REGULATORY MECHANISMS OF EUKARYOTIC TRANSLATION TERMINATION

by

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A DISSERTATION

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Translation is separated into three distinct steps: initiation, elongation, and termination. Termination is mediated by a heterodimer of eRF1 and eRF3. eRF1 (encoded by the \textit{SUP45} gene) functions to recognize the stop codon in the A-site of the ribosome and stimulate polypeptide chain release. eRF3 is a GTPase that helps eRF1 stimulate release. Translation consumes a large amount of resources and is highly regulated. Regulatory mechanisms exist to control translation at the first two steps. We made the novel observation that eRF1 is phosphorylated by the CK2-kinase at serines 421 and 432 suggesting that the final step of translation may also be regulated. Phosphorylation of eRF1 was found to be dynamic and dependent on active cellular metabolism. Phosphorylation played little role in mediating stop codon recognition and no role in NMD or binding to eRF3. During our studies of eRF1 phosphorylation, we made a mutant of eRF1 where the C-terminal 19 amino acids were deleted (eRF1-\textit{CΔ19}). This mutant has a reduced affinity for eRF3 and a readthrough phenotype. In addition, the eRF1-\textit{CΔ19} steady-state protein and \textit{sup45-CΔ19} mRNA levels are elevated 5-fold by a mechanism that increases the half-life of the \textit{SUP45} transcript. We furthered our study and found that eRF1 levels were elevated in a strain expressing a GTPase deficient mutant, eRF3-H348Q, and in a strain expressing a hybrid eRF1 protein, Eo1/Sc23-eRF1-C124S. The increase in eRF1 levels was not due to a defect in stop codon recognition or
a decrease in the catalytic efficiency of the GTPase activity of eRF3. We concluded our study showing that the eRF1-CΔ19 and eRF3-H348Q mutants have a slow rate of peptide release. When we combine these data with the cold sensitive phenotype of the Eo1/Sc23-eRF1-C124S mutant, our results indicate that steady-state levels of eRF1 are most likely coupled to a defect in polypeptide chain release. This study reveals a novel regulatory mechanism to control eRF1 levels in a eukaryote.
DEDICATION

To my mother whose support, love, and prayers kept my spirit up during difficult times and to my father whose advice of, “Improvise, adapt, and overcome”, should be made the creed of all graduate students.
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INTRODUCTION

Translation Initiation

The dogma of modern molecular biology holds that messenger ribonucleic acids (mRNA) are synthesized from a deoxyribonucleic acid (DNA) template. The mRNA is modified in the nucleus and exported into the cytoplasm. The mRNA then serves as a template for protein synthesis in a process known as translation. Translation is separated into three distinct steps: initiation, elongation and termination. The process of translation is paramount to the cell’s survival and utilizes a copious amount of resources. Translation has evolved to couple maximal speed and accuracy with optimal utilization of the cellular resources. Because of its central role in the cell, it is a highly regulated process able to sense conditions in the cell and adjust accordingly to diverse environmental stimuli and conditions inside the cell. The three steps of translation will be described here as well as their regulatory mechanisms. Most of the emphasis will be placed on eukaryotic translation. The differences in the mechanism of translation between eukaryotes and prokaryotes will be pointed out in the termination step.

Mechanisms of translation initiation. The initial studies on translation came from work in prokaryotic organisms. Translation initiation occurs in a prokaryote by assembling the preinitiation complex on a Shine-Dalgarno sequence; a sequence on the mRNA that has complementary to a region of the 18S ribosomal RNA (rRNA) in the 30S subunit of the ribosome. Eukaryotes did not appear to have obvious Shine-Dalgarno sequences. Vast biochemical and genetic evidence have suggested a different mechanism described as the scanning model. In this mechanism, 43S ribonucleoprotein complexes
assemble at the capped 5’ end of the mRNA and scan down to the first AUG codon (50). The 43S complexes consist of the 40S ribosomal subunit, eukaryotic initiation factor 2 (eIF2) bound to guanosine 5’-triphosphate (GTP) and Met-tRNA\textsubscript{i}, as well as eIF1A and eIF3 (Figure 1). Binding to the cap requires the heterotrimer eIF4F that is made up of eIF4A, eIF4G, and eIF4E. Following formation of this complex, eIF4B and eIF1A act in concert to enable scanning of the 43S complex in the 5’ to 3’ direction to the first AUG start codon (Figure 1). Upon recognition of the start codon, the GTP molecule bound to eIF2 is hydrolyzed to guanosine 5’-diphosphate (GDP) by eIF5 which displaces eIF2 from the 48S initiation complex. eIF2-GDP is recycled to the eIF2-GTP form by eIF2B (79). With eIF2 removed, eIF5B can now promote joining of the large 60S ribosomal subunit to form the 80S ribosome (67). In this complex, the Met-tRNA\textsubscript{i} occupies the peptidyl site (P-site) while the aminoacyl site (A-site) is ready to accept the next aminoacylated tRNA and begin the elongation step of translation (2).

Within the last 20 years, it was discovered that there are alternative mechanisms of initiation that do not involve ribosomal scanning (63). It was determined that some viral genomes of RNA viruses can adopt complex secondary structures that can recruit or position ribosomes at the start codon and initiate translation with a reduced (or even absent) activity of eIF4F. These regions of RNA were termed internal ribosome entry sites (IRES) and were found initially in picornaviruses such as poliovirus and foot-and-mouth disease virus (33).

**Regulatory mechanisms of translation initiation.** The large commitment of resources to translation is vital to the cell and must be regulated. The initiation step is
Figure 1. Model for eukaryotic translation initiation. Assembly of the ternary complex, eIF3, and the 40S ribosomal subunit make up the 43S complex. This complex assembles on the mRNA at the 5’end and scans down to the first AUG with the help of the trimer eIF4F. Proper recognition of the AUG triggers assembly of the 60S ribosomal subunit to form the 80S complex.
regulated through its protein eIF2α, a subunit of the trimer eIF2, which as noted above plays a central role in formation of the 43S complex. At least four kinases may directly phosphorylate eIF2α at serine 51 in humans (71, 19). Phosphorylation at this residue causes eIF2α to act as a competitive inhibitor of eIF2B and thus prevent recycling of eIF2-GDP to the eIF2-GTP form (72). In a mechanism conserved from yeast to humans, the GCN2 kinase senses uncharged transfer RNA molecules (tRNA). Uncharged tRNA molecules usually indicate a nutrient poor environment lacking amino acids or precursors for their synthesis. In response, the GCN2 kinase phosphorylates eIF2α at serine 51 and shuts down translation. In humans, there are multiple kinases that can phosphorylate eIF2α. The PERK kinase phosphorylates eIF2α in response to an accumulation of misfolded proteins in the cell. The cell can also shut down translation through the PKR pathway that is activated by double stranded RNA molecules. This pathway is thought to protect against viral infection. Finally, the HRI kinase can phosphorylate eIF2α in response to heme depletion in erythroid cells (71). With multiple pathways and conservation of pathways from yeast to human, it is apparent the vital role translation plays in the cell.

Translation Elongation

**Mechanism of translation elongation.** The next step of translation begins when the next aminoacylated tRNA brings in the second amino acid of the nascent polypeptide. The charged tRNA, along with GTP and eukaryotic elongation factor 1A (eEF1A), binds to the A-site of the ribosome. The anticodon of the tRNA molecule makes base pairs with at least two bases of the codon on the mRNA (5, 65). Following recognition of the
cognate tRNA, GTP bound to eEF1A is hydrolyzed and the tRNA is released to the ribosome. The growing polypeptide chain loaded onto the P-site tRNA is transferred to the tRNA located in the A-site increasing the length of the polypeptide chain by one amino acid (Figure 2). Following the transfer, the P-site tRNA enters the exit site (E-site) of the ribosome and then leaves the ribosome completely. The A-site tRNA is translocated into the P-site, moving the ribosome down to the next codon in a GTP dependent process mediated by eEF2 (6, 41).

**Regulation of translation elongation.** It has been determined that the elongation step of translation is also regulated by mechanisms independent of that seen for translation initiation (11). With the elegant regulatory mechanisms that control initiation, it was pondered if regulatory mechanisms would even be required for elongation. However, it is logical that in response to external cell stimuli (i.e. growth factors or insulin), the cell would have to increase the activities or abundance of the elongation factors if they must match the increase in initiation factors. Furthermore, an excess of elongation factors might lead to stop codon readthrough since elongation factors are in competition with the termination factors for codon decoding. Also, a dearth of elongation factors might lead to aberrant premature chain termination or slow down the translation process. All of these scenarios point towards a requirement for elongation rates and factor abundance to be regulated.

Regulation of translation elongation occurs via controlling the activity of eEF2 by phosphorylation (64, 70). In mammals, the eEF2-kinase phosphorylates eEF2 and prevents its association with the ribosome (12). As a result, the translocation step of
Figure 2. Model for eukaryotic translation elongation. eEF1A in complex with an aminoacylated tRNA and GTP binds to the ribosome. The tRNA is either accepted or rejected in a proofreading step. The peptide chain bound to the P-site of the ribosome is transferred onto the A-site tRNA, increasing the length of the nascent polypeptide chain by one amino acid. The ribosome then translocates to the next codon with the aid of eEF2.
elongation is inhibited. The eEF2-kinase is activated by calcium/calmodulin in the cell, however it is not completely understood why intracellular calcium levels would control protein synthesis levels. The current hypotheses are limited to cell type specific control. In cardiac muscle tissue, increased calcium levels might inhibit translation so the cell’s resources may be diverted away from translation for utilization in muscle contraction or other processes (58). In another mechanism, regulation of elongation appears to be controlled by nutrient availability similar to that seen for initiation. Treatment of cells with 2-deoxyglucose, a metabolic poison, led to an increase in phosphorylation of eEF2 (35). These two mechanisms show how important translation and control of translation is to the cell.

**Translation Termination**

**Class I release factors.** Two classes of release factors mediate the final step of translation, termination. Class I release factors function to recognize the stop codon in the A-site of the ribosome and stimulate the release of the nascent polypeptide bound to the P-site tRNA completing the synthesis of the peptide (36). The class I release factors differ in structure, primary sequence, and stop codon recognition from prokaryotes to eukaryotes (46). Release factor 1 (RF1) from prokaryotes recognizes UAG and UAA stop signals while RF2 recognizes UGA and UAA stop signals. In contrast, the eukaryotic release factor (eRF1) can decode all three stop codons (except where noted below). The crystal structure of eRF1 has been ascertained and contains three distinct domains (28, 80). Domain 1, encompassing approximately amino acids 1-140, serves to recognize and decode the stop codon. Domain two stimulates the peptidyl transferase
center (PTC) and contains a universally conserved Gly-Gly-Gln motif that is thought to protrude into the PTC (34, 61). Domain three contains the binding site for the class II release factor (24, 37, 59, 80). While little or no sequence conservation exists between prokaryotic and eukaryotic class I release factors, there is strong conservation among eukaryotes. An amino acid alignment between eRF1 from the yeast *Saccharomyces cerevisiae* and human eRF1 shows over 60% sequence identity over the entire primary sequence with most of the disparity at the extreme C-terminus (80).

Recent experiments in some eukaryotes show that some organisms have diverged from the universal code where UAA, UAG, and UGA serve as stop codons (13, 44, 76). This group of organisms known as ciliates has reassigned one or more of the universal stop codons to sense codons. In the ciliate *Tetrahymena thermophila*, the UAA and UAG have been reassigned to sense codons leaving UGA as the only stop codon. In *Euplotes octocarinatus*, UGA has been reassigned to a sense codon leaving UAA and UAG as the only stop signals in the organism. This group of organisms appears to be unique and offers a tool for understanding the termination mechanism in a eukaryote.

**Class II release factors.** Class II release factors are GTPases that play a role in the termination process. Their roles are different in prokaryotes than in eukaryotes (32, 60, 68). In prokaryotes, RF3 (the class II release factor) serves to recycle the class I factors off of the ribosome following termination. In eukaryotes the activity of eRF3 is still not fully elucidated. It has been shown that eRF3 functions in recognition of the stop codon as mutations in the GTPase domain of eRF3 give rise to a readthrough phenotype (75). In addition, RF3 is dispensable for viability in prokaryotes while absolutely
required for growth in budding yeast. Using in vitro release assays, RF1 and eRF1 are able to stimulate release alone however, the rate of release for eRF1 is greatly enhanced by the addition of eRF3 again suggesting a role for eRF3 in the actual peptide release step (3, 27, 29, 38). The crystal structure of the essential C-terminal portion of eukaryotic eRF3 has also been determined and like eRF1 reveals a 3 domain structure (49). Domain 1 (amino acids 237-467) encompasses the GTPase domain where GTP/GDP can bind and be hydrolyzed. Domain 3 (amino acids 555-662) contains the eRF1 binding site while domain 2 (amino acids 468-554) remains uncharacterized (81, 59, 23). The crystal structure of eRF3 does not include the amino acids 1-236 which are dispensable for viability in yeast (75). In the yeast S. cerevisiae, this domain has been implicated in prion formation (47, 66). Sequence conservation between yeast eRF3 and human eRF3 is strong with 56% similarity over the entire protein. This is in stark contrast with RF3 that has 17% amino acid similarity to that of yeast eRF3 (49). These observations emphasize a lack of conservation of the termination process from prokaryotes to eukaryotes for both the class 1 and class II release factors.

**Mechanism of translation termination.** As described above, the mechanisms of translation termination are very different in eukaryotes when compared to prokaryotes. The termination process begins when a stop codon enters the A-site of the ribosome. eRF1 and eRF3 act as a heterodimer to decode the stop codon. In contrast to elongation where recognition of the codon involves proper formation of hydrogen bonds between the codon of the mRNA and the anticodon of the tRNA, the termination reaction involves a protein-mRNA interaction. Two models of how the stop codon is decoded by the
termination machinery have been hypothesized. One hypothesis suggests that the class I release factors contains a protein “anticodon” that can recognize the stop codon. This is how prokaryotes recognize a stop codon. RF1 contains a protein “anticodon” in the form of a Pro-Ala-Thr motif while RF2 contains a Ser-Pro-Phe motif to decode the stop codon (39). Extensive mutational analysis in domain 1 of eRF1 has not supported a conservation of the protein “anticodon” into eukaryotes (10, 62). An alternative to this hypothesis suggests decoding of the stop codon involves cooperation of two motifs that have some conservation in eukaryotes. The YxCxxxF (amino acids 122 to 128) and the TASNIKS motifs (amino acids 55-61) are believed to decode the stop codon in eukaryotes (38). Recent data have shown that the first uridine in the stop codon can crosslink with the lysine residue in the TASNIKS motif (14). In addition, substitution of the tyrosine residue in the YxCxxxF motif abolished stop codon recognition (48). These two motifs, although separated in the primary sequence, are located in close proximity of each other in the eRF1 crystal structure (80).

