J. Microbiol. Biotechnol. (2010), **20**(9), 1283–1287
doi: 10.4014/jmb.1004.04020
First published online 23 July 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

GTPase Activity Analysis of eRF3 in *Euplotes octocarinatus*

Song, Li[1], Jun-Li Dong[1], Ya-Qin Zhao[2], Bao-Feng Chai[1], and Ai-Hua Liang[1]*

[1]*Institute of Biotechnology, and* [2]*Institute of Molecular Science, Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Shanxi University, Taiyuan 030006, China*

Received: April 15, 2010 / Revised: June 2, 2010 / Accepted: June 3, 2010

In eukaryotes, eRF3 participates in translation termination and belongs to the superfamily of GTPases. In this work, the dissociation constants for nucleosides bound to *Euplotes octocarinatus* eRF3 in the presence and absence of eRF1a were determined using fluorescence spectra methods. Furthermore, a GTP hydrolyzing assay of eRF3 was carried out using an HPLC method, and the kinetic parameters for GTP hydrolysis by eRF3 were determined. Consistent with data from humans, the results showed that eRF1a promoted the binding of GTP to eRF3 and the GTP hydrolyzing activity of eRF3. However, in contrast to the lack of GTP binding in the absence of eRF1 in human eRF3, the *E. octocarinatus* eRF3 was able to bind GTP by itself. The nucleotide binding affinity of the *E. octocarinatus* eRF3 also differed from the human data. A structure model and amino acid sequence alignment of potential G domains indicated that these differences may be due to valine 317 and glutamate 452 displacing the conserved glycine and lysine involved in GTP binding.

Keywords: Dissociation constants, eRF3, *Euplotes octocarinatus*, GTPase, kinetic parameter

The termination of protein synthesis is mediated by two classes of polypeptide chain-release factors (RFs). The class 1 RFs are a codon-specific factor [8, 11, 19]. In prokaryotes, the class 1 RFs, RF1 and RF2, recognize the stop codons UAG/UAA and UGA/UAA, respectively [8, 11], whereas in eukaryotes, the class 1 RFs (eRF1s) recognize all three stop codons UAG/UAA/UGA, except in ciliates [10, 11].

The class 2 RFs are a codon-nonspecific factor. The class 2 RFs belong to the superfamily of G proteins: translation factors involved in various steps of protein biosynthesis [27]. G proteins are activated by the binding of GTP and deactivated by the hydrolysis of GTP to GDP. After the dissociation of GDP, the active G protein is regenerated by the rebinding of GTP. For many, but not all G proteins, the GTPase activity requires GTPase-activating proteins (GAP) to promote catalysis. The substitution of GTP for GDP in the G protein is very slow and usually catalyzed by a guanine-nucleotide exchange factor (GEF) [12].

In humans, *Xenopus laevis*, and rabbits, the eRF3 is a ribosome-dependent and eRF1-dependent GTPase [5, 6]. eRF3 as a GTPase can bind GTP and hydrolyze GTP. The presence of eRF1 greatly increases the affinity of GTP for eRF3 in humans [7, 18, 21]. The GTPase activity of eRF3 is required to couple the recognition of the translation termination signals by eRF1 into an efficient polypeptide chain release [22]. An *in vitro* reconstitution of the translation termination also revealed the cooperative action of eRF1 and eRF3 in ensuring the fast release of nascent polypeptides [1]. Moreover, recent studies have shown that the M domain of eRF1 is in contact with the GTPase domain of eRF3, and that arginine 192 in the M domain is important for the stimulatory effect of eRF1 on the GTPase activity of eRF3 [3, 14].

In contrast to other higher eukaryotes, ciliates have evolved to reassign stop codons as sense codons [9, 10, 16, 17]. To understand this event of condon reassignment, a functional study of the release factors is essential. In *Euplotes octocarinatus*, two kinds of class 1 release factors, eRF1a and eRF1b [15], and one eRF3 have been reported [26]. The eRF1a binding domain of the *E. octocarinatus* eRF3 is similar to that of the yeast eRF3, yet different to that of the human eRF3 [24]. Chai *et al*. [2] reported that the GTPase activity of eRF3 is dependent on ribosome in *E. octocarinatus*, whereas the role of eRF1 involved in eRF3 GTPase activity remains unclear. The constants for GTP binding and the kinetic parameters for GTP hydrolysis by eRF3 are also unknown. Accordingly, in this study, we cloned an N-terminally truncated *E. octocarinatus* eRF3 (corresponding to the G region and eRF1a binding region), named eRF3C.

