sole source of eRF1 results in a defect in translation termination, with increased readthrough occurring at UAA, UAG and UAA codons (Table 1).

### Table 1. eRF1-CΔ19 causes readthrough of stop codons

<table>
<thead>
<tr>
<th>Stop Signal</th>
<th>Percent Readthrough</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>CΔ19</td>
</tr>
<tr>
<td>UAAA</td>
<td>0.30±0.03</td>
<td>1.00±0.09</td>
</tr>
<tr>
<td>UAAC</td>
<td>0.51±0.03</td>
<td>2.88±0.30</td>
</tr>
<tr>
<td>UAGG</td>
<td>0.37±0.05</td>
<td>1.18±0.05</td>
</tr>
<tr>
<td>UAAU</td>
<td>0.23±0.02</td>
<td>1.38±0.16</td>
</tr>
<tr>
<td>UAGA</td>
<td>0.21±0.01</td>
<td>0.77±0.11</td>
</tr>
<tr>
<td>UAGC</td>
<td>0.49±0.04</td>
<td>4.44±0.61</td>
</tr>
<tr>
<td>UAGG</td>
<td>0.23±0.02</td>
<td>1.28±0.08</td>
</tr>
<tr>
<td>UAGU</td>
<td>0.16±0.01</td>
<td>1.03±0.11</td>
</tr>
<tr>
<td>UGAA</td>
<td>0.67±0.09</td>
<td>2.95±0.48</td>
</tr>
<tr>
<td>UGAC</td>
<td>1.33±0.14</td>
<td>23.04±2.09</td>
</tr>
<tr>
<td>UGAG</td>
<td>0.58±0.06</td>
<td>5.10±0.52</td>
</tr>
<tr>
<td>UGAU</td>
<td>0.22±0.03</td>
<td>1.62±0.21</td>
</tr>
</tbody>
</table>

*aAll measurements were carried out in the sup45Δ strain YDB447 carrying pDB800 (SUP45) or pDB0843 (sup45-CΔ19).

*bPercent readthrough is expressed as mean ± standard deviation.

*c*p≤0.01, as determined by Mann-Whitney statistical test.

Unexpectedly, western analysis revealed that steady-state levels of eRF1-CΔ19 were elevated six to seven-fold relative to a strain expressing wild type eRF1. This increase was observed when eRF1-CΔ19 was expressed either from a chromosomal insertion or a centromeric plasmid (Figure 1A). This increased abundance of eRF1-CΔ19 was not simply due to increased protein stability, since we found that eRF1-CΔ19 had a significantly shorter half-life (t½=3.0 hours) that wild-type eRF1 (t½=25.8 hours) (supplemental Figure 1). Northern analysis found that sup45-CΔ19 mRNA levels were
also elevated ~six-fold whether expressed from the chromosome or from a centromeric plasmid (Figure 1A). Since eRF3 is known to play a pivotal role in translation termination and also binds directly to eRF1, we also examined the steady-state abundance of eRF3 protein and its encoding SUP35 mRNA levels in isogenic wild type and eRF1-CΔ19 strains. Steady-state eRF3 protein levels were increased 1.3 to 1.7-fold, while SUP35 mRNA levels were elevated 1.7 to 2.6-fold in those strains (Figure 1A). Thus, eRF1-CΔ19 protein and mRNA levels increased were increased to a much greater extent than eRF3 protein and mRNA.

*Variable stability of SUP45 mRNA regulates eRF1 abundance.*

In the experiments described above we observed a coordinate increase in the steady-state level of eRF1 protein and mRNA. This suggests that translational efficiency was not increased, and regulation was mediated through either the synthesis or stability of the SUP45 mRNA. To distinguish between these possibilities, we first asked whether the SUP45 promoter was responsible for up-regulating eRF1 levels. To do this, we place the wild-type SUP45 and sup45-CΔ19 ORFs under GAL1 promoter control. Transcriptional shut-off was achieved by shifting the carbon source from galactose to glucose, and total RNA was isolated at the indicated times to determine mRNA abundance by northern analysis. We found that sup45-CΔ19 mRNA displayed a 1.4-fold increase in half-life ($t_{1/2}=5.0$ min) compared to SUP45 wild-type mRNA ($t_{1/2}=3.6$ min) (Figure 1B). This suggests that at least a significant portion of the increased abundance of sup45-CΔ19 mRNA was due to altered mRNA half-life.
Figure 1. eRF1 levels are elevated and stabilized in the eRF1-CΔ19 strain. A) The abundance of eRF1-CΔ19 and sup45-CΔ19 mRNA is increased relative to eRF1-WT and SUP45 mRNA, respectively. B) Half-life analysis of SUP45 and sup45-CΔ19 mRNAs expressed under GAL1 promoter control. Transcriptional inhibition of SUP45 and sup45-CΔ19 mRNAs was achieved by carbon source shift from galactose to glucose. C) Half-life analysis of SUP45 and sup45-CΔ19 mRNAs in strains grown in minimal (SD) medium. Transcriptional inhibition was achieved by the addition of 3µg/ml thiolutin.
We next asked whether the increased stability of sup45-CΔ19 mRNA could be observed (relative to SUP45 mRNA in an isogenic control strain) when both were expressed under SUP45 promoter control. The strains were grown in synthetic dextrose (SD) medium and mRNA half-life was measured following transcriptional shut-off using the RNA polymerase inhibitor thiolutin. We found that wild-type SUP45 mRNA exhibited a half-life of 10.8 minutes while sup45-CΔ19 mRNA half-life was 26.7 minutes, a 2.5-fold increase (Figure 1C). Finally, we examined the half-life of these mRNAs in cells grown in rich (YPD) medium. We observed a four-fold increase in sup45-CΔ19 mRNA stability (57.7 minutes) compared to wild-type SUP45 mRNA (13.3 minutes) (supplemental Figure 2). These results suggest that a major component for the increase in steady-state eRF1 levels is due to an increase in sup45-CΔ19 mRNA stability.

