The Effect of Mutation at Thr\(^{295}\) of *Saccharomyces cerevisiae* eRF1 on Suppression of Nonsense Codons and eRF1 Structure

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The termination of translation in *Saccharomyces cerevisiae* is controlled by two interacting polypeptide chain release factors, eRF1, and eRF3. Two regions in eRF1, at position 281-305 and 411-415, were proposed to be involved in the interaction to eRF3. In this study we have constructed and characterized eRF1 mutants at position 295 from threonine to alanine and serine residues resulting in eRF1(T295A) and eRF1(T295S) respectively. The mutations did not affect the viability or temperature sensitivity of the cells. The stop codons readthrough of the mutants were analyzed *in vivo* using PGK-stop codon-LACZ gene fusion and the results showed that the suppression of the mutants was increased in all of the codon terminations. The suppression of the UAG codon was the high for both mutants, with a 7-fold increased for eRF1(T295A) and a 9 fold increase for eRF1(T295S). The suppressor activity of eRF1(T295S) was higher compared to that of eRF1(T295A), suggesting that the accuracy of translational termination in eRF1(T295S) was lower than that of eRF1(T295A). Computer modeling analysis using Swiss-Prot and Amber version 9.0 programs revealed that the overall structure of eRF1 mutants has no significant difference with the wild type. However, substitution of threonine to serine on eRF1(T295S) triggered a secondary structure change on the other motif of the C-terminal domain of eRF1. This observation did not occur for on eRF1(T295A). This suggests that the high stop codon suppression on eRF1(T295S) is probably due to the slight modification of the structure of the C-terminal motif.

Keywords: *Saccharomyces cerevisiae*, eRF1, mutation, nonsense codon suppression.

Protein biosynthesis 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 eukaryotic system, codon-specific RF’s, and codon-non specific RF’s (Kisselev et al. 2003). Eukaryotic release factor 1 (eRF1), encoded by 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 (Bertram et al. 2000). Eukaryotic release factor 3 (eRF3), encoded by SUP35 gene, is a class II release factor that facilitates stop codon recognition and stimulates the termination reaction in a GTP dependent manner (Frolova et al. 1996; Salas-Marco and Bedwell 2004). Following stop codon recognition, eRF1 also induces polypeptide chain release by activating the peptidyltransferase center of the ribosome.

Both eRF1 and eRF3 are essential for the viability of yeast cells and deletion of the C-terminal part of each protein separately, lead to lethality (Inge-Vechtomov et al. 2003). The crystal structure of human eRF1 has been determined and found to be a protein is arranged in three domains. The N-terminal domain (domain 1) has been proposed to be responsible for stop codon recognition (Song et al. 2000). This proposal was supported by investigations using the mutational approach (Bertram et al. 2000) and by crosslinking experiments (Chavatte et al. 2001). The middle domain (domain 2) is responsible for peptidyl-tranferase hydrolytic activity and includes a GGQ motif that has been highly conserved through evolution (Frolova et al. 1999).

Mutations in GGQ (G residues) are dominant-negative *in vitro* and lethal *in vivo* in *S. cerevisiae* cells (Song et al. 2000). The domain 3 corresponds to the C-terminal part of eRF1 that is necessary for the interaction with eRF3 although there are some discrepancies in the precise location of the region of eRF1 which interacts with eRF3. Progressive deletion of the C-terminal region of eRF1, 6-19 amino acids in *S. cerevisiae* (Eurwilaichitr et al. 1999) and 17 amino acids of Schizosaccharomyces pombe (Ito et al. 1998) resulted in a corresponding loss of eRF3 binding affinity. In any case, the core eRF3-binding region identified for *Homo sapiens* eRF1 (by yeast two-hybrid and deletion analysis), showed that two regions in each release factor are critical for mutual binding. These are position 281-305 and 411-415 (GILRY) of eRF1, and position 478-530 and 628-637 of eRF3 (Merkulova et al. 1999). Although deletion of residues within domain 3 of eRF1 resulted in the loss of eRF3 interaction, detail of the position and amino acid residues for this interaction remain unclear.