**Corresponding author*
Phone: +86-351-7018731; Fax: +86-351-7011499;
E-mail: aliang@sxu.edu.cn

Furthermore, we determined the dissociation constants for the nucleosides bound to eRF3 in the presence and absence of eRF1a and the kinetic parameters (V_{max}, K_m, and k_{cat}) of eRF3. The results showed that eRF1a promoted the binding of GTP to eRF3 and the hydrolyzing of GTP by eRF3 in *E. octocarinatus*. In contrast to the lack of GTP binding without eRF1 in the human eRF3, the *E. octocarinatus* eRF3 was able to bind GTP by itself. Thus, the eRF3 binding affinity to nucleotides differed from that in humans.

Materials and Methods

Expression and Purification of eRF3C and eRF1a

The *E. octocarinatus* eRF3C was expressed and purified as described previously [25]. The *E. octocarinatus* eRF1a gene was amplified using a PCR with a forward primer (5'-CGACA***GGATCC***ATGT CAATAATTGATAGT-3', *Bam*HI site underlined) and reverse primer (5'-AGCTC***CTCGAG***TTATAAATTTTTATGAGC-3', *Xho*I site underlined). The PCR product was gel-purified and subcloned into the *Bam*HI–*Xho*I sites of pGEX-6p-1, and the clones were confirmed by sequencing.

The recombinant plasmid pGEX-6p-1-eRF1a was transformed into strain *E. coli* BL21 (DE3). The transformants were grown to OD_{600}=0.4–0.6 at 37°C and then induced with 0.1 mM isopropyl-1-thio-β-D-galactoside (IPTG) for 16 h at 16°C. The cells were harvested, lysed by sonication on ice, and centrifuged at 12,000 ×*g* for 15 min. The eRF1a protein was purified as described previously [25], and the protein concentrations were measured spectrophotometrically using molar extinction coefficients at 280 nm of 41,130 $M^{-1}cm^{-1}$ for eRF3C and 31,370 $M^{-1}cm^{-1}$ for eRF1a. The extinction coefficients of eRF3C and eRF1a were estimated as previously described [20].

Fluorescence Spectroscopy Assay

The fluorescence spectra were measured using a Hitachi F-2500 spectrometer. The samples were irradiated at 295 nm to selectively excite the indole side chains of the tryptophan residues, and the emission spectra were recorded between 310 nm and 410 nm. To measure the dissociation constants of eRF3C or eRF3C–eRF1a and nucleotides in the *E. octocarinatus*, the intrinsic fluorescence of the protein was quenched by adding small aliquots of nucleotides (60 μM GTP or 70 μM GDP) to the eRF3C or eRF3C–eRF1a complex at a 2 μM concentration in the presence of 2 mM $MgCl_2$.

GTP Hydrolysis Assay

The rabbit reticulocyte ribosomal subunits were isolated as previously described [4–6]. For the GTPase assay of the components of the termination complex, the reaction mixture contained 50 pM eRF3C, 50 pM eRF1a, 1 μg of rabbit ribosomes, 0.95 nM GTP, and 2 mM $MgCl_2$ in a reaction buffer (10 mM Hepes, pH 7.4, 50 mM NaCl). The GTP hydrolysis assay was carried out at 30°C and the enzymatic reaction stopped by heating for 3 min at 100°C. For the kinetic analysis of the GTPase activity of eRF3, the rates of GTP hydrolysis were calculated for the above reaction mixture containing several GTP concentrations (0.03 mM, 0.04 mM, 0.06 mM, 0.13 mM, 0.16 mM, 0.32 mM, and 0.63 mM). The GTP hydrolysis reaction was continued for 3 min at 30°C and then stopped as described above. The GTP hydrolysis was then measured based on an HPLC experiment, where 30-μl aliquots were applied to a C18 reverse-phase HPLC column and an isocratic run of 7.5% acetonitrile in 10 mM tetrabutylammonium bromide and 100 mM K_2HPO_4/KH_2PO_4, (pH 6.5) was performed at a flow rate of 1 ml/min. The GTP and GDP eluted from the column were detected using a calibrated detector (Beckman). Finally, the data were analyzed using Lineweaver–Burk plots. The V_{max} (the inverse of the y-intercept) and K_m (negative inverse of the x-intercept) for the eRF1a-stimulated reactions were both calculated, and the k_{cat} was then derived from the V_{max} ($k_{cat}=V_{max}$/pmol eRF3C).