Interestingly, the SUP45 (and sup45-CΔ19) mRNA half-lives increased in protein to growth rates (and the metabolic demand for higher protein synthesis rates).

**Increases in eRF1 levels are due to loss of the last 19 amino acids of eRF1.**

Through sup45-CΔ19 mRNA half-life analysis, we found that sup45-CΔ19 mRNA is more stable than wild-type SUP45 mRNA. Since the sup45-CΔ19 mutation contains a deletion of the 57 nucleotides, we next asked whether the deletion removed a cis-regulatory destabilizing element that induces SUP45 mRNA turnover (Figure 2A). To test this model, we introduced a UAA stop codon into the full-length SUP45 gene at the same position where the sup45-CΔ19 ORF ends (19 codons upstream of the normal SUP45 termination codon). This construct resulted in the production of a full-length SUP45 ORF (referred to as eRF1-1stop) but a truncated eRF1 protein. Western and
northern analysis on cultures grown in SD medium revealed that the eRF1-1stop mutation resulted in an elevated steady-state eRF1 protein (4-fold) and SUP45 mRNA (10-fold) (Figure 2B, 2C). These results suggest that the elevated eRF1-CΔ19 levels are due to an absence of the last 19 amino acids of eRF1 protein, rather than deletion of the last 19 codons of the sup45-CΔ19 mRNA. To confirm this finding, we asked whether the eRF1-1stop mutation resulted in an increase in the stability of the sup45-1stop transcript as observed with the sup45-CΔ19 mutation. We found that sup45-1stop mRNA displayed a similar increase in stability to sup45-CΔ19 mRNA (Figure 2D). These results indicate that the removal of the last 19 codons did not remove a destabilizing element in SUP45 ORF. We conclude that the truncated eRF1 protein causes a defect in translation termination that increases eRF1 abundance through an increase in SUP45 mRNA stability.

The sup45-CΔ19 mutation does not increase the abundance of other mRNAs.

To confirm the specificity of the increase in SUP45 mRNA abundance, we next examined the levels of several other mRNAs. We measured the abundance of ATP2 mRNA, which encodes the β subunit of the mitochondrial ATP synthase; TOM70 mRNA, involved in mitochondrial protein recognition and import; and ACT1 mRNA, which encodes the cytoskeletal protein actin. None of these mRNAs exhibited an increase in abundance in the sup45-CΔ19 strain (Figure 3A). These results suggest that the sup45-CΔ19 mutation specifically increases abundance of the sup45-CΔ19 mRNA.

We also examined the influence of the sup45-CΔ19 mutation on the CYH2 mRNA, which encodes the ribosomal large subunit protein L28. CYH2 mRNA is inefficiently
spliced in yeast, and pre-mRNA that escapes to the cytoplasm is normally degraded by the nonsense-mediated mRNA decay (NMD) pathway. This makes the steady-state level of CYH2 pre-mRNA a sensitive indicator of NMD efficiency. We found that the sup45-CΔ19 mutation caused a 1.8-fold increase in pre-CYH2 mRNA, an intermediate level compared to the 3.5-fold increase conferred by a upf1Δ mutation. In contrast, we did not
observe an increase in the mature CYH2 mRNA in the sup45-CΔ19 strain (Figure 3B). These results suggest that the sup45-CΔ19 mutation partially compromises the efficiency of NMD. This observation is consistent with the critical role of the termination complex in inducing NMD of mRNAs at nonsense codons [31], and further supports the model that eRF1-CΔ19 is defective in its ability to assemble into termination complexes with eRF3. Interestingly, we also consistently observed a two-fold enhancement of pre-CYH2 accumulation when the upf1Δ and the sup45-CΔ19 mutations were combined. This suggests that the sup45-CΔ19 mutation may also partially inhibit the RNA turnover pathway that degrades NMD substrates in the absence of NMD.

![image]

**Figure 3.** Other mRNAs do not increase under conditions that increase SUP45 mRNA. The abundance of A) ATP2, TOM70, ACT1, and B) CYH2 mRNAs were determined by northern blot analysis. The designations “pre-“ and “mature” denote the position of the unspliced and spliced forms of CYH2 mRNA.
The inability of eRF1-CΔ19 to form a termination complex with eRF3 leads to inefficient termination.

To gain more direct evidence of the specific step of translation termination defective in yeast strains expressing eRF1-CΔ19 that leads to the up-regulation of SUP45 mRNA, we evaluated the ability of eRF1-CΔ19 to mediate polypeptide release using an in vitro polypeptide release assay (Figure 4). This assay allows us to examine the rate of polypeptide release mediated by purified eRF1 (or eRF1-CΔ19) and eRF3. Pre-termination complexes were formed in an in vitro translation reaction that synthesized a radiolabeled tetrapeptide ([35S]-MVHL) from an mRNA template. Translation was carried out by the addition of purified mammalian components, including 40S and 60S ribosomal subunits, eIF1A, eIF1, eIF2, eIF3, eIF4A, eIF4B, eIF4F, eIF5, eIF5B and elongation factors eEF1 and eEF2. In addition, charged methionyl-, valyl-, histidyl-, and leucyl-tRNAs were present. Release factors were omitted from the reaction to allow formation of pre-termination complexes (pre-TCs), which are stalled ribosomes with the termination codon positioned in the ribosomal A-site. The pre-TCs were then purified over a sucrose gradient to remove unassociated factors. The availability of these pre-TCs allowed us to measure the rate of peptide release upon the addition of eRF1 and eRF3 (either separately or together). While the peptide release assay uses all mammalian components [29], we previously demonstrated that yeast release factors function similarly to the mammalian release factors in this assay [11].

