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The Effect of Mutation at Thr [295] of *Saccharomyces cerevisiae* eRF1 on Suppression of Nonsense Codons and eRF1 Structur

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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 on 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 peptidyl-transferase 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 (domain1) 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 of 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.

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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) yeast nitrogen base, 2% (w/v) glucose, 0.1% (w/v) 5-Fluorootic 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 rei A1*] 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 *S. 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 1 400 bp *Bgl*II/*Hin*dIII fragment from plasmid pEPES-T295S and a fragment from pEPES-T295A containing the entire *sup45* mutant genes, were cloned into *Bgl*II and *Bam*HI sites of plasmid pUKC1901 (a gift from M F 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-T295S and LE-T295A). The transformants were grown in plasmid-selective medium and 3 x 10^6 cells were inoculated into 5 mL YPD respectively. Triplicate samples from cultures grown, to a density of OD_{600nm} ~1.0, were treated essentially as described by Coligan *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 were calculated as $(OD_{420})-(OD_{550} \times 1.75)$ (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 (RSMD) was calculated for

Table 1 Yeast strains used in this study

Strain	Genotype	Source
ΔLE2(803)	*SUQ5, ade2-1, his3-11, 15, ura3-1 leu2-1, can1-100,* [*psi*$^-$], *SUP45::HIS3*	University of Kent at Canterbury, UK
ΔLE2(*SUP45*)	*SUQ5, ade2-1, his3-11, ura3-1, leu2-1 can1-100,* [*psi*$^-$], *SUP45::HIS3,* [*LEU2-1-SUP45*]	This study
ΔLE2(T295A)	*SUQ5, ade2-1, his3-11, 15, ura3-1, leu2-1, can1-100,* [*psi*$^-$], *SUP45::HIS3,* [*LEU2-1-SUP45-T295A*]	This study
ΔLE2(T295S)	*SUQ5, ade2-1, his3-11, 15, ura3-1, leu2-1, can1-100,* [*psi*$^-$], *SUP45::HIS3,* [*LEU2-1-SUP45-T295S*]	This study

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

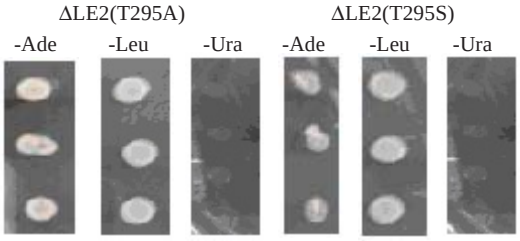

Fig 2 The viability of mutant yeast strains on variation media. (-Ade) minimum media without adenine, (-Leu) minum media without leucine, (-Ura) minimum media without uracil.

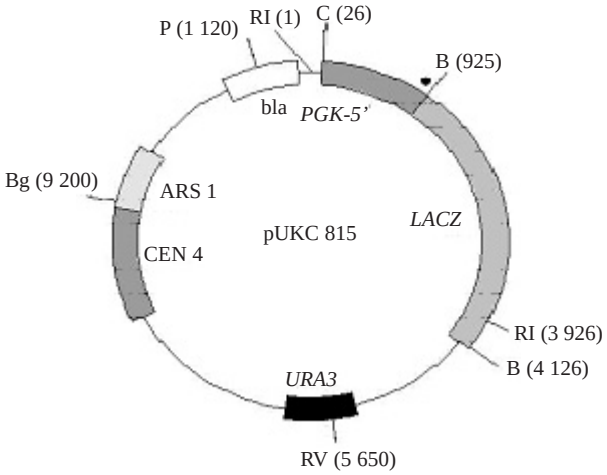

Fig 3 Plasmid used for suppression assay. pUKC815, a *URA3* based plasmid carrying *PGK-LACZ* gene fusion without codon termination in between; pUKC817, 818, and 819 are *URA3* based plasmids carrying *PGK*-stop codon-*LACZ* gene fusion TAA, TAG, and TGA on oligonucleotides respectively; (●), PUKC 817: GAT CTA *TAA* GCT TTG GAT, PUKC 818: GAT CTA *TAG* GCT TTA AAG GAT, PUKC 819: GAT CTA *TGA* GCT TTA AAG GAT.