Results and Discussion

Dissociation Constants for eRF3 Binding to GTP

Fluorescence spectra methods were used to measure the dissociation constants of eRF3 binding to GTP or GDP. The fluorescence spectra of eRF3C in the native state and with the addition of GTP are shown in Fig. 1A. The fluorescence emission maximum of eRF3C was seen at 334 nm, and this maximum emission intensity decreased with the addition of GTP. The same observation was also seen with the addition of GDP in the presence of 2 mM $MgCl_2$ (data not shown). This decrease was due to the fluorescence change of tryptophan in eRF3C when located in a different hydrophobic environment. eRF3C includes four tryptophan residues (W352, W353, W460, W519) (Fig. 3A), and their position in close vicinity to the G domains could explain the observed fluorescence changes. To calculate the dissociation constants of eRF3C and GTP/GDP, it was assumed that eRF3C had n GTP/GDP-binding sites that were independent and identical. The dissociation constants were then fitted using Eqs. (1)–(5).

$$E+nG \leftrightarrow E[G]_n \tag{1}$$

$$\frac{F-F_\infty}{F_0-F_\infty}=\frac{[E]_b}{[E]_t} \tag{2}$$

$$\frac{[E]_f \cdot [G]_f}{[E]_b}=K_D \tag{3}$$

$$[G]_f=[G]_t-n[E]_b \tag{4}$$

$$\frac{[G]_t}{[E]_b}=\frac{K_D}{[E]_f}+n \tag{5}$$

where E is eRF3C; G is GTP/GDP; $[E]_b$, $[E]_f$, and $[E]_t$ are the bound, free, and total concentrations of eRF3C, respectively; $[G]_b$, $[G]_f$, and $[G]_t$ are the bound, free, and total concentrations of GTP/GDP, respectively; F_0 and F are the fluorescence intensities in the absence and presence of GTP/GDP, respectively; and F is the fluorescence intensity when the eRF3C/eRF3C–eRF1a and GTP/GDP binding