The addition of either yeast eRF1-WT or eRF1-CΔ19 to pre-TCs without eRF3 resulted in very slow peptide release. The addition of yeast eRF1 and eRF3 caused a dramatic increase in the rate of peptide release (Figure 4A). In contrast, the addition of eRF1-
CA19 and eRF3 to pre-TCs resulted in only a slight stimulation of peptide release. This demonstrated that a significant defect in polypeptide release is associated with eRF1-CA19, consistent with previous studies showing that eRF1-CA19 is unable to form a stable complex with eRF3 [8,21]. To examine this point further, we titrated increasing amounts of eRF3 into the eRF1-CA19 release assay to determine whether driving termination complex formation might increase the rate of peptide release (Figure 4B). Upon increasing the abundance of eRF3 by 5 and 25-fold, we saw a dose-dependent increase in the efficiency of peptide release. The 25-fold excess of eRF3 with eRF1-CA19 resulted in rate of peptide release indistinguishable from the rate observed with eRF1-WT and eRF3. These results indicate that the termination defect associated with eRF1-CA19 is due to incomplete formation of the termination complex. This suggests that this defect may be responsible for the stabilization of SUP45 mRNA, and ultimately, the increased abundance of eRF1 when termination is inefficient.

To further explore this interaction, we examined the consequences of co-expressing eRF1-WT and eRF1-CA19 in vivo. We pulsed-labeled cultures for 10 minutes with [35S]-methionine followed by immunoprecipitation with eRF1-specific antiserum to confirm that both proteins were expressed at similar levels (Figure 4C). We next performed a western analysis and found that only eRF1-WT was detectable in the strain expressing both eRF1-WT and eRF1-CA19, and it was present at normal levels. These results suggest that eRF1-CA19 undergoes preferential degradation when co-expressed with wild-type eRF1. When taken together with our other data, these results suggest that eRF1-CA19 may be unable to form termination complexes with eRF3 in competition with eRF1-WT, which leads to its preferential degradation in vivo.
Figure 4. Excess eRF3 rescues the polypeptide release defect associated with eRF1-CΔ19 in vitro. A) The kinetics of peptide release mediated by eRF1-CΔ19 or eRF1-WT. Pre-termination complexes were incubated with 0.5 pmol eRF1 alone, 0.5 pmol eRF1-CΔ19 alone, 0.5 pmol eRF1 plus 0.2 pmol eRF3, or 0.5 pmol eRF1-CΔ19 plus 1.0 pmol eRF3. B) Addition of excess eRF3 rescues peptide release by eRF1-CΔ19. Peptide release mediated by 0.5 pmol eRF1-CΔ19 with 1.0 pmol eRF3 (1x), 5.0 pmol eRF3 (5x), or 25 pmol eRF3 (25x) are shown. The peptide release curve obtained with wild-type eRF1 and eRF3 (from panel A) is shown as a dashed line for comparison. C) eRF1-CΔ19 is unstable when co-expressed with eRF1-WT in vivo. eRF1 synthesis was measured by pulsed-labeling cells with [35S]-methionine/cysteine for 10 minutes. Steady-state levels of eRF1 protein were determined by Western analysis.
Overexpression of eRF3 in vivo decreases sup45-CΔ19 mRNA and protein levels.

Since excess eRF3 rescued the defect in termination associated with eRF1-CΔ19 in the in vitro polypeptide release assay, we next asked whether overexpression of eRF3 in vivo had a similar effect. To do this, we overexpressed eRF3 in eRF1-WT and eRF1-CΔ19 yeast strains. The presence of an eRF3 overexpression plasmid resulted in a 7 to 9-fold increase in eRF3 protein levels and a 18- to 20-fold increase in SUP35 mRNA levels (Figure 5A, 5B). This increase in eRF3 levels coincided with a two to three-fold decrease in eRF1-CΔ19 protein and sup45-CΔ19 mRNA levels. These results suggest that overexpression of eRF3 partially rescued the defect in termination caused by eRF1-CΔ19 which, in turn, reduced the overproduction of SUP45 mRNA in strains carrying sup45-CΔ19 allele. Furthermore, we found that wild-type eRF1 levels were also slightly reduced when eRF3 was overexpressed (Figure 5A).