While it is established that eRF3 plays a role in stop codon recognition, the exact mechanism has not been determined. eRF1 and eRF3 are known to form a stable heterodimer through interactions of their C-terminal regions and the interaction can occur independently of the ribosome in vitro (24, 42). It remains to be determined whether formation of the heterodimer of eRF1 and eRF3 occurs on the ribosome or in the cytoplasm. It has been demonstrated that eRF1 and eRF3 can be independently targeted to the ribosome (31, 75). eRF3 binds to eRF1 when bound to GTP and current models suggest GTP hydrolysis occurs prior to polypeptide release but following stop codon recognition (Figure 3) (75). This is supported by mutational analysis of the GTPase
domain of eRF3 as well as the crystal structure and recent biochemical data of the release factors (49).

Failure to terminate efficiently at the stop codon will cause readthrough of the stop codon by the ribosome. The ribosome will continue to translate until another stop codon enters the A-site of the ribosome or it reaches the end of the mRNA molecule. Readthrough can occur since the release factors are in constant competition with near cognate tRNAs for decoding the codon in the A-site of the ribosome (74).

In summary, eRF1 and eRF3 form a heterodimer that binds to the ribosome and recognizes the stop codon, stimulating hydrolysis of the GTP molecule bound to eRF3. The Gly-Gly-Gln motif of eRF1 stimulates the PTC causing release of the nascent polypeptide from the P-site of the ribosome. eRF1 and eRF3-GDP are removed from the ribosome by a yet uncharacterized mechanism and the ribosome is recycled for additional rounds of translation.

**Regulation of translation termination.** No regulation of translation termination in a eukaryote has been demonstrated. However, with data showing that initiation and elongation are regulated, it remains likely that termination is regulated as well. A recent copurification of human eRF1 and protein phosphatase 2A suggest that it might be regulated (7). Steady-state levels of RF2 are regulated in prokaryotes. The RF2 transcript contains a premature stop codon and production of full-length RF2 requires a +1 ribosomal frameshift (1, 20). When levels of RF2 are high in the cell, the ribosome terminates at this premature stop codon, yielding a truncated and nonfunctional form of RF2. When levels of RF2 are low, the ribosome stalls at this premature stop codon causing a +1 frameshift by the ribosome yielding full-length RF2. Like other aspects of
translation termination, this mechanism does not appear to be conserved in eukaryotes since no premature stop codons have been found in the transcripts of class I release factors in eukaryotes (with the exception of ciliates as noted in the previous section).

**Nonsense mediated mRNA decay (NMD).** NMD is a eukaryotic surveillance process that degrades mRNAs containing premature stop codons and appears to act in concert with translation termination (18). Translation of mRNAs containing premature stop codons would lead to truncated proteins that might have serious consequences in the cell. Recent work has focused on how the cell recognizes the mRNA stop codon as premature and how the cell differentiates between premature and normal stop codons. The mechanism in mammals appears to be coupled to remodeling of the mRNA as it is being exported from the nucleus. When messages are spliced together, proteins known collectively as the exon junction complex (EJC), are deposited 20-24 nucleotides upstream of each exon-exon junction site. These proteins are later removed during the first round (termed the “pioneer round”) of translation by the ribosome (15, 52, 53).

When the translation machinery encounters a premature stop codon during the pioneer round of translation, the EJC components act to signal the termination machinery that the transcript is aberrant and is then targeted for degradation. Current models suggest that it is hUpf3 (human upframeshift 3) that is bound to the EJC and signals through its interactions with hUpf2 to mark the message as premature (43, 45). hUpf2 is bound to hUpf1 which is hypothesized to bind the termination complex of eRF1 and eRF3 based on biochemical and genetic data from yeast and supported somewhat by data in higher eukaryotes (17, 43). Termination at a normal stop codon will not form the
Figure 3. Schematic of eukaryotic translation termination. eRF1 and eRF3 act in concert to recognize the stop codon in the A-site of the ribosome. GTP is hydrolyzed to GDP by eRF3 prior to peptide release. Release of the nascent peptide chain is achieved by hydrolyzing the ester bond between the peptide and the tRNA in the P-site by eRF1.
interaction between the EJC and the termination machinery since all the EJC components would be removed when the ribosome encounters the correct stop codon and thus would not be susceptible to NMD. Strong evidence for this model stems from the observation that transcripts from HSP70 (heat-shock protein 70) and histone mRNAs are naturally intronless and thus not spliced, are immune to NMD (57).

NMD in yeast shares some similarities to that seen in humans but has several mechanistic differences. Splicing is a rare event in the budding yeast *S. cerevisiae* and thus EJC complexes are not present on the majority of messages. In addition, NMD appears to be an omnipresent process in yeast rather than being restricted to the pioneer round of translation as seen in mammals (55). In other words, the NMD machinery can still recognize a nonsense containing message that has undergone multiple rounds of translation. This cannot occur in the pioneer round model of NMD. According to the faux 3′-untranslated region (UTR) model of NMD, mRNA stability and translation termination are coupled together (4). To prevent NMD, termination must occur adjacent to a properly configured 3′UTR. A “properly configured” 3-UTR consists of interactions between a terminating ribosome and the protein factor(s) bound to the 3′UTR. One such interaction that is vital to proper termination is the interaction between eRF3 and poly(A) binding protein 1 (Pap1p). The failure to terminate near a properly configured 3′UTR activates the NMD pathway. It is believed that Upf1p, bound to the termination complex, actively targets nonsense containing messages to cytoplasmic mRNA decay foci known as P-bodies (78). Once in the P-bodies, Upf2p and Upf3p aid in the rapid decapping and turnover of the message that is the hallmark of NMD.
Translation termination and disease. 30% of all genetic disease is a result of nonsense mutations (26). The inframe premature stop codon may either lead to a dysfunctional protein that exhibits a dominant-negative affect or result in complete loss of the protein. The best example of the former case is in patients with a certain type of β-thalassemia. In these patients, the gene contains a premature stop mutation in the last exon. As a result of being located so close to the normal stop codon, the NMD machinery does not recognize the message and a truncated protein is made from the aberrant message. This defective protein then dimerizes with the α-globin chain to make a dominant-negative protein leading to anemia. When the stop mutation occurs outside of the final exon, little or no protein is synthesized since the message is recognized and degraded by the NMD machinery (51).

The scientific community has attempted to treat these genetic mutations with drugs that stimulate readthrough of the premature stop in an attempt to make enough full-length protein to alleviate the phenotypes associated with the disease. Recent work has shown promise for a group of drugs known as aminoglycosides (54, 69, 22, 83). Treatment of a bronchial epithelial cell line from a patient containing a W1282X mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene with aminoglycosides G418 or gentamicin can restore protein expression and function (9). In another study, aminoglycoside treatment of mice carrying the G542X mutation in the human CFTR gene show an increase in CFTR expression in their intestinal glands (21). These results are only the beginning to a new method of treating not just one genetic disease but most or even all disease caused by nonsense mutations. There are some limitations to this type of treatment. Addition of these drugs causes relatively small
levels of readthrough leading to only a small percentage of full-length protein being made. In some diseases, 50% or more of the functional protein is required to correct the disease phenotype. Additionally, it is not known what affect a 50% level of readthrough of the normal stop codons might have in the cell. It is paramount for the scientific community to continue its research into fully understanding the translation termination mechanism and its regulatory mechanisms for the development of new therapeutic strategies to treat these genetic disorders using this strategy.

In this work, we sought to expand our knowledge of the termination step of translation with particular interest into discovery of the regulatory mechanisms of termination. We began with the novel observation that eRF1 is phosphorylated in the budding yeast *S. cerevisiae*. We propose a model where the phosphorylation is dynamic and may play role in fine-tuning the termination machinery. During our experiments to ascertain the metabolic role of phosphorylation of eRF1, we found another potential regulatory mechanism that seems to couple polypeptide chain release efficiency to steady-state levels of eRF1 protein and mRNA. This work will serve as the basis for future studies of translation termination regulation.
eRF1 PHOSPHORYLATION BY CK2 PROTEIN KINASE IS DYNAMIC BUT HAS LITTLE AFFECT ON THE EFFICIENCY OF TRANSLATION TERMINATION IN
SACCHAROMYCES CEREVISIAE

by

ADAM K. KALLMEYER, KIM M. KEELING, AND DAVID M. BEDWELL

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Abstract

Protein synthesis requires a large commitment of cellular resources and is highly regulated. Previous studies have shown that a number of factors that mediate the initiation and elongation steps of translation are regulated by phosphorylation. In this report, we show that a factor involved in the termination step of protein synthesis is also subject to phosphorylation. Our results indicate that eukaryotic release factor 1 (eRF1) is phosphorylated in vivo at serine 421 and serine 432 by the CK2 protein kinase (previously casein kinase II) in the budding yeast Saccharomyces cerevisiae. Phosphorylation of eRF1 has little affect on the efficiency of stop codon recognition or nonsense mediated mRNA decay (NMD). Phosphorylation also is not required for eRF1 binding to the other translation termination factor, eRF3. In addition, we provide evidence that the putative phosphatase Sal6p does not dephosphorylate eRF1, and the state of eRF1 phosphorylation does not influence the allosuppressor phenotype associated with a sal6Δ mutation. Finally, we show that phosphorylation of eRF1 is a dynamic process that is dependant upon carbon source availability. Since many other proteins involved in protein synthesis have a CK2 protein kinase motif near their extreme C-termini, we propose that this represents a common regulatory mechanism that is shared by factors involved in all three stages of protein synthesis.
**Introduction**

Protein synthesis is carried out in three distinct steps: initiation, elongation, and termination. While the first two steps have been extensively studied, our understanding of the termination process has lagged behind. Two classes of release factors mediate translation termination in the budding yeast *Saccharomyces cerevisiae* and other eukaryotes. eRF1 (encoded by the *SUP45* gene) is a class I release factor that recognizes any of the three stop codons when they are located in the ribosomal A site (5, 10). Following stop codon recognition, eRF1 also induces polypeptide chain release by activating the peptidyl transferase center of the ribosome. eRF3 (encoded by the *SUP35* gene) is a class II release factor that facilitates stop codon recognition and stimulates the termination reaction in a GTP dependent manner (17, 37).

Protein synthesis requires a major commitment of cellular energy and resources. It is not surprising that translation is regulated by multiple mechanisms in response to external stimuli such as nutrient abundance or environmental stress. One mechanism of modulating translational efficiency is through the phosphorylation of various translation factors. Probably the most well characterized example of this regulation is the phosphorylation of serine 51 of the α-subunit of the mammalian translation initiation factor eIF2. When eIF2α is phosphorylated, it competitively inhibits GTP exchange by eIF2B (39). As a result, the ternary complex of eIF2•Met-tRNA<sub>Met</sub>•GTP cannot be replenished and translation initiation is inhibited. The importance of this regulatory mechanism is underscored by its strong conservation throughout essentially all eukaryotic species.
The elongation step of translation is also subject to regulation by phosphorylation in eukaryotes. For example, the elongation factor eEF1A can be phosphorylated on threonine 431 by protein kinase Cδ in mammalian cells (26). Insulin can also increase the phosphorylation of eEF1A, as well as the phosphorylation of the α and γ subunits of its exchange factor eEF1B, through the action of the multipotential S6 kinase (9). These modifications are thought to enhance translation rates. In contrast, phosphorylation of threonine 56 in eEF2 by the novel eEF2 kinase strongly inhibits translation in mammalian cells (8). These examples illustrate the complexity of phosphorylation of specific translation factors on the overall efficiency of the translation process.

In this report, we describe the novel observation that eRF1 is phosphorylated in the budding yeast *S. cerevisiae*. Since human eRF1 was previously found to reside in a stable complex with the catalytic subunit of PP2A phosphatase (2), these results raise the possibility that the termination step of translation, like initiation and elongation, may be subject to regulation by phosphorylation. Here, we characterize the site of yeast eRF1 phosphorylation, show that it is phosphorylated by the CK2 protein kinase, and demonstrate that this post-translational modification is dependant upon carbon source availability.
Materials and Methods

Strains and growth conditions. The *Saccharomyces cerevisiae* strains used in this study are described in Table 1. Strains YDB447 and YDB640 were derived from YDB340 using standard genetic techniques. The strain YDB640 was constructed using a one step gene replacement strategy. The entire *SAL6* ORF was removed by transforming yeast strains YDB340 and YDB447 with a PCR fragment containing the *TRP1* gene from *Candida glabrata* with flanking homology upstream and downstream of *SAL6*. The *C. glabrata TRP1* gene was amplified from pCGTRP1 (27). The *UPF1* gene was deleted in strain YDB641 using a similar strategy. Candidate deletion strains were screened by PCR to verify the insertion. Construction of strains YDB340 and YDB447 have been described elsewhere (37). Strains YDH6 and YDH8 were kind gifts from Claiborne V. C. Glover III, and their construction has been described (21).

YPD is rich medium supplemented with 2% glucose; SMD is synthetic medium supplemented with 2% dextrose (glucose) and amino acids. Wickerham’s minimal medium is a defined minimal medium (43). For $[^{32}P]$–orthophosphate labeling, the phosphate concentration in Wickerham’s minimal medium was reduced to 100 μM. Other specific nutritional supplements were added as needed.

Plasmids. The centromeric plasmid pDB800 (with a *LEU2* selectable marker) carries the wild type *SUP45* gene with 1568 bp upstream of the AUG start codon and 1036 bp downstream of the UAA stop codon. pDB800 was also used as template for site-directed mutagenesis using the QuikChange® mutagenesis kit (Stratagene). The S421A mutation was introduced into the *SUP45* gene and verified by sequencing and
Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>YDB340</td>
<td>$MAT^\alpha$ ura3-52 leu2-3,112 his3Δ-200 trp1-Δ901 lys2-80 suc2Δ-901 $[\psi^-]$</td>
<td>(37)</td>
</tr>
<tr>
<td>YDB447</td>
<td>$MAT^\alpha$ ura3-52 leu2-3,112 his3Δ-200 trp1-Δ901 lys2-80 suc2-Δ901 sup45::HIS3 $[\psi^-]$</td>
<td>(37)</td>
</tr>
<tr>
<td>YDB640</td>
<td>$MAT^\alpha$ ura3-52 leu2-3,112 his3Δ-200 trp1-Δ901 lys2-80 suc2-Δ901 sup45Δ::HIS3 sal6Δ::TRP1 $[\psi^-]$</td>
<td>This study</td>
</tr>
<tr>
<td>YDB641</td>
<td>$MAT^\alpha$ ura3-52 leu2-3,112 his3Δ-200 trp1-Δ901 lys2-80 suc2-Δ901 sup45Δ::HIS3 upf1Δ::TRP1 $[\psi^-]$</td>
<td>This study</td>
</tr>
<tr>
<td>YDH6</td>
<td>$MAT^\alpha$ cka1-Δ1::HIS3 cka2-Δ1::TRP1 CKA2::LEU2</td>
<td>(21)</td>
</tr>
<tr>
<td>YDH8</td>
<td>$MAT^\alpha$ cka1-Δ1::HIS3 cka2-Δ1::TRP1 cka2-8ts::LEU2)</td>
<td>(21)</td>
</tr>
</tbody>
</table>
then a SpeI-HindIII fragment was subcloned back into pDB800 to generate pDB841. Using a similar approach, plasmids encoding the following SUP45 mutant alleles were constructed: S432A (pDB859), S421A/S432A (pDB902), S421D (pDB857), S432D (pDB860), and S421D/S432D (pDB858). Similarly, the I222S mutation was introduced into the SUP45 gene and subcloned back into pDB800 using a Blpl-SpeI fragment to generate pDB970.