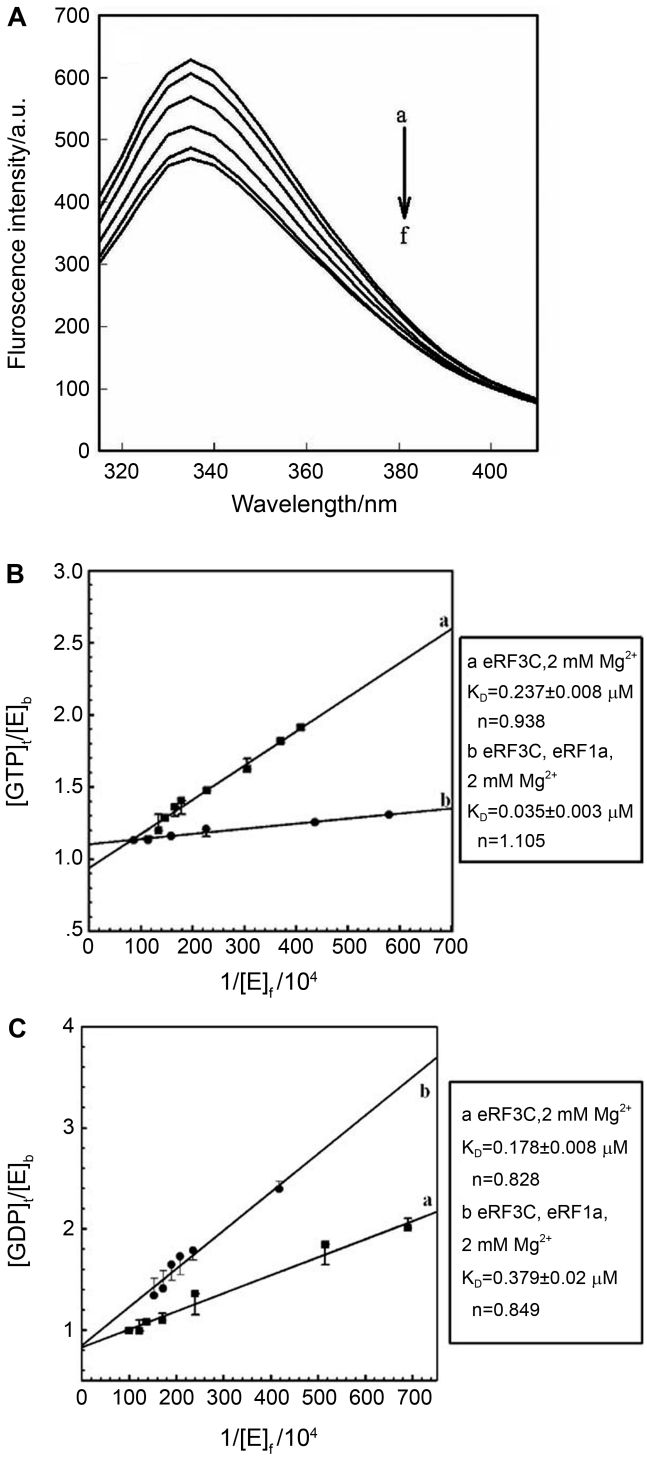

Fig. 1. Dissociation constants for eRF3C binding GTP/GDP in the presence and absence of eRF1a.

A. Fluorescence spectra of eRF3C binding GTP. (a) [GTP]/[eRF3]=0, (b) [GTP]/[eRF3]=0.2, (c) [GTP]/[eRF3]=0.4, (d) [GTP]/[eRF3]=0.6, (e) [GTP]/[eRF3]=0.9, (f) [GTP]/[eRF3]=1.1. **B**. Plot of $[GTP]_f/[E]_b$ vs. $1/[E]_f$. The dissociation constants of eRF3C and GTP in the absence and presence of eRF1a were calculated from curves (a) and (b). **C**. Plot of $[GDP]_f/[E]_b$ vs. $1/[E]_f$. The dissociation constants of eRF3C and GDP in the absence and presence of eRF1a were calculated from curves (a) and (b).

reaches saturation. The dissociation constants were determined using a plot of $[G]_f/[E]_b$ vs. $1/[E]_f$, whereas $[E]_b$ and $[E]_f$ were obtained using Eq. (2). The dissociation constants of the eRF3C–eRF1a complex bound to GTP or GDP were determined as described above. The fluorescence emission maximum of the eRF3C–eRF1a complex was also seen at 334 nm, and the maximum emission intensity decreased with the addition of GTP/GDP (data not shown). No fluorescence change was detected for eRF1a after adding GTP/GDP (data not shown). Thus, the emission intensity decrease of the eRF3C–eRF1a complex was due to the fluorescence change of tryptophan in eRF3C. As shown in Fig. 1B and 1C, eRF1a increased the affinity of eRF3C to GTP by 7-fold, while decreasing the affinity of eRF3C to GDP by 2-fold at 2 mM $MgCl_2$. The affinity of the eRF3C–eRF1a complex to GTP was about 11-times higher than that of the eRF3C–eRF1a complex to GDP at 2 mM $MgCl_2$. When compared with the human data, the affinity of eRF3 binding to GTP or GDP in *E. octocarinatus* was higher than that [K_D (eRF3-GTP)=200 μM, K_D (eRF3-eRF1-GTP)=0.7 μM, K_D (eRF3-GDP)=1.3 μM, K_D (eRF3-eRF1-GDP)=1.9 μM] of eRF3 binding to human nucleotides [7]. Furthermore, virtually no GTP binding (K_D=200 μM) was observed in the absence of eRF1 in humans, whereas a K_D of 0.237 μM was seen for eRF3 alone in *E. octocarinatus* (Fig. 1B).