Our working model is that defects in translation termination lead to stabilization of sup45-CΔ19 mRNA and an increase in eRF1-CΔ19 expression. Since we observed reductions in both eRF1-CΔ19 protein and sup45-CΔ19 mRNA levels, our model predicts that the efficiency of stop codon recognition should also be improved. To test this prediction, we performed dual luciferase readthrough assays. We found that eRF3 overproduction reduced readthrough in the eRF1-CΔ19 strain at UAA, UAG, and UGA stop codons by 12, 14, and 11-fold, respectively (Table 2). Interestingly, overexpression of eRF3 in the wild-type eRF1 strain also reduced readthrough at the three stop codons by roughly two-fold. These findings are consistent with the model that eRF3 overexpression in vivo can drive complex formation between eRF1-CΔ19 and eRF3, resulting in increased stop codon recognition and reduced sup45-CΔ19 mRNA levels.
Figure 5. The abundance of eRF3 influences eRF1 protein and SUP45 mRNA levels. A) Overexpression of eRF3 decreases eRF1-CΔ19 protein abundance. B) Overexpression of eRF3 decreases sup45-CΔ19 mRNA abundance. C) Depletion of eRF3 increases eRF1 protein abundance. D) Depletion of eRF3 increases SUP45 mRNA abundance.
Together, this data provides evidence of the importance of eRF3 in this feedback loop controlling eRF1 abundance.

**Table 2. eRF3 overproduction reduces readthrough**

<table>
<thead>
<tr>
<th>Stop signal</th>
<th>WT</th>
<th>WT+ eRF3</th>
<th>Fold Decrease</th>
<th>CΔ19</th>
<th>CΔ19+ eRF3</th>
<th>Fold Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAAC</td>
<td>0.32±0.10</td>
<td>0.18±0.06</td>
<td>1.8</td>
<td>3.35±0.08</td>
<td>0.27±0.04</td>
<td>12.4</td>
</tr>
<tr>
<td>UAGC</td>
<td>0.20±0.01</td>
<td>0.12±0.008</td>
<td>1.7</td>
<td>4.24±0.25</td>
<td>0.30±0.04</td>
<td>14.1</td>
</tr>
<tr>
<td>UGAC</td>
<td>0.51±0.10</td>
<td>0.25±0.04</td>
<td>2.0</td>
<td>19.13±1.53</td>
<td>1.78±0.32</td>
<td>10.7</td>
</tr>
</tbody>
</table>

\(^a\) All readthrough measurements were carried out in YDB340 (SUP45 +/- pDB1246 (SUP35 on 2µ plasmid) or YDB638 (sup45-CΔ19) +/- pDB1246.  
\(^b\) Percent readthrough is expressed as mean ± standard deviation.

**Depletion of eRF3 increases the steady-state level of SUP45 mRNA.**

The results discussed above demonstrated that overexpression of eRF3 decreased sup45-CΔ19 mRNA abundance. To better understand how eRF3 availability regulates SUP45 mRNA, we also evaluated eRF1 levels following eRF3 depletion. We reasoned that if eRF3 overexpression promoted termination complex formation and decreased SUP45 mRNA levels, depletion of eRF3 should decrease termination complex formation, leading to an increase in SUP45 mRNA abundance. To facilitate eRF3 depletion, we placed the SUP35 gene under GAL1 promoter control and depleted eRF3 by shifting the carbon source from galactose to glucose. After eRF3 depletion of eRF3 to 20% of normal, steady-state SUP45 mRNA levels increased 2.5-fold while eRF1 protein levels increased 1.9-fold (Figure 5C, 5D). These findings suggest that modulation of eRF1 levels are based on the availability of eRF3 and the formation of functional eRF1/eRF3 complexes.
A defect in termination complex formation is not the only signal for up-regulation of eRF1 levels.

We next asked whether strains expressing other eRF1 or eRF3 mutant alleles also displayed increased eRF1 levels. In a previous study, we constructed a yeast strain expressing a hybrid eRF1 protein containing domain 1 from *Euplotes octocarinatus* eRF1 fused to domains 2 and 3 from *Saccharomyces cerevisiae* eRF1 [26]. This hybrid eRF1 was shown to recognize UAA and UAG stop codons, but not UGA stop codons. Consequently, this hybrid eRF1 was unable to maintain cell viability as the sole source of eRF1, which allowed intragenic suppressors to be identified that restored UGA stop codon recognition and cell viability [11]. Notably, we also observed increases in the steady-state levels of several of those mutant proteins. For example, we observed a two-fold increase in the hybrid eRF1-C124S (Figure 6A). We also found similar increases in mRNA abundance of the corresponding *sup45-C124S* mRNA (Figure 6D). Thus, other eRF1 mutants besides eRF1-Δ19 result in elevated eRF1 protein and *SUP45* mRNA levels, suggesting that this up-regulation may result from a defect in eRF1 function.

We also examined an eRF3 mutant, eRF3-H348Q, that exhibits a significant defect in GTP hydrolysis, resulting in defect in stop codon recognition and overall termination efficiency [32]. We found that expression of eRF3-H348Q as only source of cellular eRF3 resulted in a two-fold increase in eRF1 protein (Figure 6A) and *SUP45* mRNA (Figure 6B). eRF3 protein levels in this strain were not increased relative to a strain expressing wild-type eRF3. Since mutations in both eRF1 and eRF3 were found to increase eRF1 abundance, these results are consistent with the existence of a regulatory mechanism increases eRF1 levels in response to a defect in translation termination.
The abundance of SUP45 mRNA is regulated via the 5′ → 3′ decay pathway.