To make the construct to express the eRF1 mutant lacking the C-terminal 19 amino acids (eRF1-CΔ19), a SpeI site was generated immediately 5’ of the stop codon of SUP45. A Blpl-HindIII fragment was then subcloned back into pDB800, digested with SpeI and ligated together to precisely delete the last 19 codons. The resulting plasmid was named pDB843. The plasmid used for protein purification of N-terminal His6-tagged eRF1-CΔ19 was made by digesting plasmid pDB843 with SpeI and HindIII and subcloned into SUP45-pET-3A vector pDB698 to create pDB897. Similarly, plasmid pDB858 was digested with SpeI-HindIII and ligated into pDB698 to introduce the eRF1 S421D/S432D double mutant into the pET-3A vector (pDB941).

**Metabolic Labeling and Western Blots.** Yeast strains were grown in Wickerham’s low phosphate minimal medium at 30°C. Cells were grown to mid-log phase (0.5 to 1.0 A_{600} units/ml) and 2.2 OD units of each strain were harvested and re-suspended at a cell density of 4 ODs/ml. Cells were then labeled with 200 μCi/ml of EXPRESS [^{35}S] protein labeling mix (Perkin Elmer) for 1 hour or 400 μCi/ml of [^{32}P]-orthophosphate (Amersham) for 2 hours unless otherwise specified. After labeling, 2.5 ODs of cells were harvested for each sample and 100% TCA was added to achieve a final...
TCA concentration of 7%. Cells were incubated on ice for 20 minutes, harvested by centrifugation, washed twice with 100% acetone and dried under vacuum. Cells were then re-suspended in 50 µl of SDS boiling buffer (1% SDS, 50 mM TRIS pH 7.5, 1 mM EDTA), lysed by agitation with glass beads, and boiled for 5 minutes. Next, 800 µl of Tween-IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween 20, 0.1 mM EDTA) was added to the samples. After a brief centrifugation, 600 µl of the supernatant were removed and combined with either 4µl (eRF3) or 6µl (eRF1) polyclonal antisera and incubated overnight at 4°C. Next, 50 µl of Protein A-agarose bead suspension was added and incubated for 1 hour at 4°C, washed twice with Tween-IP buffer and twice with a 1% solution of 2-mercaptoethanol. The samples were then boiled in SDS gel loading buffer for 5 minutes. The samples were removed with a Hamilton syringe and loaded onto an 8% SDS-PAGE gel. Gels were developed using a PhosphorImager (GE Healthcare).

Cells for western blot were grown and lysed using identical conditions as described above, but the radioisotopes were omitted from the growth medium. Protein concentrations were determined by the Lowry method (29). 25 µg of protein were loaded in each lane of an 8% SDS-PAGE gel. Protein was then transferred to a PVDF membrane using a Transblot apparatus (Bio-Rad). The membranes were blocked in 0.3% Tween 20/PBS buffer containing 5% nonfat milk. Membranes were incubated in the same buffer with a 1:400 dilution of eRF1 polyclonal antiserum for 2 hours at room temperature or overnight at 4°C. Bands were detected using [125I]-Protein A and results were visualized using a PhosphorImager (GE Healthcare).
**Glucose Depletion Metabolic Labeling.** The glucose depletion experiment was carried out as described above with the following modifications. Cells were grown in Wickerham’s low phosphate medium with 2% glucose to mid-log (0.5 to 1.0 $A_{600}$ units/ml). Equal aliquots of cells were harvested and re-suspended at 4 $A_{600}$ units/ml in Wickerham’s low phosphate medium with or without 2% glucose. After further incubation at 30°C for 2 hours, 400 µCi/ml $[^{32}P]$-orthophosphate was added and incubation was continued for two hours. Cells were then harvested and processed as described above. For the chase experiment, cells were grown in Wickerham’s low phosphate medium in the presence of 2% glucose to mid-log (0.5 to 1.0 $A_{600}$ units/ml). Cells were then harvested and labeled for 2 hours at 4 $A_{600}$ units/ml in fresh Wickerham’s low phosphate medium containing 2% glucose. After 2 hours, equal aliquots of cells were harvested, and re-suspended to 4 $A_{600}$ units/ml in Wickerham’s medium with phosphate in the presence or absence of 2% glucose to initiate the chase period. Samples were taken at 0, 0.5, 1, 2, and 4 hours after initiating the chase period and processed as described above.

To assess the role of CK2 protein kinase in eRF1 phosphorylation, strains YDH6 and YDH8 were grown to early log phase at 25°C in Wickerham’s low phosphate medium. Equal aliquots of cells were then shifted to 37°C or maintained at 25°C with shaking for an additional 12 hours (approximately 3 generations). The cells were then harvested, re-suspended at 4 $A_{600}$ units/ml in fresh medium, and aliquots were metabolically labeled as described above at either 25°C or 37°C for 2 more hours. The cells were then lysed and samples processed as described above.
**Analytical Ultracentrifugation.** Purification of N-terminal His$_6$-tagged eRF1 and His$_6$-tagged eRF3 (residues 254-685) from the soluble fraction of *E. coli* lysates has been described (37). Immediately following purification, proteins were dialyzed in phosphate buffered saline (140 mM NaCl, 20 mM NaPO$_4$, pH 7.4). Protein concentrations were determined using Beer’s law: $A=elc$, where $A$ is the absorbance, $e$ is the molar absorptivity, $l$ is the light path through the sample, and $c$ is the concentration. Protein concentrations for analysis were 29 µM for individual proteins or 58 µM total for the mixtures of eRF1 and eRF3. For complex formation, eRF1 and eRF3 were mixed in 1:1 molar ratios and incubated on ice for 20 minutes prior to centrifugation. Sedimentation values were obtained in a Beckman XL-A ultracentrifuge using a 4-channel An-60 Ti rotor. Samples were centrifuged at 50,000 rpm at 4°C for a total of 150 scans. Distributions of the sedimentation coefficients were obtained using the software program SEDFIT (downloadable from www.analyticalultracentrifugation.com).

**Readthrough Assays.** The stop codon readthrough assays were performed using a bicistronic reporter construct consisting of a *Renilla* luciferase gene followed by an in-frame firefly luciferase gene. Separating the two genes is either a tetranucleotide termination signal (e.g. UAA A) or a similar sequence containing a sense codon (e.g. CAA A). The construct is driven by the *PGK1* promoter and has a *CYC1* poly(A) addition signal. Cells were grown in SMD medium supplemented with the appropriate amino acids. Percent readthrough was determined by taking the ratio of the firefly/*Renilla* activities obtained form the termination signal construct relative to the ratio of the firefly/*Renilla* activities for the control (sense codon) construct. Samples
were processed in quintuplicate and each experiment was repeated at least twice. For further details, see (25).

Northern Blots. RNA was extracted from cells using a hot phenol-extraction method (38). Total RNA (25 µg/lane) from the indicated strains was resolved on a 1% agarose-formaldehyde gel and transferred to a nitrocellulose membrane using a Posi-Blot® pressure blotter (Stratagene). Samples were baked in a vacuum oven at 80°C and then probed with a [³²P]-labeled DNA probe for the CYH2 gene. After visualizing results using a PhosphorImager (GE Healthcare), the membrane was stripped by incubation in stripping solution (10mM TRIS-HCl pH=7.5, 0.2% SDS) for 1.5 hours at 75°C. The membrane was then probed with an ACT1 probe for loading control, and results were again visualized using a PhosphorImager (GE Healthcare).

Results

eRF1 is phosphorylated by CK2 protein kinase in vivo. Due to the frequent phosphorylation of translation initiation and elongation factors, we began this study by asking whether either of the translation termination factors, eRF1 or eRF3, are phosphorylated in the budding yeast Saccharomyces cerevisiae. Yeast cultures were metabolically labeled with [³²P]-orthophosphate or [³⁵S]-methionine for two hours, and immunoprecipitation experiments were carried out using polyclonal antibodies to either eRF1 or eRF3. Figure 1 shows that while both eRF1 and eRF3 readily incorporated [³⁵S]-methionine, only eRF1 could be labeled with [³²P]-orthophosphate. These results demonstrate that eRF1 is a phosphoprotein in yeast cells.
Figure 1. eRF1 is phosphorylated in vivo. Yeast strain YDB447 expressing wild type eRF1 (from plasmid pDB800) was labeled with $^{35}\text{S}$-methionine ($^{35}\text{S}$) or $^{32}\text{P}$-orthophosphate ($^{32}\text{P}$). eRF1 or eRF3 was immunoprecipitated from radiolabelled cells using a rabbit polyclonal antisera specific for each factor.
Table 2. Known CK2 phosphorylation sites near the C-terminus of translation factors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Phosphorylation Site&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPP2B</td>
<td>P2β</td>
<td>… KEESDDDM …</td>
<td>component of ribosomal stalk</td>
<td>(3)</td>
</tr>
<tr>
<td>RPP2A</td>
<td>P2α</td>
<td>… AEESDDDM …</td>
<td>component of ribosomal stalk</td>
<td>(3)</td>
</tr>
<tr>
<td>RPP1B</td>
<td>P1β</td>
<td>… AEESDDDM …</td>
<td>component of ribosomal stalk</td>
<td>(3)</td>
</tr>
<tr>
<td>RPP1A</td>
<td>P1α</td>
<td>… KEESDDDM …</td>
<td>component of ribosomal stalk</td>
<td>(3)</td>
</tr>
<tr>
<td>RPP0</td>
<td>P0</td>
<td>… EEESDDDM …</td>
<td>component of ribosomal stalk</td>
<td>(3)</td>
</tr>
<tr>
<td>TIF5</td>
<td>eIF5</td>
<td>… IISEEE …</td>
<td>Promotes GTP hydrolysis by eIF2</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>… AESDDDEEDDE*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUI2</td>
<td>eIF2α</td>
<td>… DSEDDEDESDDDE*</td>
<td>α-subunit of eIF2</td>
<td>(16)</td>
</tr>
</tbody>
</table>

<sup>a</sup> An asterisk (*) indicates position of stop codon.
Using the NetPhos 2.0 phosphorylation site predictor (www.cbs.dtu.dk/services/NetPhos), we found that 10 serine and 3 threonine residues reside within predicted phosphorylation sites in eRF1. We compared this information with a literature search of other proteins involved in translation that are also phosphorylated. Table 2 shows a list of 7 proteins that are phosphorylated near their C-termini in consensus CK2 protein kinase motifs. Strikingly, eRF1 also has two consensus CK2 protein kinase motifs within its last 17 amino acids (see Figure 3B). Our analysis suggested that a number of other proteins involved in translation also have CK2 protein kinase motifs near their extreme C-termini (see Discussion). The conserved nature of these potential CK2 protein kinase motifs led us to next test whether the CK2 protein kinase is responsible for eRF1 phosphorylation in vivo.

CK2 protein kinase activity is essential for cell viability in S. cerevisiae and is encoded by two genes, CKA1 and CKA2 (35). Consequently, we used a strain, YDH8, that retains only temperature-sensitive CK2 protein kinase activity due to disruption of the CKA1 gene and the presence of a temperature-sensitive mutation in the CKA2 gene (cka2ts) to test the hypothesis that eRF1 is phosphorylated by the CK2 protein kinase. This cka2ts strain was previously used to determine whether other yeast phosphoproteins are substrates for the CK2 protein kinase (4, 7, 44). Yeast cultures were grown at the permissive temperature (25°C) to mid-log phase, shifted to the restrictive temperature of 37°C and grown for an additional 12 hours (approximately 3 generations). Cells were then labeled with [32P]-orthophosphate, and eRF1 was immunoprecipitated to determine its level of phosphorylation (Figure 2). Cells from parallel cultures were harvested for western blot analysis to correct for any differences in the steady-state level of eRF1 under
Figure 2. eRF1 is phosphorylated by CK2 protein kinase in vivo. (A) Metabolic labeling of eRF1 in the wild type strain YDH6 (WT) or the CK2 temperature-sensitive mutant YDH8 (TS). Western blot data (WB) to determine steady-state protein levels and [\(^{32}\text{P}\)]-orthophosphate metabolic labeling (\(^{32}\text{P}\)) were used to generate the ratios of [\(^{32}\text{P}\)] incorporated per unit eRF1 protein shown in (B). Twenty-five micrograms of total protein were loaded in each lane for each western blot while 2.5 A\(_{600}\) units of cells were immunoprecipitated with an eRF1-specific antiserum in each \(^{32}\text{P}\) labeling experiment. The data represent the mean values (+/- the standard deviation) of multiple experiments normalized to the wild type strain grown at 25°C.
Figure 3. eRF1 is phosphorylated at serine residues 421 and 432. (A) $^{32}$P-orthophosphate labeling in strains expressing wild type eRF1, eRF1-S421A, eRF1-S432A or eRF1-S421A/S432A. (B) Amino acid sequence of the C-terminal 28 residues of eRF1. The location of serine residues that reside in consensus CK2 protein kinase phosphorylation sites (S-X-X-D/E) that were mutated in (A) are underlined (31). The asterisk (*) indicates the position of the stop codon.
these conditions. The results show a 70% decrease in the phosphorylation state of eRF1 in the temperature-sensitive mutant (relative to the wild-type strain) at the restrictive temperature. These results suggest that yeast eRF1 is an in vivo target of the CK2 protein kinase.

The experiments presented above, as well as the results from the NetPhos 2.0 analysis, suggested that eRF1 phosphorylation occurred at one or both CK2 protein kinase sites located within the C-terminal 17 amino acids of eRF1. To test this hypothesis, site-directed mutagenesis was used to change each of the serine residues at positions 421 and 432 to alanine, both individually and together, and plasmids encoding the mutant forms of eRF1 were introduced into a sup45Δ strain expressing wild type eRF1 from a plasmid carrying the URA3 gene as a selectable marker. Plasmid shuffling (6) was then carried out by plating the transformants on a medium supplemented with 5-fluoroorotic acid (5-FOA), a uracil analogue that will only allow the formation of colonies from cells that have lost the wild type SUP45 plasmid with the URA3 marker. Each strain was then tested for the ability to phosphorylate eRF1 (Figure 3). The results show that the introduction of either the S421A or S432A mutations reduced the level of eRF1 phosphorylation, while both mutations together eliminated most (if not all) eRF1 phosphorylation. These results indicate that the serine residues at positions 421 and 432 are the major sites of eRF1 phosphorylation in vivo.