Therefore, consistent with the results for humans [7, 18], eRF1a promoted the binding of GTP to eRF3, and the eRF3–eRF1a complex favored the binding of GTP over GDP at 2 mM $MgCl_2$ in *E. octocarinatus*. However, in contrast to the human data, the eRF3 in *E. octocarinatus* exhibited a higher affinity for nucleotides and was able to bind GTP by itself.

GTP Hydrolysis Assay of eRF3

All the components of the termination complex were investigated for their GTPase activity in the presence of 2 mM $MgCl_2$ (Fig. 2A). eRF3C exhibited a very low intrinsic GTPase activity. When eRF1a or ribosomes were added, the GTPase activity of eRF3C was not significantly improved [Fig. 2A, curves (b) and (d)]. However, when both eRF1a and ribosomes were added to the reaction system, the GTPase activity of eRF3C was significantly enhanced [Fig. 2A, curve (a)], indicating that eRF3 was an eRF1a- and ribosome-dependent GTPase in *E. octocarinatus*, as previously observed in *X. laevis* [5]. The kinetic parameters of eRF3C were determined from the curve in Fig. 2B. The V_{max} and K_m of eRF3C was 4.3 pmol/s and 0.5 mM, respectively, and the k_{cat} of eRF3C was 0.086/s. In contrast to the data (K_m=5 mM, k_{cat}=0.014/s) for *X. laevis* [5], the activity of eRF3 hydrolyzing GTP was higher in *E. octocarinatus*, possibly because of the higher binding affinity of eRF3 to GTP.

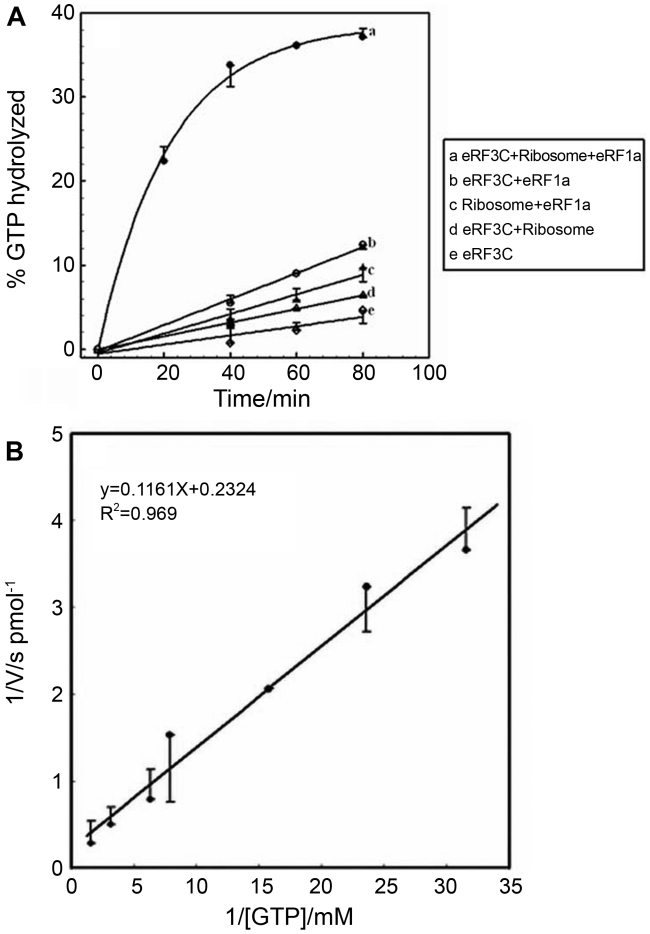

Fig. 2. GTPase activity of eRF3.
A. GTPase assay of components of the termination complex. **B**. Kinetic analysis of the GTPase activity of eRF3 in the presence of 2 mM $MgCl_2$.