The results described above suggest that the abundance of SUP45 mRNA (and eRF1 protein) is regulated by changes in SUP45 mRNA stability through a process that couples mRNA half-life to the availability of functional translation termination complexes. mRNA turnover is mediated by 5′ → 3′ and 3′ → 5′ pathways [33,34]. To gain further initial insight into the mRNA turnover pathway controlling SUP45 mRNA abundance, we examined the consequences of knockouts of factors in both pathways (supplemental figure 3A). We found that deletion of genes encoding either Ski2p or Ski7p, two factors involved in recruiting cytoplasmic mRNAs to the exosome for 3′ → 5′ turnover, had no effect on SUP45 mRNA abundance. In contrast, deletion of the gene

\[
\text{Figure 6. Other eRF1 and eRF3 mutants display elevated eRF1 levels. A) eRF1 abundance is elevated in strains expressing hybrid eRF1-C124S or eRF3-H348Q. B) The abundance of the corresponding SUP45 mRNAs is increased in strains expressing hybrid eRF1-C124S or eRF3-H348Q. In all cases, protein or mRNA abundance is normalized to an isogenic wild-type control strain.}
\]
encoding Xrn1p, the cytosolic 5′ → 3′ exoribonuclease, caused a 4-fold increase in SUP45 mRNA abundance [34]. Since our earlier results found that eRF1-CΔ19 causes a partial defect in NMD, we also examined whether a upf1Δ mutation altered SUP45 mRNA abundance. No change was observed. Finally, we analyzed SUP45 mRNA levels in an eRF3-ΔNM mutant. eRF3-ΔNM contains a deletion of the N-terminal and middle domains of eRF3, leaving the essential GTPase domain intact. The N and M domains mediate binding to other proteins, including Pab1p [35]. We expressed eRF3-ΔNM as the only form of eRF3 in the cell and found that SUP45 mRNA abundance was not altered. When taken together, these results suggest that eRF1 levels are controlled primarily by a mechanism that mediates 5′ → 3′ turnover of the SUP45 mRNA.

**DISCUSSION**

A number of studies have shown the importance of post-transcriptional control of key proteins involved in translation [36], although such studies have not included eukaryotic termination factors. Regulation of such factors coordinately works to fine-tune the overall efficiency of translation. Tight control of proteins involved in translation is essential since changes in growth rates dictate the amount of factors needed based upon metabolic demand [37]. In addition, an excess of certain translation factors can also cause deleterious effects by altering the balance of key steps in the translation process, frequently leading to disease states [1,38]. Here we describe a new post-transcriptional regulatory mechanism that governs eRF1 abundance, and ultimately the abundance of translation termination complexes. We show that steady-state eRF1 levels are significantly increased when termination is inefficient, and this mechanism is mediated
(at least in part) through an increase in the stability of its mRNA. Alternately, when translation termination is efficient SUP45 mRNA is readily degraded through the 5’ → 3’ pathway to maintain the appropriate levels of SUP45 mRNA and eRF1 protein (Figure 7).

Figure 7. Model for eRF1 regulation. Efficient translation termination carried out by functional eRF1/eRF3 complexes leads to destabilization of SUP45 mRNA and maintenance of its normal steady-state level, which limits eRF1 abundance (left). Inefficient translation termination caused by limiting functional termination complexes promotes stabilization of SUP45 mRNA and an increase in eRF1 abundance (right).

We found rich culture media that allowed faster growth rates resulted in a larger increase in sup45-CΔ19 mRNA stability than growth in minimal media. Since the proportion of total cell mass attributed to the translational machinery increases with growth rate, this suggests that translational demand controls the level of eRF1 protein by coupling SUP45 mRNA stability to the availability of functional termination complexes. We found that removal of the last 57 nucleotides of the SUP45 ORF alone is not
responsible for elevated \textit{SUP45} mRNA levels, since the eRF1-1stop construct that retained this region of the mRNA also exhibited an increase in abundance of the \textit{SUP45} mRNA. We conclude that a \textit{SUP45} mRNA stability element does not reside in the last 19 codons of the \textit{SUP45} ORF. Since both \textit{sup45-CΔ19} and \textit{sup45-1stop} alleles encode identical eRF1 proteins missing the last 19 amino acids, these results suggest that the inability to form functional translation termination complexes is responsible for this phenotype.

Other mutations that compromise the function of termination complexes also increased eRF1 abundance, since hybrid eRF1-C124S mutant resulted in an increase in the abundance of eRF1 protein and \textit{SUP45} mRNA. Furthermore, we found that the eRF3-H348Q mutant also led to an increase in eRF1 protein and \textit{SUP45} mRNA abundance. This indicated that defects in either component of the termination complex results in an increase in eRF1 abundance. When taken together, these results suggest that a regulatory mechanism monitors the efficiency of translation termination to control eRF1 abundance. We note that strains expressing eRF1-CΔ19, the hybrid eRF1-C124S, and eRF3-H348Q each increased eRF1 abundance to different extents. This may be due to differences in the specific termination defects associated with each mutation. This variable response correlates with the \textit{sup45-CΔ19} mRNA half-life analyses of strains grown in minimal medium, where \textit{sup45-CΔ19} mRNA was stabilized 2.5-fold relative to wild-type eRF1, and rich medium where translational demands are greater and \textit{sup45-CΔ19} mRNA was stabilized 4.3-fold. When taken together, these results indicate that eRF1 levels are tightly controlled in a manner that couples translational demand and
termination efficiency so the efficiency of translation termination is maintained within a relatively narrow window.