The phosphorylation state of eRF1 has little affect on the efficiency of translation termination or nonsense mediated mRNA decay (NMD). Having identified the two major sites of eRF1 phosphorylation, we used the phosphorylation mutants described above to characterize the role eRF1 phosphorylation plays in the cell.
Figure 4. The dual luciferase reporter system used to monitor the readthrough of stop codons \textit{in vivo}.
The double mutant eRF1-S421A/S432A allowed us to examine how the loss of eRF1 phosphorylation affects the function of this protein. We also constructed another mutant, eRF1-S421D/S432D, in which the phosphorylated serine residues were changed to aspartic acid to mimic constitutive phosphorylation. We first used these mutants to determine whether phosphorylation plays a role in translation termination. To do this, we used a dual luciferase reporter system (19, 25, 37). Briefly, this system utilizes tandem Renilla and firefly luciferase genes that are separated by a single in-frame stop codon (Figure 4). The activity of firefly luciferase, encoded by the distal open reading frame, provides a quantitative measure of readthrough of the stop codon that separates the two open reading frames. The activity of Renilla luciferase, encoded by the proximal open reading frame, serves as an internal control for mRNA abundance and translation initiation rates. Since not only the stop codon but also the first nucleotide after a stop codon influences the efficiency of translation termination (together referred to as the tetranucleotide termination signal), we examined the effect of the eRF1 phosphorylation mutants on all possible tetranucleotide termination signals (Table 3). We found that the mutations that eliminated phosphorylation (eRF1-S421A/S4342A) caused a small (1.2 to 1.5-fold) increase in readthrough at the UAA A, UAA U, and UAG G termination signals. Similarly, the mutations that mimicked constitutive phosphorylation (eRF1-S421D/S432D) exhibited a small (1.3 to 1.6-fold) increase in readthrough at the UAA A, UAA U, UAG C and UGA G termination signals, but had little effect on stop codon recognition at other signals. These increases are significant (p≤0.01) but relatively small when compared to an eRF1 mutant lacking the extreme C-terminal 19 amino acids (3.3 to 17.6-fold, data not shown). Taken together, these results indicate that the
Table 3: Effect of eRF1 phosphorylation status on readthrough of stop codons

<table>
<thead>
<tr>
<th>Termination Signal</th>
<th>Percent Readthrough&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fold Change (Relative to WT)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>WT</td>
<td>S421A</td>
</tr>
<tr>
<td>UAA A</td>
<td>0.30+/-0.03</td>
<td>0.41+/-0.02</td>
</tr>
<tr>
<td>UAA C</td>
<td>0.51+/-0.08</td>
<td>0.51+/-0.03</td>
</tr>
<tr>
<td>UAA G</td>
<td>0.37+/-0.05</td>
<td>0.34+/-0.05</td>
</tr>
<tr>
<td>UAA U</td>
<td>0.23+/-0.01</td>
<td>0.28+/-0.02</td>
</tr>
<tr>
<td>UAG A</td>
<td>0.21+/-0.01</td>
<td>0.23+/-0.02</td>
</tr>
<tr>
<td>UAG C</td>
<td>0.49+/-0.04</td>
<td>0.46+/-0.02</td>
</tr>
<tr>
<td>UAG G</td>
<td>0.23+/-0.02</td>
<td>0.34+/-0.02</td>
</tr>
<tr>
<td>UAG U</td>
<td>0.16+/-0.01</td>
<td>0.16+/-0.01</td>
</tr>
<tr>
<td>UGA A</td>
<td>0.67+/-0.09</td>
<td>0.58+/-0.08</td>
</tr>
<tr>
<td>UGA C</td>
<td>1.33+/-0.14</td>
<td>1.32+/-0.25</td>
</tr>
<tr>
<td>UGA G</td>
<td>0.58+/-0.06</td>
<td>0.62+/-0.05</td>
</tr>
<tr>
<td>UGA U</td>
<td>0.22+/-0.03</td>
<td>0.21+/-0.03</td>
</tr>
</tbody>
</table>

<sup>a</sup> All measurements were carried out in the sup45Δ strain YDB447 carrying pDB800 (eRF1-WT), pDB902 (eRF1-S421A/S432A), or pDB858 (eRF1-S421D/S432D).

<sup>b</sup> Percent readthrough is expressed as mean ± standard deviation.

<sup>c</sup> Significantly different from the wild type strain (P≤0.01), as determined by the Mann-Whitney statistical test.
phosphorylation state of eRF1 has little affect on the efficiency of stop codon recognition 
in vivo.

Nonsense mediated decay (NMD) rapidly degrades mRNAs that contain
premature stop mutations in a process that is intimately linked with translation
termination (1, 25). The induction of NMD in yeast requires a termination complex
(eRF1 and eRF3 bound to the 80S ribosome at a stop codon), three NMD factors (Upf1p,
Upf2p/Nmd2p, and Upf3p) and an aberrant 3´ untranslated region (3´-UTR) in the
mRNA (1, 18). Furthermore, eRF1 interacts with Upf1p (12). Deletion of any of the
three Upf factors will abrogate NMD and prevent the rapid turnover of mRNAs that
contain premature stop codons. We used the CYH2 mRNA to test the hypothesis that
phosphorylation of eRF1 plays a role in NMD. This mRNA has an intron that is spliced
inefficiently. The intron results in an in-frame premature stop codon that normally makes
this pre-mRNA a substrate for the NMD pathway. Furthermore, it has been shown that
defects in the NMD pathway prevent the rapid turnover of the CYH2 pre-mRNA (13). To
determine whether the phosphorylation of eRF1 plays a role in NMD, we carried out a
northern blot using a probe that recognized both the precursor and mature forms of the
CYH2 mRNA (Figure 5). We observed a significant accumulation of the unspliced CYH2
transcript in a upf1Δ strain, but not in the eRF1-S421A/S432A or the eRF1-
S421D/S432D double mutants. These results indicate that the phosphorylation status of
eRF1 does not adversely affect the NMD pathway.

**Phosphorylation of eRF1 does not affect its ability to bind eRF3.** The release
factor eRF1 interacts with its functional partner eRF3 via a binding site near its C-
Figure 5. The phosphorylation state of eRF1 does not affect NMD. Northern blot analysis of the CYH2 transcript was carried out on total RNA from the following strains: wild type (WT), upf1Δ, eRF1 S421A/S432A, and eRF1 S421D/S432D. For northern blots, 25 µgs of total RNA from the indicated strains were loaded in each lane.
terminus (14, 34). Since our data indicate that phosphorylation sites are also located near the C-terminus of eRF1, it is possible that the phosphorylation state of eRF1 may influence its ability to interact with eRF3. To test this hypothesis, we examined the association between eRF1 and eRF3 by performing sedimentation velocity experiments using analytical ultracentrifugation (Figure 6). His-tagged forms of full-length eRF1 or a fragment of eRF3 consisting of amino acid residues 254 to 685 were purified from *E. coli* extracts using Ni$^{2+}$-NTA affinity chromatography. Since *E. coli* does not contain protein kinases that recognize CK2 protein kinase sites, the wild type eRF1 purified from *E. coli* was assumed to be in the de-phosphorylated form. As a negative control for eRF1-eRF3 complex formation, we used an eRF1 mutant lacking the last 19 amino acids (eRF1-CΔ19) that does not efficiently bind eRF3 (15). Preliminary experiments found that eRF1, eRF1-CΔ19, and eRF3 each have sedimentation coefficients ranging from 2.07S to 2.09S (Figure 6). When wild type (un-phosphorylated) eRF1 and eRF3 were incubated together for 20 minutes at 4°C before centrifugation, we observed a new 3.06S complex that represented the eRF1-eRF3 complex. This indicated that a lack of phosphorylation did not prevent formation of the eRF1-eRF3 complex. As expected, this 3.06S peak was not observed when eRF1-CΔ19 was incubated with eRF3, indicating that deletion of the C-terminal 19 amino acids of eRF1 disrupted eRF1-eRF3 complex formation. Incubation of eRF3 with the eRF1 mutant that mimics constitutive phosphorylation (eRF1-S421D/S432D) resulted in the appearance of the 3.06S peak, indicating that complex formation still occurred when acidic residues were present at the sites of eRF1 phosphorylation. Taken together, these results indicate that the phosphorylation state of eRF1 does not play a significant role in eRF1-eRF3 heterodimer formation.
Figure 6. Phosphorylation status of eRF1 does not affect its association with eRF3. Sedimentation coefficients of the indicated protein(s) (indicated in boxes) were generated by analytical ultracentrifugation. The values indicated above the peaks represent the S values derived using the SEDFIT program. S, Svedberg units; c(S) Distribution, differential distribution (fringes/Svedberg).
The state of eRF1 phosphorylation is not affected by the putative phosphatase encoded by SAL6 and does not affect the allosuppressor phenotype associated with a sal6Δ mutant. Mutations in the SUP45 and SUP35 genes, encoding eRF1 and eRF3, respectively, were originally identified based on their ability to cause readthrough of stop mutations in various biosynthetic genes of S. cerevisiae (22). These mutations were termed omnipotent suppressors because they cause readthrough at all three stop codons. Later, the SAL6 gene was identified in a screen for mutants that enhanced the readthrough phenotypes associated with omnipotent suppressor mutations (40). Mutations in the SAL6 gene alone did not result in a suppressor phenotype, but they exacerbated the readthrough associated with omnipotent suppressor mutations in the SUP35 or SUP45 genes. This enhanced suppression phenotype is referred to as an allosuppressor effect. The SAL6 gene was later sequenced and found to encode a protein with significant homology to PP1-like protein phosphatases (11, 42). However, it is presently still not clear how the protein encoded by the SAL6 gene mediates the allosuppressor effect.

Given our finding that eRF1 is a phosphoprotein, we next tested the hypothesis that the allosuppressor phenotype associated with the sal6Δ mutation is mediated through its effect on the phosphorylation state of eRF1. To do this, we used the dual luciferase reporter system to compare termination efficiencies in strains expressing the phosphorylation mutants in SAL6 and sal6Δ backgrounds (Figure 7). Since the allosuppressor effect requires the presence of an omnipotent suppressor mutation, the phosphorylation site mutations were combined with the eRF1-I222S mutation, which was originally identified as the sup45-2 omnipotent suppressor allele (41). When these eRF1 mutant proteins were expressed from a low copy plasmid in a sup45Δ strain, we found
Figure 7. The SAL6 allosuppressor phenotype is unaffected by the phosphorylation state of eRF1. (A) Stop codon readthrough in SAL6 or sal6Δ strains was measured using the dual luciferase reporter with the UAA U termination signal. These strains expressed either wild type eRF1 (WT), eRF1-I222S (the sup45-2 omnipotent suppressor allele), eRF1-I222S/S421A/S432A, or eRF1-I222S/S421D/S432D. (B) [\(^{32}\)P]-orthophosphate labeling of eRF1 in wild type (WT) and sal6Δ strains and western blots (WB) of total protein indicate that [\(^{32}\)P] incorporated into eRF1 per unit of steady-state eRF1 protein is unaffected by the sal6Δ mutation.
that expression of the eRF1-I222S allele as the sole source of eRF1 resulted in a 3.3-fold increase in readthrough (Figure 7A). When eRF1-I222S was expressed in the sal6Δ strain, we observed a 6.6-fold increase in readthrough, indicating that the presence of the sal6Δ mutation caused an additional 2-fold increase in readthrough. This observation recapitulated the previously observed allosuppressor effect associated with SAL6 mutations (40). However, we did not detect any significant change in readthrough when either the S421A/S432A or the S421D/S432D phosphorylation site mutations were combined with the eRF1-I222S mutation in the sal6Δ strain. If Sal6p were the phosphatase for eRF1, the sal6Δ mutation might increase the phosphorylation state of eRF1. This hypothesis predicts that the mutant protein that mimics constitutive phosphorylation, eRF1-S421D/S432D, will phenocopy the allosuppressor effect in a SAL6 strain since the presence of the aspartic acid residues mimic the maximal phosphorylation state of eRF1. However, such an allosuppressor effect was not observed in the SAL6 strain expressing eRF1-I222S/S421D/S432D (Figure 7A). These results suggest that the allosuppressor effect associated with the sal6Δ mutation is not mediated by its effect on the phosphorylation state of eRF1.

The absolute level of eRF1 phosphorylation might also be expected to increase in a sal6Δ strain if Sal6p is the phosphatase responsible for the dephosphorylation of eRF1 (assuming it is not already fully phosphorylated under normal conditions). To test this hypothesis, we used our metabolic labeling assay to determine whether any change in the phosphorylation state of eRF1 could be detected in the sal6Δ mutation. As shown in Figure 7B, we did not observe any significant difference in the phosphorylation state of eRF1 between the wild type and sal6Δ strains. We cannot rule out the possibility that
Figure 8. Phosphorylation of eRF1 is reduced upon carbon source starvation. (A) Growth curve of sup45Δ strain expressing wild type eRF1 (YDB447 carrying pDB800) during experiment in the presence or absence of glucose. (B) $^{32}$P metabolic labeling and western blot analysis of eRF1 from cells harvested after incubation in media with or without glucose. Cells were harvested at t = 4 hrs as shown in (A).
eRF1 is completely phosphorylated under the growth conditions in which this experiment was carried out. If that were the case, the deletion of its phosphatase would not be expected to show a detectable increase in phosphorylation. However, when considered together with the allosuppressor readthrough data, these results argue that Sal6p is not the phosphatase responsible for the dephosphorylation of eRF1, and the state of eRF1 phosphorylation does not appear to be important for the allosuppressor affect associated with sal6 mutations.

**eRF1 phosphorylation and dephosphorylation is a dynamic process that requires cell growth.** The results above indicate that the phosphorylation state of eRF1 does not significantly influence its termination activity. Consistent with this finding, mutations that eliminated the CK2 phosphorylation sites in the five proteins that form the ribosomal stalk shown in Table 1 also did not affect either ribosome activity or formation of the stalk structure (3, 36). Similarly, studies that altered the CK2 phosphorylation sites in eIF2α and eIF5 did not have any detectible effects on translation initiation (16, 30). However, the existence of CK2 phosphorylation sites in these and other proteins involved in translation suggests that this modification may carry out some common function. To gain further insight into the nature of eRF1 phosphorylation, we next asked whether this modification required active cell growth. Wild type cells were grown to mid-log phase, harvested, and identical aliquots were re-suspended in the same growth medium in the presence or absence of 2% glucose. The cultures were incubated for two hours at 30°C to deplete any residual glucose in the glucose-depleted culture, and [³²P]-orthophosphate was then added to each culture. Incubation at 30°C was continued for an additional two hours, and the cells were harvested. As expected, glucose depletion under
these conditions effectively blocked cell growth (Figure 8A). When we examined the phosphorylation state of eRF1, we found that growth inhibition by glucose depletion essentially eliminated the phosphorylation of eRF1 (Figure 8B). These results indicate that ongoing cell growth is required for eRF1 phosphorylation to occur.