Preliminary studies using computer modeling analysis of the structure of eRF1, especially of the above two regions, showed that tyrosine at position 410 (Y410) and threonine at position 295 (T295) of eRF1 are exposed to the molecular surface and are predicted to be involved in its interaction with eRF3. Mutation on tyrosine to serine at position 410 in eRF1 has been reported to decrease the binding affinity of the protein to eRF3 protein (Subandi 2002; Akhmaloka et al. 2008). In order to further probe the role of T295 in yeast eRF1 protein, we report here the effect of mutation T295 of yeast eRF1 on the termination of protein biosynthesis. The nonsense codons read-through process has been used to assay the accuracy of the termination process.
MATERIALS AND METHODS

Microbial Strains, Plasmids, and Growth Conditions. Saccharomyces cerevisiae strains used in this study are listed in Table 1. All strains were SUQ5 and [psi]. Yeast cultures were grown in a standard-rich-medium, YPD: 1% (w/v) bacto peptone, 0.5% (w/v) yeast extract; and 2% (w/v) glucose or in minimal medium, SM: 0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose, supplemented with appropriate amino acids, at 30°C with gentle shaking. For the plasmid shuffling, the strains were grown on medium YNB-FOA: 0.67% (w/v) amino acids, at 30°C with gentle shaking. For the plasmid base, 2% (w/v) glucose, supplemented with appropriate glucose or in minimal medium, SM: 0.67% (w/v) bacto peptone, 0.5% (w/v) yeast extract; and 2% (w/v) 5-Fluoroic acid (5-FOA), 20 μg ml⁻¹ uracil.

The Escherichia coli strain used in this work was DH5α [supE44 lacU169 (Q80lacZ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1] for the cloning experiments. The E. coli culture was grown in Luria Bertani medium LB: 1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl, supplemented with relevant antibiotics for selection (e.g. 50 μg ml⁻¹ ampicillin).

Plasmid pUKC1901, a shuttle vector between E. coli and Saccharomyces cerevisiae cells, carrying sup45-Y410S gene was used for the construction of plasmid carrying sup45 mutants. The other plasmids, pUKC815, 817, 818, and 819 were used in the nonsense codon read-through assay on S. cerevisiae.

DNA-Manipulation and Plasmid Constructions. All DNA manipulation and plasmid constructions were carried out according to standard protocols (Sambrook et al. 1989). To generate plasmids pSPES-T295S and pSPES-T295A, a 1400 bp BglII/HindIII fragment from plasmid pEPES-T295S and a fragment from pEPES-T295A containing the entire sup45 mutant genes, were cloned into BglII and BamHI sites of plasmid pUKC1901 (a gift from MF Tuite, University of Kent at Canterbury, UK), which is a shuttle vector of E. coli and S. cerevisiae. The recombinant plasmids were used to transform E. coli DH5α for propagating all plasmids.

Plasmid Shuffling. The plasmid shuffling was carried out based on the standard method of Akhmaloka (1991). The haploid LE(803) (SUP45::HIS3, URA3, SUP45) was used in plasmid shuffling. The strain was transformed with (LEU2 sup45) plasmids (pSPES-T295S and pSPES-T295A). The transformants were selected on media without uracil and leucine (-Ura-Leu) and then checked by replicating onto 5-FOA medium to counter select against URA3 plasmids (Kaiser et al. 1994). Growth was also assayed using serial dilution overnight culture with OD(600nm) = 1.0. Serially (10-fold) diluted yeast cell cultures were spotted on plates containing 5-FOA to determine the ability of sup45 mutant alleles to support cell growth. The wild type yeast SUP45 gene carried by the URA3 plasmid is eliminated since 5-FOA is toxic to cells expressing the URA3 gene.

