The relation between the stop codon reassignment and the specificity of eukaryotic release factor 1 (eRF1) in *Blepharisma*

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SUMMARY

In *Blepharisma*, the universal stop codon UGA has been known to reassign to sense codon (Trp). The reassignment of the codon UGA implies that eRF1 (the stop codon-recognizing protein) may loses its binding specificity toward UGA. In this study, we examined the stop codon recognizing capacity of eRF1 domain 1, (the stop codon-binding domain), infrom *Blepharisma musculus* by an in vivo assay system in yeast. Unexpectedly, the chimeric eRF1, which containsg *Blepharisma* domain 1, was able to complement a defect in yeast eRF1, i.e. *Blepharisma* eRF1 and recognizeds all three stop codons (UAA, UAG and UGA). Our result leads to a speculation that the stop codon reassignment in Blepharisma has occurred by a mechanism that might require the appearance of new tRNA able to read the reassigned codon UGA.

INTRODUCTION

The genetic code of nuclear genes in some ciliates has been known to deviate from the universal genetic code. There are two types of stop codon reassignment have been found in ciliates: 1) UAA/UAG reassigned to sense codons (Gln) and only UGA used as stop codon; 2) UAA/UAG used as stop codons and UGA reassigned to sense codon (Cys in *Euplotes*). In *Blepharisma*, UAA has been found to be a stop codon (Liang and Heckmann, 1993), and the UGA codon is reassigned to tryptophan (Lozupone *et al.*, 2001). Although the assignment of UAG codon is still unknown in *Blepharisma*, this genus was supposed to be in the same group with *Euplotes*.

Eukaryotic release factor (eRF1) is believed to play an important role in the stop codon reassignment in ciliates. In most eukaryotes, a single eRF1 recognizes all three stop codons. However, in ciliates, the specificity of the stop codon recognition by eRF1 has been altered. For examples, in *Euplotes*, eRF1 recognizes UAA and UAG as stop codons, but not UGA codon, which instead encodes cysteine (Kervestin *et al*., 2001). The crystal structure of human eRF1 has been determined, which is organized into three domains (Song *et al*., 2000). The functional importance of the three domains of eRF1 has been characterized by genetic and biochemical studies. Especially, *in vivo* genetic analysis of yeast eRF1 and *in vitro* site-directed mutagenesis of human eRF1 revealed that stop codon-binding sites are localized in domain 1. To clarify whether the reassignment of UGA codon in *Blepharisma* relates to the stop codon recognition of eRF1, we have examined the stop codon recognizing capacity of eRF1 from *Blepharisma musculus* whose sequence has been determined previously (Kim *et al.*, 2005). This study is expected to enable us to understand the stop codon usage and the molecular basis of genetic code deviation in this species.

MATERIALS AND METHODS

Chimeric eRF1 construction

Domain 1 of eRF1 cDNA from *B. musculus* (amino acid sequence positions: 1-140, human eRF1 numbering) which was marked with restriction sites *Nde*I at 5'-end and

*Xho*I at 3'-end was cloned using a TOPO TA Cloning Kit (Invitrogen). Domain 2-3 of eRF1 cDNA from *Saccharomyces cerevisiae* which was marked with restriction sites *Xho*I at 5'-end and *Sal*I at 3'-end was separately cloned using pT7Blue vector (Novagen). MCS of pT7Blue vector also contains *Nde*I restriction site at upstream of the insert.

Purified plasmid DNAs of TOPO vector containing domain 1 of *Blepharisma* eRF1, and pT7Blue vector containing domain 2-3 of yeast eRF1 were treated with *Nde*I and *Xho*I. Chimeric eRF1 was constructed by inserting domain1 of *Blepharisma* eRF1 into the pT7Blue vector containing domain 2-3 of yeast eRF1.

***In vivo* complementary tests**

Three low-copy-number CEN/ARS vectors containing marker gene URA3 (p416CYC1, p416ADH and p416GPD which are composed of a weak CYC1 promoter, a medium ADH promoter and a strong GPD promoter, respectively) were used in this study. The chimeric eRF1 was cloned in the *Spe*I-*Sal*I site of the vectors.

Two *S. cerevisiae* strains were used in this study: temperature sensitive eRF1 mutant (*SUP45 ts*) strain, and tetracycline-regulated eRF1 (P_{tet}*SUP45*) strain. The wild-type yeast eRF1 (*SUP45*$^+$) and the chimeric eRF1 were transformed into these *S. cerevisiae* strains by using Frozen_EZ yeast transformation kit (ZYMO Research). The transformed yeasts were screened by cell growth on plates lacking uracil. *In vivo* complementary test of transformants of *SUP45 ts* strain were monitored by cell growth at permissive (30^{o}C) and non-permissive (37^{o}C) temperatures. *In vivo* complementary test of transformants of P_{tet}*SUP45* (tet-OFF system) strain were monitored by cell growth on plates with and without Doxycyclin (10 μg/ml), a tetracycline derivative.