The \textit{in vitro} peptide release assay demonstrated that excess eRF3 was sufficient to rescue the termination defect caused by eRF1-CΔ19. This indicated that excess eRF3 drives termination complex formation between eRF3 and eRF1-CΔ19, which restores the normal rate of peptide release. Our \textit{in vivo} data allowed us to confirm and extend that observation. We showed that overexpression of eRF3 reduced the abundance of \textit{sup45-CΔ19} mRNA and eRF1-CΔ19 protein. This effect correlated with an increase in termination complex formation, since readthrough of termination codons was reduced 10 to 14-fold in the \textit{sup45-CΔ19} strain when eRF3 was overproduced. Interestingly, overexpression of eRF3 in a wild-type strain also modestly suppressed readthrough reduced the abundance of wild-type eRF1 protein. These results indicate that eRF3 overproduction can drive termination complex formation enough to enhance termination efficiency, leading to a reduction in eRF1 abundance. Finally, we explored the opposite side of the regulatory circuit, showing that limitation of termination complexes by eRF3 depletion led to a modest up-regulation of eRF1 protein and \textit{SUP45} mRNA. Since eRF3 is normally present in a six-fold molar excess relative to eRF1, it is likely that the small increase in \textit{SUP45} mRNA observed was due to an inability to adequately deplete the excess pool of eRF3 that normally exists in yeast cells. When taken together, these results demonstrate the tight regulation that controls eRF1 levels in response to functional termination complexes in yeast. It will be interesting to determine whether a similar regulatory mechanism exists in mammalian cells.
In summary, our results describe a new regulatory mechanism that couple SUP45 mRNA (and eRF1 protein abundance) to the availability of functional translation termination complexes. A recent study used mathematical modeling to suggest that eRF1 levels may be fine-tuned at the translational level in yeast [39]. Our results indicate that post-transcriptional eRF1 abundance occurs, but is manifested through differential SUP45 mRNA stability that results in eRF1 protein levels that are proportional to SUP45 mRNA abundance. It is interesting to compare this to the post-transcriptional mechanism that regulates the synthesis of the prokaryotic class I release factor, RF2 [40]. Unlike eRF1, RF2 regulation occurs at the translational level through a mechanism of translational frameshifting. When RF2 is in excess, its translation terminates prematurely due to the presence of an in-frame UGA termination codon in its ORF. When RF2 is limiting ribosomes pause at the UGA codon. That leads to a ribosomal frameshift that allows translation to resume in the +1 reading frame, resulting in production of additional full-length RF2 protein. Together, the existence of these post-transcriptional mechanisms to control release factor abundance in these diverse organisms illustrates the importance of carefully controlling the efficiency of translation termination. Delineation of the cis-regulatory element(s) that control SUP45 mRNA abundance will provide a more complete mechanistic view of how the efficiency of translation termination is controlled in yeast.

ACKNOWLEDGMENTS

We thank Heflin Center Genomics Core Facility for DNA sequencing. This study was supported by NIH grant R01 GM 68854 to D.M.B.
REFERENCES

15. Frolova LY, Tsvikovskii RY, Sivolobova GF, Oparina NY, Serpinsky OI, Blinov VM, Tatkov SI, Kisselev LL: Mutations in the highly conserved GQG motif of class 1 polypeptide release factors abolish ability of human eRF1 to trigger peptidyl-tRNA hydrolysis. RNA 1999, 5:1014-1020.


<table>
<thead>
<tr>
<th>Strain</th>
<th>Designation</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>YDB340</td>
<td>MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-80 suc2-Δ901 GAL+mel [psi-]</td>
<td>[32]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB1246 YEplac112 (2µ, TRP1, ampª) carrying SUP35</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td>YDB405</td>
<td>MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901ade2-101 suc2-Δ9 sup35::HIS3 GAL+ mel [psi-]</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB764 YCplac111 (CEN4 LEU2, ampª) carrying SUP35</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB1244 YCplac22 (CEN4, TRP1, ampª) carrying Pgal-&gt;SUP35</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td>YDB447</td>
<td>MATa ura3-52 leu2-3,112 lys2-80 his3-Δ200 trp1-Δ901 suc2-Δ901 sup45::HIS3 [psi-]</td>
<td>[32]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB800 pRS315 (CEN4, LEU2, ampª) carrying SUP45</td>
<td>[32]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB843 pRS315 (CEN4, LEU2, ampª) carrying sup45-CA19</td>
<td>[21]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB967 YCplac22 (CEN4, TRP1, ampª) carrying Pgal-&gt;HA-SUP45</td>
<td>[26]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB972 YIplac128 (LEU2, ampª) carrying sup45-CA19</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB974 YCplac111 (CEN4 LEU2, ampª) carrying En/Sc sup45-C124S</td>
<td>[11]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB1037 YCplac111 (CEN4 LEU2, ampª) carrying En/Sc sup45-E57S S58N</td>
<td>[11]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB1243 YIplac128 (LEU2, ampª) carrying sup45-1stop</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB1245 YCplac22 (CEN4, TRP1, ampª) carrying Pgal-&gt;sup45-CA19</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pUKC802 YEp24 (2µ, URA3, ampª) carrying SUP45</td>
<td>[41]</td>
<td></td>
</tr>
<tr>
<td>YDB498</td>
<td>MATa leu2-3,112 his3-11,15 trp1-1 ura3-1ade1-14 sup35::HIS3 [psi-]</td>
<td>[32]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB663 YCplac22 (CEN4, TRP1, ampª) carrying SUP35</td>
<td>[32]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB670 YCplac22 (CEN4, TRP1, ampª) carrying sup35- H348Q</td>
<td>[32]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB1006 YCplac22 (CEN4, TRP1, ampª) carrying sup35-ΔNM</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td>YDB499</td>
<td>MATa leu2-3,112 his3-11,15 ura3-1 ade1-14 ski7::TRP1</td>
<td>[42]</td>
<td></td>
</tr>
<tr>
<td>YDB630</td>
<td>MATa leu2-3,112 his3-11,15 trp1-1 ura3-1 ade1-14 [psi-] (\times) Candida HIS3</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td>YDB632</td>
<td>MATa leu2-3,112 his3-11,15 trp1-1 ura3-1 ade1-14 [psi-] (\times) Candida TRP1</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td>YDB638</td>
<td>MATa ura3-52 leu2-3,112 lys2-80 his3-Δ200 trp1-Δ901 suc2-Δ901 sup45::HIS3 sup45-CA19::LEU2 [psi-]</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDB1246 YEplac112 (2µ, TRP1, ampª) carrying SUP35</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td>YDB668</td>
<td>MATa ura3-52 leu2-3,112 lys2-80 his3-Δ200 trp1-Δ901 suc2-Δ901 sup45::HIS3 sup45-1stop::LEU2 [psi-]</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td>YJW614</td>
<td>MATa leu2-3, 112 his3-11, 15 trp1-1 ura3-1 ade1-14 [psi-]</td>
<td>[42]</td>
<td></td>
</tr>
</tbody>
</table>
**Supplemental Fig 1.** eRF1 and eRF1-CA19 proteins display similar half-lives when expressed as the sole source of eRF1. Strains expressing eRF1 and eRF1-CA19 proteins under GAL promoter control were used. Protein turnover rates were measured after shifting cultures from galactose to glucose to terminate SUP45 (or sup45-CA19) transcription. Under these growth conditions, SUP45 and sup45-CA19 mRNA half-lives were found to be very short (3.5 minutes and 5.0 minutes, respectively). Thus, the contribution of continued translation from pre-existing mRNAs was considered to be negligible 2 hours post-shift and thereafter.
Supplemental Figure 2. *sup45-CΔ19* mRNA is more stable than *SUP45* mRNA. Half-life analysis of *SUP45* and *sup45-CΔ19* mRNAs in strains grown in rich (YPD) medium. Transcriptional inhibition was achieved by the addition of 3μg/ml thiolutin.
Supplemental Figure 3. Deletion of the cytosolic 5′ → 3′ exoribonuclease (xrn1Δ) increases steady-state SUP45 mRNA levels. A) Abundance of SUP45 mRNA was measured in a ski2Δ strain and ski7Δ strains (both defective in cytosolic 3′ → 5′ decay), eRF3-ΔNM (defective in Pab1p association), xrn1Δ (defective in cytosolic 5′ → 3′ exoribonuclease activity) and upf1Δ (defective in nonsense-mediated decay).
SUMMARY