There are two possible explanations for this result. First, eRF1 may only be phosphorylated once during (or immediately after) its synthesis. By subjecting the cells to glucose depletion, we are inhibiting de novo protein synthesis, and consequently the phosphorylation of newly synthesized eRF1. Alternatively, eRF1 may cycle between phosphorylated and dephosphorylated forms during normal growth. When cell growth is inhibited by glucose depletion, this phosphorylation cycle may arrest with cells that contain predominantly the dephosphorylated form of eRF1. To distinguish between these possibilities, we performed a metabolic chase experiment (Figure 9). Cells were labeled for two hours with $^{32}$P-orthophosphate, then shifted to growth medium with excess unlabeled orthophosphate (with or without glucose) to monitor the persistence of the $^{32}$P incorporated into eRF1. We found that the eRF1 phosphorylation decreased more than 10-fold during the first four hours in cells incubated with glucose. Since the doubling time for this strain under these conditions is ~3.5 hours, the large decrease in pre-existing phosphorylated eRF1 we observed was much greater than the two-fold decrease expected following one round of cell division. In contrast, eRF1 phosphorylation decreased only ~two-fold in cells incubated in the absence of glucose (most of which occurred during the first 30 minutes while residual glucose was being metabolized). From these results, we conclude that eRF1 phosphorylation is a dynamic process that is dependent upon active cell growth.
Figure 9. eRF1 undergoes dynamic phosphorylation during cell growth. (A) Cells grown in Wickerham’s low phosphate medium were metabolically labeled with $^{32}$P-orthophosphate for 2 hours. To initiate the chase period, cells were harvested and resuspended in Wickerham’s medium with excess unlabeled orthophosphate. The chase was carried out four 4 hours in the presence or absence of glucose, and samples were collected at the indicated times for immunoprecipitation with an eRF1-specific antiserum ($^{32}$P). Samples were harvested at the same times from unlabeled cultures (but otherwise grown under identical conditions) for western blot analysis (WB). (B) Logarithmic plot of $^{32}$P incorporation per unit of eRF1 from (A). The data shown are representative of two independent experiments.
Discussion

Previous studies have shown that several factors involved in the initiation and elongation steps of translation undergo post-translational modification by phosphorylation. In this study we show that the translation termination factor eRF1 is also a phosphoprotein in yeast, showing for the first time that all three steps in translation are modified in this manner. We found that eRF1 is phosphorylated in two sites near its C-terminus by the CK2 protein kinase as previously shown for several other yeast proteins involved in translation, including all five proteins of the ribosomal stalk (3), and initiation factors eIF5 (30) and eIF2α (16) (Table 2). Surprisingly, we found that the introduction of mutations that either eliminated eRF1 phosphorylation or mimicked constitutive eRF1 phosphorylation had little affect on the efficiency of stop codon recognition in vivo (as measured by a translational readthrough assay). The level of eRF1 phosphorylation was also unaffected by disruption of the SAL6 gene, which encodes a putative PP1-like phosphatase. Furthermore, the allosuppressor phenotype associated with the sal6Δ mutation was insensitive to the state of eRF1 phosphorylation. These results indicate that the phosphorylation of eRF1 does not play a significant role in its ability to mediate efficient translation termination under the growth conditions used in the current study.

We also found that the phosphorylation state of eRF1 did not influence its ability to bind eRF3, even though phosphorylation occurred within the portion of eRF1 that is responsible for eRF3 binding. In addition, our results indicate that phosphorylation of eRF1 does not influence nonsense mediated mRNA decay. From these data, we can infer that the phosphorylation state of eRF1 probably does not affect Upf1p binding, since the
association of Upf1p with the termination complex is required for optimal translation termination efficiency (12, 25).

Based on the results summarized above, the physiological role for the CK2-mediated phosphorylation of eRF1 remains obscure. Previous studies of other proteins involved in translation that undergo CK2 phosphorylation near their C-termini have also had difficulty establishing a function for this modification. A study of the P1β protein of the ribosomal stalk suggests that phosphorylation, in conjunction with an N-terminal signal, may stimulate protein degradation. This hypothesis is consistent with the previous observation that phosphorylation can function as a degradation signal for the vacuolar and proteosome turnover pathways (23). However, it was subsequently shown that P1β was not degraded by either of these pathways. Instead, it was shown that failure of the P1 dimer to associate with P2 causes a rapid turnover of P1 (33). Therefore, phosphorylation of P1 may simply play a role in its association with P2, with enhanced degradation occurring due to a lack of assembly. In another example, yeast eIF2α is phosphorylated by the CK2 protein kinase at three serine residues near its C-terminus (amino acids 292, 294, and 301). Although mutation of these serine residues to alanine did not cause a detectible phenotype, they did exacerbate the growth defects observed when these mutations were combined with other mutations that reduced the efficiency of nucleotide exchange, suggesting that phosphorylation of the CK2 protein kinase sites in eIF2α may be required for optimum eIF2 activity (16). Similarly, we found that changes in the phosphorylation state of eRF1 resulted in only subtle affects on stop codon recognition that may help to fine-tune the termination process at certain termination signals (Table 3). Finally, it was shown that eIF5 is phosphorylated at multiple sites near
its C-terminus by the CK2 protein kinase (30), but a role for this modification was not found. When taken together, these results suggest that phosphorylation of various translation factors by the CK2 protein kinase does not play a strong role in their specific functions related to translation under steady-state growth conditions. Thus, the purpose of this common post-translational modification remains somewhat obscure.

The database searches carried out during this study also revealed that at least two other yeast proteins involved in translation, eIF2Bε and eIF4γ, also have CK2 phosphorylation motifs near their extreme C-termini. In addition, a number of other translation factors, including eEF1β, eIF1A, Tif35p, eIF2Bγ, and eIF4B have consensus CK2 phosphorylation motifs within 50 amino acids of their C-termini. We hypothesize that these proteins are also phosphorylated by the CK2 protein kinase. Based on the results of phosphorylation studies described above, the phosphorylation of these factors (if it occurs) may not play a dramatic role in regulating their activities. Instead, they may be phosphorylated by the CK2 protein kinase in response to favorable growth conditions in a manner similar to the 5 ribosomal stalk proteins, eIF2α, eIF5 and eRF1. Here, we have provided evidence that: 1) the phosphorylation of eRF1 is dynamic and goes through a phosphorylation / dephosphorylation cycle rather then being constitutively phosphorylated following its synthesis; 2) active cell growth is required for the phosphorylation / dephosphorylation cycle, and 3) inhibition of this phosphorylation cycle by eliminating the sites of phosphorylation does not have adverse affects on translation or steady-state growth when cells are grown with glucose as carbon source. It is possible that this phosphorylation pathway is important when the cells are grown under adverse conditions where subtle changes in the activity of the translational apparatus or
the function of other related factors may be important. It is also possible that this conserved modification may act to down-regulate translation during conditions that are not favorable for cell growth.

A significant body of evidence also indicates that many factors have alternate cellular functions. For example, eEF1A not only plays a role in translation elongation, but has also been shown to bind actin and influence the distribution of the actin cytoskeleton (20, 24, 32). Ccr4p, a subunit of the major cytoplasmic deadenylase complex, also acts as a transcription factor that becomes active in response to DNA damage (28). It is currently not known whether a post-translational modification such as phosphorylation regulates any (or all) of the alternate functions of these factors. Further studies will be required to examine this possibility.

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EUKARYOTIC REALASE FACTOR 1 (eRF1) STEADY STATE mRNA LEVELS ARE COUPLED TO EFFICIENT POLYPEPTIDE CHAIN RELEASE

by

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Abstract

Protein synthesis in a cell is separated into three distinct steps and requires a large commitment of the cellular resources. At least two proteins, eRF1 and eRF3, mediate the final and least characterized step, termination. In this report, we demonstrate that the steady-state levels of eRF1 are coupled to the efficiency of translation termination. We found that steady-state eRF1 protein and mRNA levels were elevated in three release factor variants: eRF1-CA19, eRF3-H348Q, and Eo1/Sc23-eRF1-C124S. This increase was not observed in a strain carrying a knockout of the *TPA1* gene and prion form of eRF3 (*tpa1Δ[PSI+]*) despite having stop codon readthrough levels comparable to eRF1-CA19 and eRF3-H348Q. This showed that the increase in steady-state eRF1 levels is not simply coupled to a mechanism that responds to heightened stop codon readthrough in the cell. We utilized two in vitro assays to look at the GTPase activity and rate of peptide release of the mutants to find a translation termination defect common to the three mutants where eRF1 levels are elevated. From the in vitro results, we show that while the eRF1-CA19 and eRF3-H348Q mutants exhibit a defect in GTP hydrolysis, Eo1/Sc23-eRF1-C124S does not. In an in vitro termination release assay, we show that the mutants eRF1-CA19 and eRF3-H348Q have a slow rate of peptide release. When we combine these results with the cold sensitive growth phenotype of Eo1/Sc23-eRF1-C124S, our results indicate that steady-state levels of eRF1 are most likely coupled to a slow rate of peptide release and not to stop codon readthrough or GTP hydrolysis. We hypothesize that a slow rate of peptide release will trigger a regulatory mechanism to increase steady-state levels eRF1 by increasing the half-life of the *SUP45* mRNA.
Introduction

Proteins are synthesized by a process known as translation. Translation is separated into three distinct steps: initiation, elongation, and termination. Our knowledge of all three steps has grown significantly in the last 10 years, including crystal structures for some of the protein components of eukaryotic translation (3, 17, 24). Translation requires a large amount of energy to be put into the system in the form of proofreading so it should be expected to be a highly regulated process.

Our understanding of the mechanism of the final step of translation, termination, has lagged behind the other two and it remains to be seen whether the process is regulated in a eukaryote. Recent evidence has suggested that it may be regulated in some manner by phosphorylation (4). It has been shown that eRF1 (encoded by the SUP45 gene) is phosphorylated by the CK2 kinase in a manner similar to eIF2, eIF5, and 5 proteins of the ribosomal stalk (P0, P1α, P1β, P2α, and P2β) (14).

The crystal structure of human eRF1 revealed a partitioning of the three known functions of eRF1 into three distinct domains (24). The function of domain 1 of eRF1 is to recognize the stop codon in the A-site of the ribosome. Upon correct recognition of a stop codon, domain 2 stimulates the peptidyl transferase center of the ribosome to release the nascent polypeptide bound to the P-site. The third and final domain of eRF1 binds to eRF3 (encoded by the gene SUP35) to stimulate the GTPase activity of eRF3. We recently made a mutant of eRF1 where the C-terminal 19 amino acids of eRF1 were removed (hereafter referred to as eRF1-CΔ19). Consistent with previous results, we showed that this mutant has a defect in forming a heterodimer with eRF3 (7, 14). In this
report, we show that the steady-state levels of eRF1 protein and mRNA, but not eRF3 levels, are elevated in this mutant due to an increase in SUP45 mRNA stability. In addition to the eRF1-CΔ19 mutant, we show that eRF1 levels are also elevated in other eRF1 and eRF3 mutants with characterized termination defects including, the eRF3 GTPase mutant, eRF3-H348Q (21). The eRF3-H348Q mutant showed defects in stop codon recognition, GTP hydrolysis, and peptide release.

Recently, our lab has made a hybrid eRF1 protein consisting of domain 1 from the ciliate *Euplotes octocarinatus* and domains 2 and 3 from the yeast *Saccharomyces cerevisiae* (hereafter referred to as Eo1/Sc23-eRF1) (22). While this Eo1/Sc23-eRF1 hybrid mutant cannot support viability as the only source of eRF1 in the cell, we isolated a suppressor that restores viability, Eo1/Sc23-eRF1-C124S (Fan-Minogue unpublished results). This mutant is cold sensitive despite the absence of a defect in stop codon recognition and normal levels of eRF3 dependent GTPase activity. Using a recently developed in vitro peptide release assay, we found a peptide chain release defect present in the eRF1-CΔ19 and eRF3-H348Q mutants (2). eRF1 protein and mRNA levels were elevated in both of these mutant strains as well as the Eo1/Sc23-eRF1-C124S mutant. Together, these results suggest that eRF1 abundance is regulated at the level of mRNA stability by a mechanism that is coupled to the efficiency of polypeptide chain release.

**Materials and Methods**

**Strains and growth conditions.** The *Saccharomyces cerevisiae* strains used in this study were YJW619 (*MATα leu2-3, 112 his3-11, 14 trp1-1 ura3-1 ade1-14 tpa1Δ::LEU2 [PSI+]*), YDB447 (*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-80*).
suc2-Δ901 sup45Δ::HIS3), YDB498 (MATa leu2-3,112 his3-11,14 trp1-1 ura3-1 ade1-14 sup35Δ::HIS3), YDB638 (MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-80 suc2-Δ901 sup45Δ::HIS3 sup45-Δ19::LEU2). All strains were grown in either synthetic medium or yeast peptone broth with glucose as the carbon source.

**Plasmid construction and gene disruptions.** pDB800 is a centromeric plasmid containing the SUP45 (eRF1) ORF with 1568bp upstream of the AUG start codon and 1036bp downstream of the UAA stop codon from its location in the chromosome. Two forms of the eRF1-Δ19 mutant were constructed. pDB843 is a centromeric plasmid containing the sup45-Δ19 mutation and its construction had been described elsewhere (14). The sup45-Δ19 chromosome integration construct was made by removing a SalI-XbaI fragment from pDB800 and cloning it into YIplac128 to create pDB972. A SpeI-XhoI fragment was cut out of pDB843 and subcloned into pDB972 to create pDB973. pDB973 was digested with BlpI and used for integration into YDB447/pUKC802. pUKC802 contains SUP45 and is required to maintain viability in the strain YDB447. Integration of pDB973 into the chromosome of YDB447 was confirmed by initial plate selection and subsequent western blot. pUKC802 was shuffled out using 5-FOA selection, leaving the integrated eRF1-Δ19 as the only source of eRF1 in the cell.

**Immunoprecipitations and western blots.** Metabolic labeling of eRF1 was carried out in SMD medium lacking methionine by adding 200μCi/mL of EXPRESS [35S] protein labeling mix (Perkin Elmer) to 2.5 OD600 of cells for 10 minutes. The cells were then precipitated in 7% trichloroacetic acid, pelleted, and then washed twice with
cold 100% acetone and dried under vacuum. The yeast were then lysed with glass beads in lysis buffer (50 mM TRIS pH=7.5, 1% SDS, 1 mM EDTA) and mixed with 800µL of IP buffer (50 mM Tris-HCl, pH=7.5, 150 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA). 600µL of this mixture was removed, combined with 4µL of eRF1 polyclonal antibody and incubated overnight at 4°C. Protein-antibody complexes were pulled down using 50µL of Protein A coupled to sepharose beads and incubated at 4°C for an hour. The beads were then washed twice with IP buffer followed by a wash with a 1% solution of 2-mercaptoethanol. The complexes were then boiled in SDS loading buffer and separated on an 8% SDS-PAGE gel. The gels were fixed, dried, and developed using a phosphorimager (GE Healthcare). Western blots were performed as described previously using 25µg of protein per lane as determined by the Lowry method (14, 18). A 1:500 dilution of eRF1 polyclonal antiserum was used to probe the blots and 125I-Protein A was used to detect the antibody (14).