RESULTS AND DISCUSSION

Domain swapping between *B. musculus* and *S. cerevisiae* eRF1

The functions of the three domains of eRF1 have been characterized: domain 1 recognizes stop codons, domain 2 takes part in peptidyl-tRNA hydrolysis, and domain 3 has been known to interact with eRF3. Moreover, eRF1 domain 2 was supposed to interact with ribosome since mutations in domain 2 reduced the ribosome binding ability of human eRF1. Taking these findings into consideration, we constructed the chimeric eRF1 containing *Blepharisma* eRF1 domain 1 to examine the stop codon recognizing capacity of the *Blepharisma* eRF1. The restriction site *Xho*I was introduced into the linker between domain 1 and domain 2, which do not change any conserved amino acid, as well as topologies of the domains.

***In vivo* complementation activity of the chimeric eRF1**

The wild-type *S. cerevisae* eRF1 (*SUP45*$^+$) and the chimeric eRF1 were cloned into yeast expression vectors (p416CYC1, p416ADH and p416GPD), and transformed into *SUP45 ts* strain and P_{tet}*SUP45* strain. URA$^+$ transformants were selected at permissive temperature 30^{o}C. The *in vivo* complementation test of transformants of *SUP45 ts* strain was examined for their growth at non-permissive temperature 37^{o}C, while that of P_{tet}*SUP45* strain was examined for their growth in the presence of Doxycyclin (10μg/ml).

If the eRF1 expressed from the plasmid (yeast eRF1 and chimeric eRF1s) can recognize all three stop codons correctly and terminate translations normally, i. e. the chimera is able to complement the defect in the endogenous yeast eRF1, the transformants (*SUP45 ts* strain and P_{tet}*SUP45* strain) maintain their growth in non-permissive conditions (at 37^{o}C and in the presence of Doxycyclin, respectively), otherwise the transformants will fail to grow.

Our results showed that the chimeric eRF1 expressing transformants grew normally at 37^{o}C (*SUP45 ts* strain) and also in the presence of Doxycyclin (P_{tet}*SUP45* strain) as well as the wild-type *SUP45*$^+$ transformants. These two lines of genetic analyses consistently showed that the hybrid eRF1s containing *Blepharisma* domain 1 retained omnipotent stop codon decoding activity and could substitute the endogenous yeast eRF1.

The stop codon reassignment is surprisingly frequent in ciliates. The key phenomena to give such permissibility to ciliates have been proposed to be the altered specificity of stop codon recognition by eRF1 and/or the appearance of new tRNA able to read the reassigned codons. *Euplotes octocarinatus* has only one copy of cysteine tRNA gene, which codes for normal cysteine tRNA ($tRNA_{GCA}^{Cys}$). The deviated codon UGA was translated by the normal $tRNA_{GCA}^{Cys}$ despite of G:A mispairing at the first anticodon position (Grimm *et al.*, 1998) *Euplotes* eRF1 had lost its binding specificity toward UGA, resulting in the reassignment of universal stop codon UGA in this species (Kervestin *et al*., 2001). In *Tetrahymena*, in addition to the normal glutamine tRNA, two unusual glutamine tRNAs ($tRNA_{UUA}^{Gln}$ and $tRNA_{CUA}^{Gln}$) which recognize UAA and UAG were isolated (Hanyu *et al.*, 1986). For *Tetrahymena* eRF1, the data are controversial. *Tetrahymena* eRF1 was shown to be UGA-specific *in vitro*, but omnipotent *in vivo*. The codon reassignment in *Tetrahymena* might not require an UGA-specific eRF1 because the new $tRNAs^{Gln}$ seems to be able to win efficiently the competition with eRF1. In this study, our result clearly showed that *Blepharisma* eRF1 recognizes all three stop codons. We suggest that the reassignment of UGA codon in *Blepharisma* would become possible if the new $tRNA_{UCA}^{Trp}$ is existed and

quite abundant. Even with the same type of stop codon reassignment, the genetic code in *Blepharisma* has changed by a mechanism that is different from the mechanism occurred in *Euplotes*. Further studies of tRNA in *Blepharisma* will be required to test this hypothesis.

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