As we have gained a better understanding of the mechanism of translation termination, what once was thought of as discrete steps has merged into a continuous and interwoven process of termination, recycling and initiation. New roles for factors once thought to only be involved in termination have been shown to also be important for ribosome recycling. They have also provided more structural insight into the termination complex which is composed of eRF1 and eRF3. Crystallographic studies of Dom34 and Hbs1 (eRF1 and eRF3 paralogs) have granted mechanistic details that were not seen in the eRF1/eRF3 complex. New roles for eRF1 have been established with studies showing its importance for ribosome recycling. Taken together, these findings begin to highlight the vital roles of eRFs and the requirement for their interaction. Although tremendous progress was achieved, numerous questions remain unanswered.

One such question is how does eRF1 mediate efficient stop codon recognition through its N-terminal domain? This question was addressed by exhaustively probing the TASNIKS and YxCxxxF motifs located in domain one. It was found that replacing \textit{S. cerevisiae} YxCxxxF motif with that of a variant-code organism most often led to altered stop codon specificity that correlated with the original host. However, changes in the TASNIKS motif did not directly affect stop codon specificity. These findings are in agreement with a previous study from our lab and others which suggested that the TASNIKS motif only indirectly influences stop codon recognition while the YxCxxxF motif played a more prominent role [39]. Consistent with the cavity model, the YxCxxxF
motif extends from cavities two and three while the TASNIKS motif lies outside of this region [2].

However, better understanding how eRF1 is able to mediate stop codon recognition did not provide further insight into the required conformational changes of eRF1 that is important for efficient termination. Multiple studies have suggested that accommodation of eRF1 into the ribosomal A-site is aided by GTP hydrolysis of eRF3. New crystallographic studies showing the 3D structure of eRF1 bound to the ribosome with a mRNA template containing either UAA, UAG or UGA stop codons are needed to provide a better understanding of the necessary conformational changes of eRF1 to mediate stop codon recognition for specific stops and whether a different conformation is required to distinguish UAA/UAG from UGA.

In the second study, a post-transcriptional regulatory mechanism was uncovered that controlled cellular eRF1 levels through increasing the stability of eRF1 (SUP45) mRNA, thereby leading to higher eRF1 protein levels. Stability of SUP45 mRNA was modulated by the availability of functional termination complexes. SUP45 mRNA and eRF1 protein steady-state levels and SUP45 mRNA stability were increased in other eRF1 mutants and even eRF3 mutants that displayed various defects in termination. After looking at several other transcripts it appeared that only SUP45 mRNA was being up-regulated. To uncover the specific defect in termination that is responsible for altering eRF1 levels, in vitro and in vivo studies were performed. Those results suggested that eRF1-CΔ19 exhibited a defect in functional termination complexes which was rescued with excess eRF3. Furthermore, overexpression of eRF3 in vivo decreased eRF1-CΔ19 mRNA and protein levels. Consistent with eRF1 levels being controlled by the
availability of functional termination complexes, an increase in termination efficiency in eRF1-CΔ19 seen with excess eRF3 correlated with a decrease in eRF1-CΔ19 steady-state levels.