Dual luciferase assays. The plasmids bearing the two luciferases have been described elsewhere (Fig 2) (11, 15). They were performed using a kit available from Promega and completed as described previously (15, 21). Briefly, 1 OD600 of a log phase yeast culture were collected and assayed. The samples were assayed in quintuplicate and readthrough values are expressed as the mean ± the standard deviation. Statistical analysis was carried out using a Mann-Whitney test for significance.

mRNA half-life experiments and northern blots. mRNA synthesis was terminated by adding 25µg/mL of thiolutin to cells in mid log phase. Aliquots of RNA
were removed at time points following the transcription block and processed as previously described (14, 16). For WT-eRF1 and eRF1-CΔ19, 30µg or 10µg respectively, were loaded in a formaldehyde-agarose gel per time point. Half-lives were found by determining the percent eRF1 remaining relative to the t=0 time point. eRF1 mRNA levels were normalized to rRNA to control for differences in amount of RNA loaded onto the gel. The data plotted are a representative sample and data reported are the average ± the standard deviation of the mRNA half-life from five independent experiments. For the northern blots other than the half-life experiments, 10µg of mRNA were loaded into each lane and mRNA from the ACT1 gene (actin) served as the loading control. The numbers indicate the fold change relative to wildtype ± the standard deviation and were repeated at least three times.

**GTPase assays.** N-terminal 6XHIS tagged proteins were expressed and purified from the soluble fraction of an *E. coli* lysate. They were purified as previously described (14, 21). Yeast ribosomes were grown in YPD medium and purified from a 9L culture. They were purified as previously described except that the final centrifugation was performed at 170,000g for two hours at 4°C in a TLA100.1 ultracentrifuge rotor (21). 3 pmol of eRF1 (WT, CΔ19 or Eo1/Sc23-C124S), eRF3 (WT or H348Q) and ribosomes were used in each reaction. GTP hydrolysis was determined by monitoring conversion of [α-32P]-GTP to GDP and inorganic phosphate. The three species were separated on thin-layer chromatography sheets and are resolved in TLC buffer (0.5M LiCl and 2M formic acid) for 90 to 120 minutes at room temperature. The rates of GTP hydrolysis were determined from several concentrations of GTP (1, 1.5, 2.5, 5, 10, and 15µM). These
data were analyzed using Lineweaver-Burk plots. The $K_m$ (-1/x intercept) and $V_{max}$ (1/y intercept) were determined using the plots. From these data, we calculated the $K_{cat}$ ($K_{cat} = V_{max}/\text{pmol of eRF3}$) and the catalytic efficiency of eRF3 ($K_{cat}/K_m$).

**Polypeptide release assay.** The assay was performed as previously described with following modifications for utilization of the yeast release factors (2). Pre-termination complexes (pre-TCs; essentially a ribosome with the peptidyl tRNA in the P-site and the termination codon in the A site poised for the termination factors to bind and stimulate release) were formed with the mammalian initiation and elongation factors and ribosomes. Yeast release factors (purified as above in the GTPase assays) were added to stimulate release. Polypeptide chain release was monitored by measuring TCA precipitable counts following incubation. In the initial characterization of the yeast factors (Fig 7B), 0.02pmol of pre-TC were combined with 10 pmol yeRF1 and 20 pmol yeRF3 and incubated for 20 min. To determine the kinetics of peptide release by the yeast release factors, yeRF1 and yeRF3 were reduced to 1 pmol and 2 pmol respectively and the amount of pre-TC remained at 0.02 pmol.

**Results**

**Steady-state levels of eRF1 mRNA and protein are elevated in the eRF1-CA19 mutant.** We made a mutant of eRF1 via a precise deletion of the last 19 sense codons of the $SUP45$ gene (eRF1-CΔ19). The 3’UTR of the $sup45$-CΔ19 allele remained unchanged (see materials and methods). The $sup45$-CΔ19 mutation was integrated into the chromosome using either the vector pDB0973 or expressed on a centromeric plasmid,
Table 1. Percent readthrough of eRF1-CΔ19

<table>
<thead>
<tr>
<th>Stop signal</th>
<th>WT&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CΔ19&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAAA</td>
<td>0.30 ± 0.03</td>
<td>1.00 ± 0.09</td>
<td>3.33&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>UAAC</td>
<td>0.51 ± 0.03</td>
<td>2.88 ± 0.30</td>
<td>5.65&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>UAAG</td>
<td>0.37 ± 0.05</td>
<td>1.18 ± 0.05</td>
<td>3.19&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>UAAU</td>
<td>0.23 ± 0.02</td>
<td>1.38 ± 0.16</td>
<td>6.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>UAGA</td>
<td>0.21 ± 0.01</td>
<td>0.77 ± 0.11</td>
<td>3.65&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>UAGC</td>
<td>0.49 ± 0.04</td>
<td>4.44 ± 0.61</td>
<td>9.06&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>UAGG</td>
<td>0.23 ± 0.02</td>
<td>1.28 ± 0.08</td>
<td>5.56&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>UAGU</td>
<td>0.16 ± 0.01</td>
<td>1.03 ± 0.11</td>
<td>6.45&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>UGAA</td>
<td>0.67 ± 0.09</td>
<td>2.95 ± 0.48</td>
<td>4.40&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>UGAC</td>
<td>1.33 ± 0.14</td>
<td>23.04 ± 2.09</td>
<td>17.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>UGAG</td>
<td>0.58 ± 0.06</td>
<td>5.10 ± 0.52</td>
<td>8.79&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>UGAU</td>
<td>0.22 ± 0.03</td>
<td>1.62 ± 0.21</td>
<td>7.36&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> All measurements were carried out in the sup45Δ strain YDB447 carrying pDB800 (eRF1-WT) or pDB0843 (eRF1-CΔ19)

<sup>b</sup> Percent readthrough is expressed as mean ± standard deviation.

<sup>c</sup> p ≤ 0.01, as determined by Mann-Whitney statistical test
Fig 1. eRF1 protein and mRNA levels are elevated in the eRF1-CΔ19 mutant while eRF3 protein and mRNA levels remain unchanged. (A) Western blot of YDB447/pDB800 (WT) and YDB639 (eRF1-CΔ19 integrated into the chromosome) with rabbit polyclonal antisera to Sup45p (eRF1), Sup35p (eRF3), or Tom70p. Tom70p serves as the loading control. (B) Northern blot analysis of eRF1 or eRF3 levels in WT and the eRF1-CΔ19 mutant. Actin (ACT1) serves as the loading control. The number below each blot corresponds to the change in protein/mRNA levels relative to WT +/- the standard deviation. (*) p-value≤0.05, Mann-Whitney statistical test.
Fig 2. Diagram of bicistronic reporter to measure readthrough in vivo. Renilla and firefly luciferases are separated by a cassette containing a stop tetranucleotide signal (e.g. UAAA) or a sense signal (e.g. CAAA). The renilla firefly expression serves as the internal control for mRNA abundance and turnover.
Fig 3. Coexpression of WT-eRF1 and the eRF1-CΔ19 mutant reveals that the eRF1-CΔ19 mutant is unstable in the presence of WT-eRF1. Western blot of eRF1 (WB) and $^{35}$S-methionine labeling for 10 minutes followed by immunoprecipitation using rabbit polyclonal antisera to eRF1.
leaving the eRF1-CΔ19 mutant as the sole source of eRF1. This mutant was previously shown to have a defect in eRF3 binding and exhibited a readthrough phenotype indicating a defect in stop codon recognition (Table 1) (7, 14).

When we performed western blots to verify expression of eRF1-CΔ19, we found that steady-state levels of eRF1-CΔ19 protein were elevated approximately 5-fold relative to a strain expressing wildtype eRF1 (WT-eRF1). We found a similar 5-fold increase in sup45-CΔ19 mRNA levels (Fig 1). This similar increase in eRF1 protein and mRNA levels indicates that this increase is due to an increase in mRNA synthesis or stability. In contrast, steady-state levels of eRF3 protein and SUP35-mRNA were unchanged.

We next analyzed eRF1 levels in cells expressing both WT-eRF1 and eRF1-CΔ19. Western blot analysis indicated that only WT-eRF1 was detectable. In addition, the steady-state level of eRF1 protein was present at the wildtype level (Fig 3). To show that eRF1-CΔ19 was still expressed, we performed immunoprecipitations of eRF1 from cells that had been pulse labeled for 10 minutes with 35S-methionine (Fig 3). The results indicated that the eRF1-CΔ19 protein continues to be synthesized in the presence of eRF1-WT cell but is unstable. Previous studies have shown that the C-terminal region of eRF1 contains the binding site for eRF3 (6-8, 14). These results suggest that the increased efficiency of eRF3 binding by WT-eRF1 may displace eRF1-CΔ19, thus making it more susceptible to degradation.

The increase in the eRF1 level is caused by an increase in the half-life of the sup45-CΔ19 mRNA. eRF1 protein and mRNA levels could be elevated by increasing the transcription synthesis rate or by increasing the half-life of the existing SUP45
Fig 4. *sup45-CΔ19* mRNA levels in the eRF1-CΔ19 mutant are increased due to an increase in half-life of the eRF1 mRNA. Transcription in the cell was shut down by addition of 25µg/mL thiolutin and aliquots were taken at the indicated time points. Data were plotted on a logarithmic scale expressed as amount of eRF1 remaining. The rRNA served as a loading control. The numbers on the graph indicated the half-life of the mRNA in minutes +/- the standard deviation.
transcripts in the cell. To distinguish between these two possibilities, we performed half-life experiments on the SUP45 transcript. The addition of thiolutin to yeast cultures inhibits transcription and allows us to monitor the stability of the SUP45 transcript (13). The experiments indicated a half-life of SUP45 mRNA of 5.5 minutes while the half-life of the sup45-CΔ19 transcript was 18.9 minutes (Fig 4). We conclude that the elevated eRF1-CΔ19 protein is attributable to an increase in the mRNA level caused by an increase in mRNA half-life.

While the studies to further characterize this novel phenotype for the sup45-CΔ19 mutation were being conducted, we made the observation that eRF1 protein and mRNA levels were also elevated in the Eo1/Sc23-eRF1-C124S termination mutant and the GTP hydrolysis mutant eRF3-H348Q (Fig 5) (21) and Fan-Minogue unpublished results). Since these three mutations are in the release factors, we hypothesized that they share a common defect in translation termination that triggers a mechanism to increase the half-life of the SUP45 transcript.

**Increases in steady-state levels of eRF1 are not coupled to readthrough of stop codons.** There are three known functions of eRF1. First, eRF1 functions to recognize stop codons in the A-site of the ribosome. Failure to appropriately recognize a stop codon leads to the incorporation of a near cognate tRNA molecule that allows the ribosome to continue translating the mRNA until it encounters another stop codon. This failure to terminate properly is known as stop codon readthrough (10). Second, eRF1 acts with the ribosome to stimulate the GTPase activity of eRF3. Finally, eRF1 facilitates release of the nascent polypeptide chain attached to the P-site tRNA. It is possible that a
Table 2. Readthrough of various strains with termination defects at the UGAC stop signal relative to WT.

<table>
<thead>
<tr>
<th>Strain</th>
<th>WT</th>
<th>CΔ19^a</th>
<th>eRF3 H348Q^b</th>
<th>tpa1Δ [PSI+]^c</th>
<th>eRF1 Eo1/Sc23 C124S^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Readthrough level</td>
<td>0.4%</td>
<td>24%</td>
<td>24%</td>
<td>18%</td>
<td>0.43%</td>
</tr>
</tbody>
</table>

(a) This study (b) Salas-Marcos and Bedwell 2004 (c) Keeling et al. 2004 (d) Fan-Minogue et al. unpublished results
defect in any of these three functions of eRF1 could trigger a mechanism to increase the steady-state levels of eRF1.

To test the hypothesis that eRF1 levels are coupled to stop codon recognition, we examined different strains with high stop codon readthrough. These strains, eRF3-H348Q and tpa1Δ[PSI+], cause readthrough at about the same level as that seen in the eRF1-CΔ19 mutant (Table 2). The eRF3-H348Q mutant was characterized previously and has a defect in its ability to hydrolyze GTP (21). The tpa1Δ[PSI+] strain carries two defects. The prion form of eRF3, [PSI+], causes readthrough by aggregating eRF3 so it can’t be used for termination (23). Tpa1p is involved in coupling the termination process to mRNA deadenylation. When TPA1 is knocked out in a [PSI+] background, the readthrough phenotype is more than either defect alone (16). We looked at levels of eRF1 by western blot and found that eRF1 was elevated 2.2 fold in the eRF3-H348Q mutant (Fig 5). Similar to the eRF1 protein levels, a 1.9 fold increase in SUP45 mRNA was also seen, consistent with the 5 fold increases in protein and mRNA of the sup45-CΔ19 mutation. In contrast, no increase in eRF1 protein levels was observed in the tpa1Δ[PSI+] strain indicating that a defect in stop codon recognition (readthrough) is not the trigger to increase steady-state levels of eRF1. In addition, the sup35-H348Q mutation shows eRF1 protein abundance is elevated in a strain where eRF1 is not mutated, supporting the hypothesis that eRF1 levels are coupled to a defect in the termination process and not limited to mutations in SUP45.

In addition to eRF1-CΔ19 and eRF3-H348Q, we found that steady state eRF1 protein levels and SUP45 mRNA levels in the hybrid mutant Eo1/Sc23-eRF1-C124S were elevated 2.4 fold and 2.2 fold, respectively (Fig 5). This mutant has readthrough
Fig 5. eRF1 protein and mRNA levels are elevated in the eRF3-H348Q (YDB0498/pDB0663) and Eo1/Sc23-eRF1-C124S (YDB0447/pDB0974) mutants but not in the tpa1Δ[PSI+] strain. (A) Western blot of eRF1 or Tom70p using polyclonal rabbit antisera specific for eRF1 or Tom70p. (B) Northern blots for SUP45 were normalized to actin (ACT1). Values underneath the blots indicate the average increase relative to WT +/- the standard deviation. *p-value ≤ 0.05 is significant as determined by a Mann-Whitney statistical test. aYDB498/pDB663 bYJW619 cYDB447/pDB800
levels similar to WT-eRF1 indicating that stop codon recognition is normal (Table 2). However, the Eo1/Sc23-eRF1-C124S mutant is cold sensitive and grows very slowly at 35°C (Fan-Minogue unpublished results). The growth defect suggests that it may have a defect in GTP hydrolysis or peptide release. With the observation that eRF1 protein and mRNA levels are elevated in the eRF1-CΔ19, eRF3-H348Q, and Eo1/Sc23-eRF1-C124S mutants, we hypothesize that they must share a common defect. However, these results demonstrate that a defect in stop codon recognition is not required to increase the steady-state level of eRF1.