β-Galactosidase Assays. Centromeric plasmids pUKC815, 817, 818, and 819 (Akhmaloka 1991; Stansfield et al. 1995) were transformed into suppressor strains (LE-E-T295S and LE-T295A). The transformants were grown in plasmid-selective medium and 3 × 10⁶ cells were inoculated into 5 ml YPD respectively. Triplicate samples from cultures grown, to a density of OD(420) = 1.0, were treated essentially as described by Colgan et al. (1995), with the following modifications. The cells were suspended in 500 μL Z buffer, 10 μL 0.1% (w/v) SDS, and 20 μL chloroform. The samples were then vortexed for 15 s and equilibrated for 15 min at 30°C using a water bath. After adding 100 μL 4 mg mL⁻¹ O-nitrophenyl-β-D-galactosidase (ONPG), samples were vortexed for 5 s and the reactions were carried out for 30 min at 30°C in a water bath before stopping the reaction by adding 500 μL 1.0 M sodium carbonate. After centrifugation at 5 000 x g, the optical density of supernatants were measured at both 420 and 550 nm wavelengths and the Miller unit of each of the samples was calculated as (OD 420)-(OD 550) x 1.75 (Stansfield et al. 1995). The read-through for each of the three stop codons was calculated as being the percentage of LacZ expression relative to the construct lacking a stop codon (pUKC815) in the same medium.

Computer Simulation. The initial three-dimensional-structure of eRF1 was constructed by homology protein structure modeling from Swiss-Prot using predict protein program (Rost et al. 2004). The comparative modelling program used three steps: alignment of the amino acid sequence of yeast eRF1 with the template of human eRF1 (Song et al. 2000) (PDB number 1DT9.pdb.), structure prediction based on primary sequences homology, and validation of the structure using WHAT_IF program in Swiss-Prot.

The Amber version 9.0 program package (Case et al. 2006) was used for the molecular simulation. The structure was subjected to energy minimization calculation using the steepest descent method with 500 iterations followed by the conjugate gradient method with 4 500 iteration to be used as starting lowest energy structure. The energy minimized for the protein was then submitted to molecular dynamic simulation after equilibrating for about 100 ps at 350 K. Temperature and pressure were maintained constant during the simulation. The trajectories and the coordinates of the proteins were saved every 2 fs for structural analysis. Simulation image of the protein was generated using visual molecular dynamic (VMD) software (Humphrey et al. 1996). The root mean square deviation (RMSD) was calculated for

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>ΔLE2(803)</td>
<td>SUQ5, ade2-1, his3-11, 15, ura3-1, leu2-1, can1-100, [psi], SUP45::HIS3</td>
<td>University of Kent at Canterbury, UK</td>
</tr>
<tr>
<td>ΔLE2(SUP45)</td>
<td>SUQ5, ade2-1, his3-11, 15, ura3-1, leu2-1, can1-100, [psi], SUP45::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>ΔLE2(T295A)</td>
<td>SUQ5, ade2-1, his3-11, 15, ura3-1, leu2-1, can1-100, [psi], SUP45::HIS3, [LEU2-1-SUP45-T295A]</td>
<td>This study</td>
</tr>
<tr>
<td>ΔLE2(T295S)</td>
<td>SUQ5, ade2-1, his3-11, 15, ura3-1, leu2-1, can1-100, [psi], SUP45::HIS3, [LEU2-1-SUP45-T295S]</td>
<td>This study</td>
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the backbone atoms with reference to starting structure of time zero.

RESULTS

Construction and Characterization of Yeast-Strains LE2(T295A) and LE2(T295S). LE2(T295A) and LE2(T295S) are haploid yeast strains that carry chromosomal SUP45 disrupted by the HIS3 gene (SUP45::HIS3) and sup45 gene in plasmids pSEPES-T295A and pSEPES-T295S respectively. The strains were constructed from LE2(803), a haploid strain which carries SUP45::HIS3 and SUP45 genes in a CEN-URA3-based plasmid. The LE2(803) strain was transformed with LEU2 based plasmid carrying SUP45 or sup45 genes. The transformants were then subjected to plasmid shuffle analysis to verify whether strains containing sup45 could lose cell viability (Fig 1). All transformants carrying sup45 alleles and SUP45 (control) were able to grow in the presence of 5-FOA, indicating that all tested mutations can replace the wild type of SUP45 for the viability test (data not show). However, plasmid shuffle was less efficient for sup45 mutants than for the wild type SUP45.