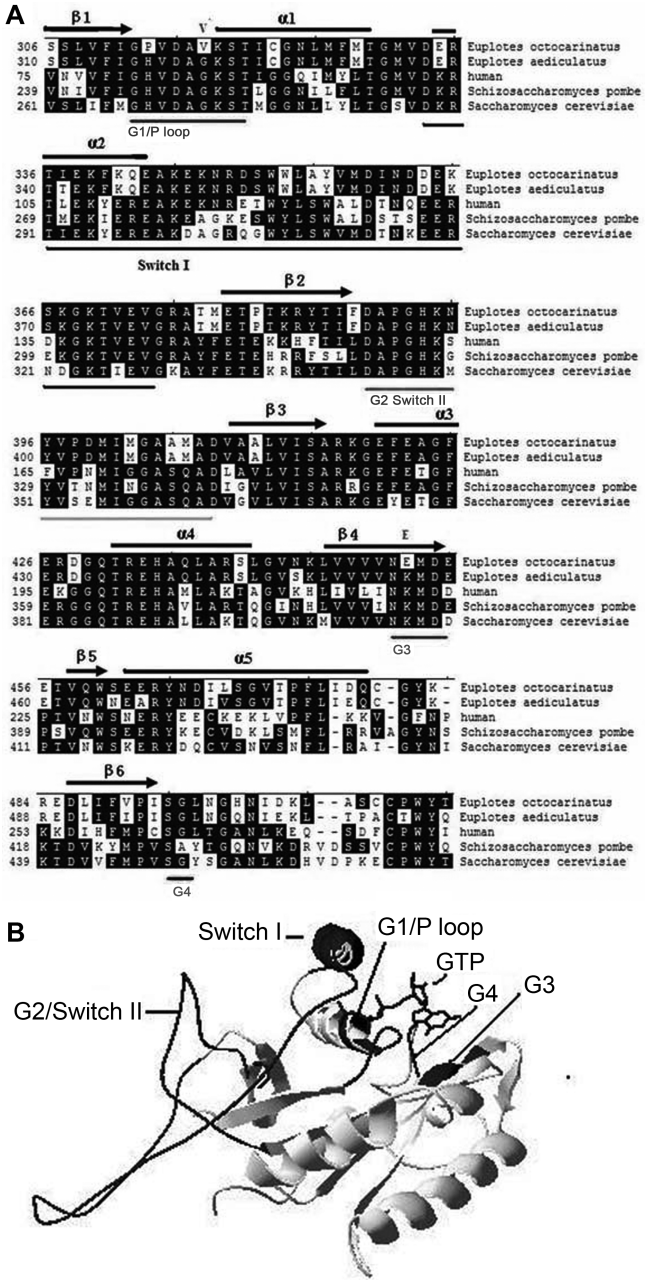

Fig. 3. Prediction of GTP binding domains in the structure of eRF3.
A. Multiple sequence alignment of *E. octocarinatus*, *E. aediculatus,* human*, S. pombe*, and *S. cerevisiae* eRF3. The invariant residues are colored in black. The secondary structure elements of the eRF3 G domain are indicated. The GTP binding motifs, and Switch I and Switch II regions are marked. **B**. Location of GTP binding domains in a structural homology model of eRF3 based on the X-ray structure of *S. pombe* eRF3 using the Swiss-Model program [23]. The GTP binding motifs, GTP, and Switch I and Switch II regions are colored in black.

Analysis of the G Domain in eRF3

When compared with the human eRF3, the eRF3 in *E. octocarinatus* exhibited a higher binding affinity for nucleotides and was able to bind with GTP by itself. Thus, to explain these observations, a search was made for potential G domains in the amino acid sequences of the eRF3 from *E. octocarinatus*, *E. aediculatus*, human, *S. pombe*, and *S. cerevisiae* (Fig. 3A), and the GTP binding domains located in the structural homology model of eRF3 based on the X-ray structure of *S. pombe* eRF3 (Fig. 3B) [13]. As a result, the GTP binding sites were located in a hydrophobic pocket (Fig. 3B), and the structures of the G domains of eRF3 were found to be similar to those in *S. pombe* eRF3. A comparison of the amino acid sequences of the potential G domains of eRF3 from the different species showed that the amino acid sequences of the four GTP binding domains were basically conserved in eRF3. The G2 and G4 sequences were DAPG and SGL, respectively, which were similar for the eRF3 from the different species (Fig. 3A). Yet, in contrast to the typical G1/P loop sequence (GxxxxGKS/T)[12], the sixth typical glycine residue was replaced with valine in eRF3 (valine317, Fig. 3A). Valine has two more methyl groups than glycine, making valine sterically larger than glycine. Furthermore, in the G3 region of eRF3,

glutamate 452 replaced the conserved lysine. Glutamate is an acidic amino acid, whereas lysine is a basic amino acid. Thus, two conserved amino acids, glycine and lysine, involved in GTP binding, were changed to valine 317 and glutamate 452, respectively, in the *E. octocarinatus* eRF3, which may explain the higher binding affinity of eRF3 to nucleotides.