**Increases in the steady-state level of eRF1 are not coupled to GTP hydrolysis.**

Previous work has shown that the eRF3-H348Q mutant protein has a defect in GTP hydrolysis. The GTPase activity of eRF3 is dependent upon binding to eRF1 and the ribosome and is required for cell viability (9, 12, 20, 21). Since eRF1 protein and SUP45 mRNA levels were elevated in the eRF3-H348Q mutant, we next tested the hypothesis that eRF1 levels were coupled to a GTPase hydrolysis by eRF3. To do this, we used a previously characterized in vitro GTPase assay consisting of eRF1 and eRF3-ΔNM (a form of eRF3 containing the GTPase and eRF1 binding domains). Salt-washed ribosomes purified from a whole cell yeast lysate were also required for optimal GTPase activity (21). While eRF3 combined with ribosomes exhibits some residual GTPase activity, maximal GTP hydrolysis is achieved only when eRF1, eRF3 and ribosomes are incubated together (Fig 6A). Using this assay, we carried out a kinetic analysis of the eRF3 GTPase activities induced by the eRF1-CΔ19, eRF3-H348Q, and Eo1/Sc23-eRF1-C124S mutants. V_{max} and K_{m} values were determined for reactions containing each of the
### Table 3. Kinetic analysis of GTPase activity

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Vmax (pmol/m)</th>
<th>Km (µM)</th>
<th>Kcat (s⁻¹)</th>
<th>Kcat/Km (s⁻¹·M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5.11</td>
<td>15.0</td>
<td>0.028</td>
<td>1873</td>
</tr>
<tr>
<td>Eo1/Sc23-C124S</td>
<td>12.8</td>
<td>34.7</td>
<td>0.071</td>
<td>2046</td>
</tr>
<tr>
<td>eRF3-H348Q</td>
<td>4.42</td>
<td>19.5</td>
<td>0.025</td>
<td>1284</td>
</tr>
<tr>
<td>eRF1-CA19</td>
<td>4.04</td>
<td>18.6</td>
<td>0.022</td>
<td>1183</td>
</tr>
</tbody>
</table>
mutant proteins. We found that the catalytic efficiency \( \frac{K_{\text{cat}}}{K_m} \) of GTP hydrolysis induced by eRF1-CΔ19 was reduced to a level similar to the eRF3-H348Q GTPase mutant, 63% and 69%, respectively, of wildtype (Table 3). It should be noted that previous work with another GTPase mutant of eRF3, eRF3-H348L, which had a catalytic efficiency reduced to 31% of wildtype, could not support viability when expressed in vivo (21). In contrast to the eRF1-CΔ19 and eRF3-H348Q mutants, the catalytic efficiency of the Eo1/Sc23-eRF1-C124S mutant was normal. While these results reveal a defect in the ability of eRF1-CΔ19 to stimulate the GTPase activity of eRF3, they do not support the hypothesis that eRF1 protein levels are coupled to the rate of GTP hydrolysis of eRF3.

The release defect of the eRF1-CΔ19 mutant can be corrected by increasing eRF3 to drive heterodimer formation of eRF1 and eRF3. We next examined the affect of the sup45-CΔ19 mutation on polypeptide release. To do this, we used a recently described in vitro termination release assay (2). This assay involves the synthesis of a radiolabeled tetrapeptide \([^{35}\text{S}]\text{-MVHL}\) from an mRNA template (Fig 7A). The synthesis of this peptide is carried out by combining 40S and 60S ribosomal subunits along with eIFs 1,1A, 2, 3, 4A, 4B, 4F, 5 and 5B and elongation factors eEF1A and eEF2 as well as aminoacylated tRNAs that were charged with purified rabbit aminoacyl-tRNA synthetases. Initially, the reaction is carried out in the absence of release factors. Once the tetrapeptide is synthesized, the ribosome stalls with a stop codon in the A-site of the ribosome forming a pretermination complex. The pretermination complexes were purified over a sucrose gradient, release factors were added, and peptide release was
Fig 6. Kinetic analysis of the GTPase activities of eRF1, eRF1-CΔ19, eRF3-H348Q and eRF1-Eo1/Sc23-C124S. (A) eRF3 GTP Hydrolysis assays. The graph indicates hydrolysis of GTP by reaction buffer alone (●), WT-eRF1 and WT-eRF3 (○), WT-eRF3 and ribosomes (■), WT-eRF1, WT-eRF3, and ribosomes (□), eRF1-CΔ19, WT-eRF3, and ribosomes (+), WT-eRF1, eRF3-H348Q, and ribosomes (△) and Eo1/Sc23-eRF1-C124S, WT-eRF3, and ribosomes (×). (B) Samples were taken from 0 to 30 minutes with GTP concentrations from 1 to 15 μM for analysis by Lineweaver-Burk plots. Every reaction mixture each contains 3 pmol of eRF1, eRF3, and salt washed ribosomes.
determined by measuring the trichloroacetic acid precipitable counts from the released
$[^{35}\text{S}]$-MVHL tetrapeptide. The previous characterization of the system used human eRF1 (heRF1) and eRF3 (heRF3) so our first experiments determined whether yeast release factors (yeRF1 and yeRF3) would work in conjunction with the mammalian system. In our initial experiments, a large excess of yeast release factors was used. We were able to achieve complete release with 10 pmol of yeRF1 alone as well as a mixture of 10 pmol of yeRF1 and 20 pmol of yeRF3. Similar to what had been previously shown for the human release factors, release was inhibited by the nonhydrolyzable GTP analog GMPNP, suggesting release was dependent upon hydrolysis of GTP. In the next set of experiments, yeRF1 and yeRF3 levels were reduced to analyze the stimulatory affect of eRF3 on the kinetics of polypeptide release. The results show that complete release was achieved by yeRF1 alone or by a mixture of yeRF1 and yeRF3 after 20 minutes. However, 70% release was achieved after only 2 minutes of incubation with yeRF1 and yeRF3, while 12 minutes were required to achieve the same level of release using yeRF1 alone (Fig 7C). This result shows that yeRF3 can increase the rate of release by yeRF1, consistent with previous experiments in this system with heRF1 and heRF3 (2).

Using this assay, we found that the eRF1-CΔ19 and eRF3-H348Q mutants had a similar release defect in polypeptide release (Fig 8). Complete release was achieved with a mixture of 0.5pmol of WT-yeRF1 and 0.2pmol of WT-yeRF3 after only 3.5 minutes while, complete release by a mixture of 0.5 pmol eRF1-CΔ19 and 1 pmol of WT-eRF3 was not achieved even after 10 minutes. Similar to the results for the eRF1-CΔ19 mutant, complete release was not achieved with a mixture of 0.5 pmol WT-yeRF1 and 1 pmol of the eRF3-H348Q mutant. In each of the mutants, only 70% release was achieved
Fig 7. Yeast release factors can trigger peptide release in a mammalian termination release assay. (A) The sequence of MVHL-Stop RNA template used for formation of pretermination complex (pre-TC) and subsequent release of the $^{35}$S-MVHL peptides. (B) Initial characterization of yeast release factors in the release assay. 10 pmol of yeRF1, 20 pmol of yeRF3 and 0.2 pmol of pre-TC were combined in the presence of 100µM GTP or the nonhydrolyzable analog GMPPNP. Graphed is the fraction of peptide released following a 20-minute incubation and measured using scintillation counting. (C) Time course of yeast release factors in the presence of GTP. The reaction contains 1 pmol yeRF1, 2 pmol yeRF3, 100µM GTP, and 0.2 pmol of the pre-TC. Key to graph: (□) yeRF1/yeRF3/GTP, (○) yeRF1 alone.
after 10 minutes under these conditions. These results indicate that the eRF1-Δ19 and
eRF3-H348Q mutant proteins have a severe release defect. The Eo1/Sc23-eRF1-C124S
mutant protein has not been analyzed yet. However, when these data are combined with
the observation that the Eo1/Sc23-eRF1-C124S mutant has a cold sensitive phenotype
not attributed to readthrough or a defect in GTPase activity, we suspect that a peptide
release defect will be observed in all three mutants. Since steady-state levels of eRF1 are
elevated in these three mutants, this would suggest that steady-state levels of eRF1 are
coupled to the rate of polypeptide release.

With the observation that eRF1-Δ19 exhibits a defect in stimulating the GTPase
activity of eRF3, we next asked if we could correct the defect in polypeptide release
associated with the eRF1-Δ19 mutant by increasing the amounts of WT-yeRF3 in the
reaction. We found that a 10-fold molar excess of WT-yeRF3 relative to the eRF1-Δ19
mutant restored release to wildtype levels (Fig 8). These results suggest that the defect in
the eRF1-Δ19 mutant is not in its enzymatic activity but rather in its inability to form a
stable complex with WT-yeRF3.

**Discussion**

Our initial characterization of the eRF1-Δ19 mutant revealed an increase in
steady-state levels of eRF1 protein and mRNA (Fig 1). We demonstrate that the increase
in steady-state levels of *sup45*-Δ19 mRNA and eRF1-Δ19 protein occurs by a
mechanism that increases the half-life of the SUP45 mRNA (Fig 4). We hypothesized
that impairment of one of the functions of eRF1, (stop codon recognition, stimulation of
the GTP hydrolysis activity of eRF3 or polypeptide chain release), activated a
Fig 8. The defect in release of the eRF1-CΔ19 mutant can be corrected by additional eRF3. 0.02 pmol of pretermination complex were paused with peptide chain attached to the tRNA_{Leu} in the P-site of the ribosome. Release of the MVHL tetrapeptide was achieved by addition of 0.5 pmol yeRF1 and 0.2-5 pmol of yeRF3 and monitored by scintillation counting (cpm). Key to the graph: 0.5 pmol yeRF1 alone (●), 0.5 pmol eRF1-CΔ19 alone (○), yeRF1 and 0.2 pmol yeRF3 (■), eRF1-CΔ19 and 0.2 pmol yeRF3 (□), eRF1-CΔ19 and 1 pmol yeRF3 (×), eRF1-CΔ19 and 5 pmol yeRF3 (△), and yeRF1 and 1 pmol eRF3-H348Q (†).
mechanism to compensate for this defect by increasing the stability of SUP45-mRNA. To test the hypothesis that eRF1 levels are coupled to the efficiency of stop codon recognition, we looked at two other strains, eRF3-H348Q and tpa1Δ[PSI+], that have previously characterized defects in stop codon recognition with readthrough levels similar to that of the sup45-CΔ19 mutation (Table 2) (15, 21). We observed an increase in eRF1 protein and mRNA levels only in the eRF3-H348Q mutant. Since eRF1 protein levels were not increased in the tpa1Δ[PSI+] strain, we conclude that a defect in stop codon recognition (readthrough) does not trigger an increase in steady-state levels of eRF1.

Having ruled out a defect in stop codon release as the trigger to increase eRF1 levels, we then turned to the other two known functions of eRF1; activating the GTPase activity of eRF3 and stimulating polypeptide chain release. We have previously characterized the GTPase defect of the eRF3-H348Q mutant (21). Using our in vitro GTPase assay, we showed that the eRF1-CΔ19 mutant has a defect in GTP hydrolysis similar to that of eRF3-H348Q (Table 3). Subsequent western blot and northern blot analysis of the eRF3-H348Q mutant showed that eRF1 protein and mRNA levels were also elevated suggesting a link between eRF1 levels and GTP hydrolysis. However, we also found that eRF1 protein and mRNA levels were elevated in another translation termination mutant, Eo1/Sc23-eRF1-C124S, which did not have a defect in GTP hydrolysis (Figs 5 and 6). These results indicated that eRF1 levels are not coupled to GTP hydrolysis.

Since the increase in steady-state eRF1 protein and mRNA levels observed in the eRF1-CΔ19, eRF3-H348Q, and Eo1/Sc23-eRF1-C124S mutants could not be linked to
GTP hydrolysis or stop codon recognition, we looked at the final known function of eRF1. Using a recently developed in vitro polypeptide release assay, we tested the hypothesis that eRF1 steady-state protein and mRNA levels are coupled to polypeptide chain release. The results from the assay show that eRF1-CΔ19 and eRF3-H348Q have a severe defect in polypeptide chain release. In addition, we have shown that the Eo1/Sc23-eRF1-C124S mutant is cold sensitive. Since the Eo1/Sc23-eRF1-C124S mutant does not have a defect in stop codon recognition or GTP hydrolysis, we hypothesize that the cold sensitive phenotype is an indication of a defect in polypeptide chain release. Studies are currently underway to ascertain the extent of the release defect in the Eo1/Sc23-eRF1-C124S mutant. Taken collectively, these data point to a mechanism where eRF1 steady-state protein and mRNA levels are coupled to the efficiency of polypeptide chain release in the cell.

In this study, we demonstrate that the eRF1 levels are elevated due to an increase in half-life of the SUP45 message (Fig 4). We propose a mechanism that could account for an increase in the half-life. In the cell, a defect in peptide release is manifested in some form, perhaps as a ribosome stalled with a stop codon in the A-site of the ribosome. The stalled ribosome may induce the expression of a putative mRNA binding protein that binds to a specific sequence element in the SUP45 transcript and stabilizes the message, thus increasing the half-life. It should be noted that the steady-state level of RF2 protein in prokaryotes is regulated by a frameshifting mechanism that is triggered by a stalled ribosome (1, 5). The ORF of RF2, which is the release factor that decodes UAA and UGA stop codons in prokaryotes, contains an inframe UGA stop codon in its coding sequence. With sufficient RF2 in the cell, the ribosome terminates efficiently at the
premature UGA stop codon and no new RF2 is made. However, when RF2 levels are low, the ribosome slips on a run of uracils adjacent to the premature stop codon causing a +1 frameshift of the ribosome leading to synthesis of full-length RF2 (1, 5). The data presented here in this study suggest that a regulatory mechanism to control the levels of eRF1 exists for eukaryotic cells but is not acting by the same mechanism since there are no stop codons in the coding region of SUP45. This is not surprising since the termination machinery and mechanism between prokaryotes and eukaryotes are very different in function, structure, and primary sequence (19). However, it is possible that expression of the putative factor we described above that controls SUP45-mRNA half-life might be controlled by a similar +1 frameshift mechanism.

Another goal in this study was to fully characterize the eRF1-CΔ19 mutant and what affect failure to form the eRF1-eRF3 heterodimer has on the termination process. Our previous studies have shown that the GTPase activity of eRF3 plays a role in stop codon decoding since mutations made in the GTPase domain of eRF3 exhibit a readthrough phenotype. Despite having similar defects in GTP hydrolysis and peptide release, the readthrough “pattern” of the GTPase mutant eRF3-H348Q is distinct from that exhibited by eRF1-CΔ19. Only a portion of the stop signals exhibit readthrough in the eRF3-H348Q mutant (UAAC, UAGC, and UGAN) while readthrough is present at all twelve possible stop signals in the eRF1-CΔ19 mutant (21). On the other hand, the readthrough “pattern” for eRF1-CΔ19 closely resembles the pattern observed in a strain with reduced eRF3 levels (21). These two deficiencies, the sup45-CΔ19 mutation and a reduction in eRF3 levels, lead to a reduction in the abundance of termination complexes causing a readthrough phenotype regardless of the stop signal. We explain these data by
suggesting that the *sup45-CΔ19* and *sup35-H348Q* mutations affect two distinct steps in the termination process. The *sup35-H348Q* mutation was made based on homology of its GTPase fold to other G proteins. The GTPase fold carries out binding and hydrolysis of GTP. Previous studies of eRF3 have shown that GTP hydrolysis must follow stop codon recognition so the eRF3-H348Q mutant affects a step following stop codon recognition (21). The eRF1-CΔ19 mutant does not bind eRF3 efficiently and therefore can’t form termination complexes required for stop codon decoding. Therefore, we hypothesize that the eRF1-CΔ19 mutant is defective in an early step of termination (i.e. preceding stop codon recognition) such as formation of the eRF1/eRF3 complex. The defect can be corrected by increasing the amount of eRF3 to drive the formation of eRF1-eRF3 complexes (Fig 8).