The viable transformants on media containing 5-FOA were characterized, including genotypes, temperature sensitivity, and the allosuppressor phenotypes. All of viable cells carrying SUP45 and sup45 mutants could grow on media without adenine (-Ade) or without -Leu, however they were unable to grow on media without -Ura (Fig 2). The viability of the cells on media -Ade showed that all transformants still possessed an allosuppressor phenotype. The viability of the transformants on media -Leu and -Ura was negative, showing that URA3 based plasmid had been replaced by LEU2 based plasmids. The data confirmed that the plasmid shuffling had been successfully carried out. Furthermore, none of the transformants were temperature sensitive mutants (data not shown).

Efficiency of LE2(T295A) and LE2(T295S) on Stop-Codon-Suppression. Stop codon read-through on LE2(T295A) and LE2(T295S) were measured based on the ability of the cells to terminate the translational process on the fusion gene between PGK-termination codon-LACZ carried by centromeric (single copy), URA3 based plasmid (Fig 3). The strains were transformed by each of four serial plasmids (pUKC815, 817, 818, and 819). Plasmid pUKC817, 818, and 819 carry UAA, UAG, and UGA termination codons respectively, while plasmid pUKC815 was used as the non-termination control.

The efficiency of all stop codon read-through in LE2(T295A) and LE2(T295S) were decreased compared to that the wild type [LE2(SUP45)]. Mutation on T295S showed a higher stop codon read-through compared to that T295A. The UAG codon showed the highest read-through among the stop codons from the results comparing mutants with the wild type. The increasing read-through of the UAG codon
was over 7 fold for LE2(T295A) and 9 fold for LE2(T295S) (Fig 4).

**Structural Modelling of eRF1(T295A) and eRF1(T295S).** Structural modelling of eRF1 mutants and the wild type were performed using the Amber 9 program followed by visualization using VMD. Based on the RMSD value, the overall structure of eRF1(T295A) and eRF1(T295S) showed no significant difference compared to the wild type. However, detailed analysis on the secondary structure of eRF1 mutants showed that eRF1(T295S) contained secondary structure changes which had not occurred on the eRF1(T295A) (Table 2). The structure of the amino acid residues change on eRF1(T295S) was not in the same motif with the position of mutation (Fig 4).

**DISCUSSION**

Domain 3 of eRF1 is considered to be responsible for the interaction with eRF3 protein (Ito et al. 1998; Ebihara and Nakamura 1999; Frolova et al. 2000). Serial deletion of this region has been reported to lose on its interaction (Eurwilaichitr et al. 1999), however there was no information concerning the effect of amino acid substitution in this region. Deletion of an amino acid usually causes an overall change of structure of a protein, while substitution of amino acid does not affect the overall structure. In order to probe the amino acids which are responsible for the function of the protein, the last strategy is preferable.

We have constructed and characterized sup45 mutants which have a mutation in codon no 295 for threonine to alanine or serine. Threonine at position 295 in yeast eRF1 was predicted to be involved on the interaction with eRF3 (Subandi 2002). Threonine at 295 is also one of the amino acids that are critical for mutual binding (Merkulova et al. 1999). Structural modeling of yeast eRF1 showed that the amino acid exposed to the surface of the protein is the one which has the requirement of amino acid residues to interact with other molecules.

**Table 2** Root mean square deviation (RMSD) value and the secondary structure change on some amino acid residues. The RMSD value was calculated using the eRF1 wild type as control

<table>
<thead>
<tr>
<th>Secondary structures of amino acid residues</th>
<th>RMSD</th>
<th>Amino acid no.</th>
<th>Amino acid no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>343-350</td>
<td>351-354</td>
</tr>
<tr>
<td>eRF1-SUP45</td>
<td>0</td>
<td>Turn</td>
<td>Turn</td>
</tr>
<tr>
<td>eRF1-T295A</td>
<td>0.0455</td>
<td>Turn</td>
<td>Turn</td>
</tr>
<tr>
<td>eRF1-T295S</td>
<td>0.0251</td>
<td>α-helix</td>
<td>Coil</td>
</tr>
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</table>