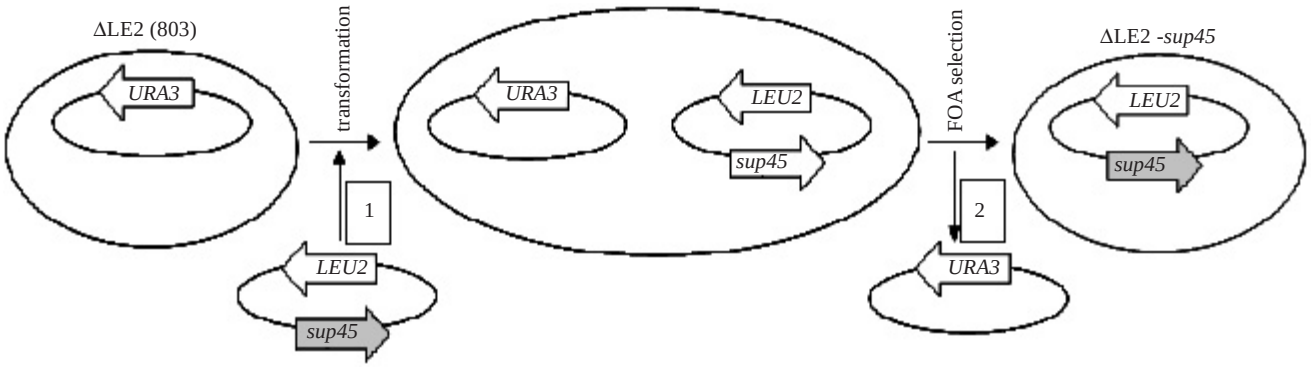

Fig 1 Strategy used for plasmid shuffling. (1) the mutagenized DNA on plasmid carryinf *LEU2* marker introduced into the yeast cell, (2) transformants carrying double plasmids (*URA3* and *LEU2* based plasmid) were grown on media containing 5-FOA to replaced *URA3* based plasmid.

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

	RMSD	Secondary stuctures of amino acid residues	
		Amino acid no. 343-350	Amino acid no. 351-354
eRF1-*SUP45*	0	Turn	Turn
eRF1-T295A	0.0455	Turn	Turn
eRF1-T295S	0.0251	α-helix	Coil

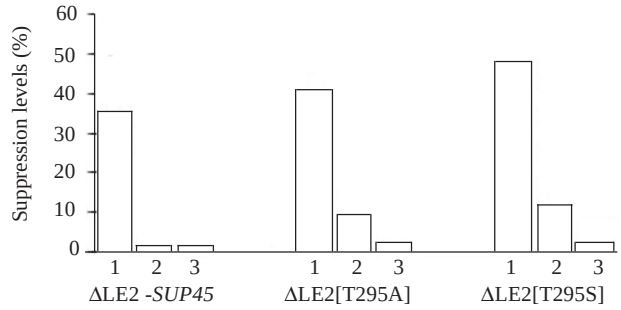

Fig 4 Codon termination read-through of yeast strains carrying *SUP45* and *sup45* mutants genes. 1, UAA; 2, UAG; 3, UGA.

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 (Chabelskaya *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 ItIp 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 (Chabelskaya *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

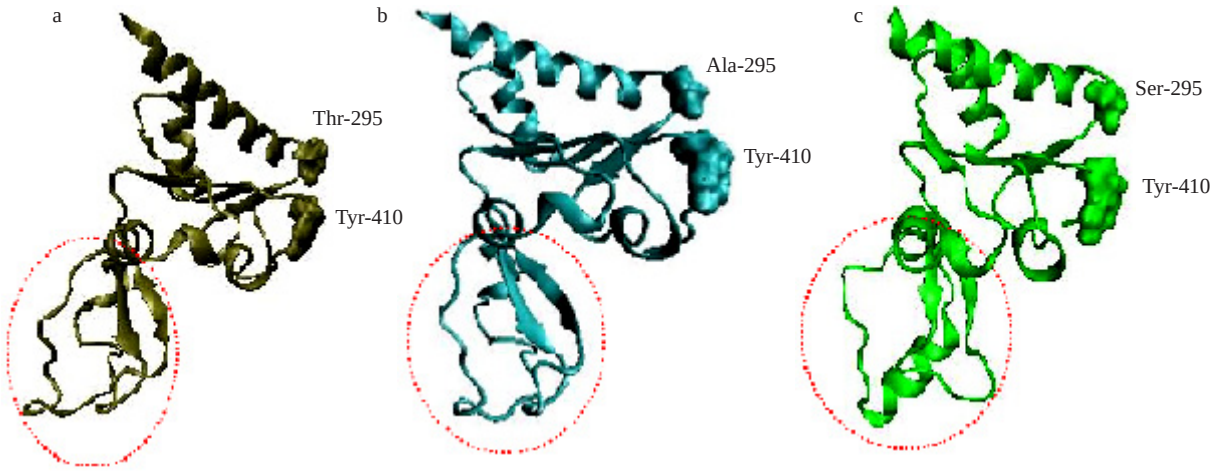

Fig 5 Three dimensional structure of domain 3 eRF1 based on molecular dynamic simulation: a, Wild type of eRF1; b, eRF1-T295A; and c, eRF1-T295S. Areas of conformational change shown within dotted circles.

analyzed using molecular dynamic simulation. The results showed that there was no significant change on the overall structure of eRF1 mutants. However, detail 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.

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