**Acknowledgements**

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References


Summary

This work began with a novel observation that the class I release factor in the yeast *Saccharomyces cerevisiae* is phosphorylated in vivo. This raised the possibility that the final step of protein synthesis in the cell is a regulated process. It was hypothesized that termination and elongation probably won’t be regulated since the rate limiting step of translation occurs during initiation (40). However, recent observations found that the second step of translation, elongation, is regulated in a manner independent of initiation. This observation combined with the data that termination is regulated in prokaryotes suggested that it could be regulated (1, 20).

The primary sequence of eRF1 has 13 total serines and threonines identified by a search algorithm as highly probable phosphorylation sites. To determine which of these site(s) are phosphorylated, we initially used a literature search to identify translation factors with characterized phosphorylation sites to further narrow our search. The CK2-kinase has been shown to phosphorylate eIF5, eIF2α, and all five proteins of the ribosomal stalk (8, 25, 56). These proteins have their CK2-kinase sites near their extreme C-termini. Similarly, eRF1 contains two serines in consensus CK2-kinase sites in its C-terminus, which served as an excellent starting place to search for the phosphorylation site(s). We made three mutants to test the hypothesis that these two sites were phosphorylated. Point mutants were made by changing the residue at serine 421 and serine 432 to alanine and a double mutant was made by changing both serines to alanine. Metabolic labeling of the mutant eRF1 proteins showed that both serines served as phosphorylation sites. The double mutant showed that most (if not all) phosphorylation of eRF1 occurred at these two sites. Using a temperature sensitive mutant of CK2-
kinase, we were able to show that eRF1 was an in vivo target of CK2-kinase (42). This added eRF1 to a growing number of translation factors that are phosphorylated in vivo by CK2-kinase.

Our next step was to characterize the metabolic affect of phosphorylation. We made two mutants for further study. The first mutant changed the serines at amino acid positions 421 and 432 to alanines and would serve as an eRF1 protein that lost the ability to be phosphorylated. The second mutant changed the serines to aspartic acids. This mutant imparts a negative charge at the two sites and mimics constitutive phosphorylation. These two mutants were subjected to a battery of biochemical assays to ascribe a function of phosphorylation to eRF1. We showed that phosphorylation does not affect binding to eRF3, plays no role in NMD, and only has a small affect on termination efficiency as determined by our dual luciferase reporter. While these results were disappointing, they were not surprising given that previous work has also failed to find a role for CK2-kinase phosphorylation for eIF5, eIF2α, and the ribosomal stalk proteins (25, 56, 73). However, our work did reveal some clues for possible roles of eRF1 phosphorylation. We showed that the phosphorylation is dynamic, suggesting a cycle of phosphorylation and dephosphorylation. Furthermore, we showed that phosphorylation is dependent on active cellular metabolism.

Our study was inconclusive as to whether phosphorylation plays a role in regulation of eRF1 activity. If it does play a role, it is likely to be restricted to yeast given that the two serines and the CK2-kinase motifs are not evolutionarily conserved in human eRF1. In addition, it has yet to be demonstrated that human eRF1 is phosphorylated. Based on our study, we suggest that phosphorylation plays a role in
fine-tuning the translation termination process as has been suggested for CK2-kinase phosphorylation of eIF2α (25). It remains to be investigated further whether phosphorylation of eRF1 may have a role in stress conditions such as osmotic stress, UV stress, amino acid starvation, or during normal cellular processes such as cell cycle progression.

Our second novel observation came from a mutant created during the phosphorylation study. We made the observation that steady-state protein levels of eRF1 were elevated in a mutant where the C-terminal 19 amino acids of eRF1 were removed. The mRNA levels of eRF1 were also elevated in the mutant. This was a phenotype only observed for eRF1 as the steady-state levels of eRF3 were not changed. We began to suspect that this increase in eRF1 levels might be a regulatory mechanism for termination and investigated it further.

Our next goal was to determine what function of eRF1 was being affected to give us a clue as to how the regulatory mechanism works. eRF1 has at least three functions in the cell: (1) stop codon recognition, (2) stimulate polypeptide chain release, and (3) act in concert with the ribosome to stimulate GTP hydrolysis by eRF3. The sup45-CΔ19 mutation causes a readthrough phenotype, increasing readthrough levels from 3-fold to 17-fold above that of SUP45. Our readthrough assay is a direct measure of how efficiently the termination complex can recognize a stop codon. A delayed recognition of the stop codon causes near-cognate tRNAs to decode the stop codons, allowing translation to continue. We first hypothesized that readthrough of stop codons was triggering the increase in eRF1 levels observed in the eRF1-CΔ19 mutant. Subsequent analysis by western blot of a tpa1Δ[PSI+] strain did not show an increase in eRF1 at the
protein level despite having readthrough levels comparable to eRF1-CΔ19. This result led to us rejecting our hypothesis that eRF1 levels were coupled to stop codon recognition (readthrough) in the cell.

Another ongoing project in our lab uses fusion proteins of class I release factors from ciliate organisms and *S. cerevisiae*. Ciliates are unique eukaryotes in that they have deviated away from the standard genetic code. In the ciliate *Euplotes octocarinatus*, UGA is not recognized as a stop signal in the organism suggesting a fundamental difference in how the class I release factor in that organism decodes a stop signal. Previously, we have shown that a fusion protein containing domain 1 from *E. octocarinatus* and domains 2 and 3 of *S. cerevisiae* showed the stop codon specificity of *E. octocarinatus* (i.e. terminates efficiently at UAA and UAG but not UGA). Because of this altered stop codon specificity, a strain expressing this protein cannot support viability as the sole source of eRF1 in yeast (76). A mutant was found in a random mutagenesis screen that could support viability (Fan-Minogue unpublished results). In this mutant, Eo1/Sc23-eRF1-C124S, eRF1 protein and mRNA levels were also elevated. This mutant has a cold sensitive growth defect and is not due to an increase in readthrough. We hypothesize that this mutant has a defect in polypeptide chain release. Experiments are currently underway to ascertain the extent of the release defect in this mutant.

eRF1 levels were also elevated in the GTPase deficient mutant eRF3-H348Q suggesting a defect in the GTPase activity of eRF3 may cause the increase in eRF1 steady-state protein levels. The eRF1-CΔ19 mutant exhibits a GTPase defect similar to that of the eRF3-H348Q mutant using our GTPase assay. However, the catalytic efficiency of the GTPase activity stimulated by the Eo1/Sc23-eRF1-C124S mutant is the
same as that stimulated by WT-eRF1. Looking at the data collectively, eRF1 levels are elevated in the eRF1-CΔ19 mutant, Eo1/Sc23-eRF1-C124S hybrid mutant, and the eRF3-H348Q GTPase mutant. Determining what these three mutants have in common could point to the trigger to increase eRF1 levels. Having ruled out stop codon recognition (readthrough) and a defect in GTPase activity, our conclusion is that the trigger to increase steady-state levels of eRF1 is coupled to the efficiency of polypeptide chain release. Such a defect in polypeptide chain release has already been shown for the strains expressing eRF1-CΔ19 or eRF3-H348Q. Similar assays will soon be undertaken for the Eo1/Sc23-eRF1-C124S mutant. If such a defect were found, it would provide correlative evidence that eRF1 levels are coupled to the efficiency of polypeptide release.

The future of this project will be focused on how a defect in polypeptide chain release is recognized in the cell. One such hypothesis is that a limitation of termination complexes (i.e. the eRF1 and eRF3 heterodimer) causes an accumulation of elongating ribosomes near the end of a message due to a slow rate of release. In the cell, additional eRF1 might alleviate this limitation by increasing the number of termination complexes. It has been suggested that eRF3 is in molar excess of eRF1 and might explain why it is only eRF1 that is increased. We showed in our mRNA half-life studies that the increase in eRF1 levels is caused by a post-transcriptional mechanism. We hypothesize that this occurs by a protein directly binding to a specific mRNA stability element on the eRF1 transcript. We envision two possible scenarios for the role the mRNA element could play in stabilizing the transcript. In the first scenario, a protein binds to an mRNA stability element in the SUP45 transcript in response to a stalled ribosome, thus increasing the steady-state level of eRF1. In a second scenario, we make an assumption that the SUP45
transcript is unstable under normal conditions due to a protein being bound to a "destabilization" element. A protein could be recruited to the SUP45 transcript in response to a stalled ribosome and remove the protein that would cause the SUP45 transcript to be unstable under normal conditions thus increasing the half-life of the SUP45 message.

The identification of the putative cis-acting mRNA stability element(s) will require a thorough analysis of the SUP45 transcript. The majority of the RNA stability proteins bind to the 3'UTR. The 3'-UTR of SUP45 contains a motif (UGUAUAUUA) predicted to be a PUF protein binding site (77). The PUF family of proteins is known to bind and affect the stability of mRNA messages via motifs with the conserved sequence UGUA (X$_{2-4}$) UA (30). It remains to be determined whether the PUF site plays a role in this mechanism. In addition to the 3'-UTR, the 5'-UTR must not be overlooked. A first step towards determining the role the 5’UTR will require the identification of the 5’ end of the transcript. This could be accomplished using a primer extension assay. A phylogenetic footprinting approach may also be of benefit to identify conserved sequences to speed the identification of any stability elements in the 5’UTR (16). Confirmation of the cis elements would involve putting them into reporter transcripts (e.g. luciferase or β-galactosidase) and see if they can stabilize these messages in an eRF1-CΔ19 mutant when they are present in a reporter construct in a context other than the SUP45 mRNA. Further confirmation could be achieved through site-directed mutagenesis.

Having identified the cis acting element, the next goal would be to identify the protein(s) that are acting in trans to stabilize the message. We would accomplish this by
Figure 1. Stabilizing model for increasing eRF1 steady-state levels in response to a ribosome stalled at a stop codon.
Figure 2. Destabilizing model for increasing eRF1 steady-state levels in response to a ribosome stalled at a stop codon.

1. Ribosome stalls at a stop codon due to a decrease in eRF1/eRF3 termination complexes.

2. Cell recognizes stalled ribosome and activates “X-factor”.

3. “X-factor” removes a “destabilizing factor” resulting in a stabilization of the SUP45 (eRF1) mRNA.

4. An increase in SUP45 mRNA leads to more eRF1 and relieves the stalled ribosome (eRF3 is in molar excess relative to eRF1 in the cell.)
synthesizing a transcript in vitro containing the putative stability element with a small amount of random flanking RNA and 6 MS2 binding sites. During the in vitro transcription process, the construct will be radiolabeled with $[^{32}\text{P}]\text{CTP}$ and prepared for protein crosslinking by incorporating 4-thio-UTP. This transcript will be incubated with a whole cell yeast lysate, exposed to ultraviolet light to crosslink the proteins, and subjected to an electric mobility shift assay (EMSA) to verify proteins can bind to it. Once the EMSA demonstrates binding, the transcript will be incubated with an MS2- MBP (maltose bind protein) heterodimer and purified over an amylase column, which binds the MBP. Proteins that bind the transcript will be identified by mass spectrometry and unique proteins will be determined by comparing the same purification of proteins using a scrambled form of the mRNA stability element (82).

In closing, complete elucidation of this regulatory mechanism will require identification of the trigger and the signaling cascade involved in responding to the trigger. It will also need to be determined what protein(s) is binding (or not binding) to the eRF1 message in response to polypeptide chain release defect in the eRF1-CΔ19, eRF3-H348Q, and Eo1/Sc23-eRF1-C124S mutants. When taken together, these studies will increase our understanding of the process of eukaryotic translation termination.
GENERAL LIST OF REFERENCES


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APPENDIX

OTHER RESULTS
To begin to identify the mRNA stability elements, we made transcript fusions where we interchanged the 5’ untranslated region (UTR) of SUP45 with the 5’-UTR of PGK1 as well as swapping the 3’-UTR of SUP45 with the 3’-UTR of CYC1 (Figure 1). The UTRs are controlling the expression and stability of a renilla reporter. We transformed these reporters into a WT-eRF1 and an eRF1-CΔ19 strain to see whether the 5’-UTR or the 3’-UTR played a role in stabilizing the SUP45 message in the eRF1-CΔ19 mutant. The results suggest that neither the 5’-UTR or the 3’-UTR can stabilize the message. Our future experiments will focus on regions of the eRF1 mRNA that are located in the coding region of SUP45. An excellent place to start would be nucleotides that correspond to the extreme C-terminus of eRF1 where the deletion was made to make the sup45-CΔ19 mutation (the final 57 nucleotides of the SUP45 mRNA). We have pondered why protein and mRNA levels of eRF1 were always higher in the eRF1-CΔ19 mutant than in the eRF3-H348Q and Eo1/Sc23-eRF1-C124S mutants, (approximately a 5 fold increase for the eRF1-CΔ19 mutant versus a 2 fold increase observed in the eRF3-H348Q and Eo1/Sc23-eRF1 mutants). These data may be explained if a putative cis acting mRNA stability element is contained in those 57 nucleotides.

A potential alternative scenario to explain these results is that the putative factor(s) responsible for increasing the steady-state levels of SUP45 mRNA in response to a decreased efficiency of polypeptide chain release might be diluted out using this assay. The PGK1 promoter is a highly active promoter. So, if we suppose that the stability element is in the 3’UTR, making massive amounts of this transcript by using the PGK1 promoter may not reveal an increase in renilla levels if the stability factor is
limiting. When these experiments are carried out, there are at least two targets for the supposed stability factor(s) to bind to; the endogenous SUP45 transcript that is normally in the cell as well as the 5’-SUP45-Renilla or the Renilla-3’-SUP45 reporter constructs. With multiple targets for the stability factor(s) to bind to, the factor(s) may be diluted out and not significantly increase the renilla reporter levels yielding the false conclusion that the mRNA stability element(s) is not located in the UTRs of SUP45. To test this hypothesis, we will rebuild the renilla reporters using a lower strength promoter and repeat the experiments.
Figure 1. Analysis of UTRs of \textit{SUP45}. The constructs contain the 5’-UTR from \textit{SUP45} or \textit{PGK1} in combination with the 3’UTR of \textit{SUP45} or \textit{CYC1}. Their output is measured by renilla activity that is normalized to total protein in the cell lysate.