**SUP45** is an essential gene in haploid *S. cerevisiae* cell (Inge-Vechtomov et al. 2003). Disruption of the gene causes lethality in haploid yeast cells. In order to assay mutation in the gene in vivo, the mutant gene was introduced into LE(803) which carries disrupted chromosomal **SUP45::HIS3** and **SUP45** gene in single copy **URA3** based plasmid. The transformants containing double plasmids (**URA3** and LEU2 based plasmids) were examined for plasmid shuffle procedure (Chabalayska et al. 2007) by growing the cells on a medium containing 5-FOA. The wild type yeast **SUP45** gene carried on the **URA3** plasmid is eliminated because 5-FOA is toxic to cells expressing the **URA3** gene (Boeke et al. 1984).

Mutation of sup45-T295A and sup45-T295S showed an allosuppressor phenotype (Fig 2). This suggested that mutation of threonine at position 295 was not essential for cell growth but caused a defect in the functioning of the mutant protein. LE2(803) carries ade2 and **SUQ5** in addition to **SUP45::HIS3** and **SUP45 CEN URA3** based plasmids. **SUQ5** is a weak tRNA suppressor (Cox 1977). This tRNA suppressor does not suppress the nonsense codon on the ade2 mutant with a [psi+] genetic background (Inge-Vechtomov et al. 1988). However, mutation on the sup45 gene can enhance the activity of **SUQ5** (Stansfield et al. 1996) to suppress the nonsense mutation on the ade2 gene with the result that the cells showed viable on the medium without supplemented by adenine (Fig 2). The viability of transformants on medium without adenine showed that the mutation enhanced the activity of **SUQ5** and thus displayed an allosuppressor phenotype.

Mutation on sup45-T295A and sup45-T295S enhanced all of the stop codon suppressions (Fig 5). However, the quantitative values were variable depending on the type of stop codons and the mutations. The UAA codon showed the most read-through in all of the strains examined, including the wild type. This was not surprising since all strains carried the **SUQ5** genetic background. **SUQ5** is a codon specific tRNA suppressor for the UAA codon (Stansfield et al. 1995). For the UAG and UGA codons, the suppression of the wild type strain was very low, or insignificant (Fig 5). However, for the mutant strains, the suppression of UAG and UGA was significantly increased. The UAG codon was the most leaky in both mutants. This is to be unexpected since eRF1 is reported to recognize all of stop codons in the same manner (Frolova et al. 1994; Zhouravleva et al. 1995; Frolova et al. 1996).

Termination of translation in eukaryotic system that involves interaction between eRF1 and eRF3 is a complex phenomenon. Detail mechanism of the process is still unclear yet. eRF1 was reported to be phosphorylated by CK2 protein but the product did not directly affect translation termination (Kallmeyer et al. 2006). Urakov et al. (2001) demonstrated that IttIp could modulate the efficiency of translation termination in yeast. Meanwhile, a recent report showed that eRF1 protein participates not only in the termination of translation but also in mRNA degradation and the initiation of translation via interaction with other proteins (Chabelayska et al. 2007).

In order to further characterize the causes of an elevation of suppression in sup45-T295A and sup45-T295S, the three dimensional structure of eRF1(T295A) and eRF1(T295S) were...
analyzed using molecular dynamic simulation. The results showed that there was no significant change on the overall structure of eRF1 mutants. However, detailed analysis of eRF1(T295S) showed that secondary structure of a few amino acids changed, from turn to α-helix form (Table 2). The structural modification of eRF1(T295S) had not directly affect on the structure of the T295 motif but it did trigger structural modification of the neighboring motif (Fig 4). This modification might affect the interaction of eRF1(T295S) with eRF3 and thus increasing the suppression of codon terminations.

ACKNOWLEDGEMENT

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Fig 5 Three dimensional structure of domain 3 eRF1 based on molecular dynamic simulation: a, Wild type of eRF1; b, eRF1-T295A; and c, eRF1-T295S. Areas of conformational change shown within dotted circles.