Although this study provided insight into the complexity of eukaryotic translation termination, it raised several unanswered questions. The overall goal of post-transcriptional regulation of eRF1 cellular levels is to maintain the appropriate levels of eRF1 in the cell. Previous studies have shown that overproduction of eRF1 does not result in an increase in termination efficiency but can actually lead to a decrease [30]. Taken together, these findings reiterate the importance of controlling eRF1 levels for its availability into termination complexes. The current study sheds light on how limitation of termination complexes resulted in increased stability of 

\[ \text{SUP45} \]

mRNA, leading to increased steady-state levels of 

\[ \text{SUP45} \]

mRNA and eRF1 protein. However, it is currently unknown how a limit for termination complexes is relayed to 

\[ \text{SUP45} \]

mRNA. Furthermore, how is 

\[ \text{SUP45} \]

mRNA up-regulated? Is a reduction in termination complexes the only signal for 

\[ \text{SUP45} \]

mRNA up-regulation? Preliminary data showing various eRF1 and eRF3 mutants that up-regulate eRF1 levels hint that limiting termination complex formation is not the only signal for 

\[ \text{SUP45} \]

mRNA up-regulation. Defects in peptide release may also be a signal for up-regulation of 

\[ \text{SUP45} \]

mRNA since eRF1-CΔ19, Eo/Sc C124S and eRF3-H348Q all contain moderate to severe defects in peptide release (Figure 1, step 1). Furthermore, preliminary data (not shown) suggest that 

\[ \text{SUP45} \]

mRNA stability is not modulated through the normal mechanism of 3’ UTR regulation. Switching 

\[ \text{SUP45} \]

3’UTR with either 

\[ \text{ACT1} \]

or 

\[ \text{CYC1} \]

3’ UTRs did not alter steady-state levels of 

\[ \text{SUP45} \]

mRNA. Studies placing different segments of 

\[ \text{SUP45} \]

ORF
to the 3’ end of a reporter and measuring the reporter mRNA steady-state levels can be performed in order to further determine how SUP45 mRNA is regulated through its cis-acting element. However, current data indicate the importance of the 5’→3’ decay machinery in governing SUP45 mRNA levels. More specifically, deletion of Xrn1p resulted in a four-fold increase in SUP45 mRNA steady-state levels. Identification of protein(s) that interact with SUP45 mRNA cis-element will provide insight on how a defect in translation termination efficiency (e.g. defect in complex formation, etc.) is relayed specifically to SUP45 mRNA. This suggests that a complex containing proteins involved in RNA turnover is recruited to SUP45 mRNA but inhibited in eRF1-CΔ19 (Figure 1, step 2). Is eRF3 directly associating with SUP45 mRNA? How is Xrn1p recruited? Are there possibly new factors involved in mediating this regulation? Are these trans-acting factors recruited to the ORF of SUP45 mRNA? Do they associate with RNA turnover machinery? A previous study suggested that Dbp5p, a DEAD-box RNA helicase, was required to bring eRF1 to the pre-termination complex [58]. Several studies, including work from our lab suggest that Dbp5p is not needed [7,27,29,59]. However, it would be interesting to see if Dbp5p is involved in regulating SUP45 mRNA. Dbp5p could be depleted from eRF1-CΔ19 cells and steady-state eRF1 levels determine to see if Dbp5p is needed for up-regulation of sup45-CΔ19 mRNA.

Although eRF3 is present in six-fold molar excess of eRF1, preliminary data suggest that eRF3 is the limiting factor for termination complex formation since overexpression of eRF3 leads to an increase in termination efficiency even in wild-type cells [60,61]. These findings suggest that excess eRF3 was able to bind to eRF1 and
Figure 1. Post-transcriptional regulation of SUP45 mRNA maintains eRF1 cellular levels depending on the efficiency of translation termination. (1) Efficient translation termination, which is either exhibited by (1) termination complex formation or (2) peptide release, is relayed to SUP45 mRNA. Factor X is able to relay the efficiency of termination either through association with the termination complex or by sensing peptide release. (2) Factor X is able to recruit Xrn1, a 5' to 3' exonuclease, which mediates the degradation of SUP45 mRNA. (3) SUP45 mRNA is unstable leading to normal steady-state levels of SUP45 mRNA and (4) eRF1 protein. However, inefficient termination as in eRF1-CΔ19 is presented by either (1) a defect in termination complex formation or (2) ineffective peptide release. This defect in termination does not recruit Factor X to SUP45 mRNA which results in increased stability and an increase in steady-state levels.
participate in translation termination. An understanding of eRF3 binding affinity to eRF1, poly(A)-binding protein (PABP), and Upf1p will provide more insight into the priority of termination complex formation. This can be determined by measuring the binding affinity for each factor to eRF3. Answers to these questions will provide a complete mechanistic view of how eRF1 levels are controlled depending on the efficiency of translation termination. Although eRF1 and eRF3 are the important factors that mediate the specific steps in termination, these studies will set the stage to better understand the additional factors that work behind the scenes to help increase the efficiency of translation termination. More importantly, future studies can address whether regulation of human eRF1 levels are maintained through a similar mechanism.

Taken together, these studies have provided further insight into understanding eRF1 function and how eRF1 protein abundance is regulated at the mRNA level. These findings also integrate the varying degree of importance of the TASNIKS and YxCxxxF motifs in eRF1 into the unifying cavity model.
GENERAL LIST OF REFERENCES