Acknowledgments

The authors are very grateful to Dr. Zhuo-Yu Li for her suggestions and revisions concerning the manuscript. This work was supported by the National Natural Sciences Foundation of China (30670282) and Natural Sciences Foundation of Shanxi (2009011059-3, 2009021035-2).

REFERENCES

1. Alkalaeva, E. Z., A. V. Pisarev, L. Y. Frolova, L. L. Kisselev, and T. V. Pestova. 2006. *In vitro* reconstitution of eukaryotic translation reveals cooperativity between release factors eRF1 and eRF3. *Cell* **125:** 1125–1136.
2. Chai, B. F., W. Wang, and A. H. Liang. 2006. Expression, characterization and immunolocalization of translation termination factor eRF3 in the ciliate *Euplotes octocarinatus*. *Res. Microbiol.* **157:** 235–240.
3. Cheng, Z. H., K. Saito, A. V. Pisarev, M. Wada, V. P. Pisareva, T. V. Pestova, *et al*. 2009. Structural insights into eRF3 and stop codon recognition by eRF1. *Gene Dev.* **23:** 1106–1108.
4. Cox, R. A. and W. Hirst. 1976. A study of the influence of magnesium ions on the conformation of ribosomal ribonucleic acid and on the stability of the larger subribosomal particle of rabbit reticulocytes. *Biochem. J.* **160:** 505–519.
5. Frolova, L., L. X. Goff, G. Zhouravleva, E. Davydova, M. Philippe, and L. Kisselev. 1996. Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase. *RNA* **2:** 334–341.
6. Frolova, L. Y., J. L. Simonsen, T. I. Merkulova, D. Y. Litvinoc, P. M. Martensen, V. O. Rechinsky, J. H. Camonis, L. L. Kisselev, and J. Justesen. 1998. Functional expression of eukaryotic polypeptide chain release factors 1 and 3 by means of baculovirus/ insect cells and complex formation between the factors. *Eur. J. Biochem.* **23:** 636–644.
7. Hauryliuk, V., A. Zavialov, L. Kisselev, and M. Ehrenberg. 2006. Class-1 release factor eRF1 promotes GTP binding by class-2 release factor eRF3. *Biochimie* **88:** 747–757.
8. Inge-Vechtomov, S. G., G. Zhouravleva, and M. Philippe. 2003. Eukaryotic release factors (eRFs) history. *Biol. Cell* **95:** 195–209.
9. Karamyshev, A. L., K. Ito, and Y. Nakamura. 1999. Polypeptide release factor eRF1 from *Tetrahymena thermophila*: cDNA cloning, purification and complex formation with yeast eRF3. *FEBS Lett.* **457:** 483–488.
10. Kervestin, S., L. Frolova, L. Kisselev, and O. Jean-Jean. 2001. Stop codon recognition in ciliates: *Euplotes* release factor does not respond to reassigned UGA codon. *EMBO Rep.* **2:** 680–684.
11. Kisselev, L., M. Ehrenberg, and L. Frolova. 2003. Termination of translation: Interplay of mRNA, rRNAs and release factors? *EMBO J.* **22:** 175–182.
12. Kjeldgaard, M., J. Nyborg, and B. F. Clark. 1996. The GTP binding motif: Variations on a theme. *FASEB J.* **10:** 1347–1368.
13. Kong, C., K. Ito, M. A. Walsh, M. Wada, Y. Liu, S. Kumar, D. Barford, Y. Nakamura, and H. Song. 2004. Crystal structure and functional analysis of the eukaryotic class II release factor eRF3 from *S. pombe*. *Mol. Cell* **14:** 233–245.
14. Kononenko, A. V., V. A. Mitkevich, V. I. Dubovaya, P. M. Kolosov, A. A. Makarov, and L. L. Kisselev. 2008. Role of the individual domains of translation termination factor eRF1 in GTP binding to eRF3. *Proteins* **70:** 388–393.
15. Liang, A. H., C. Brunen-Nieweler, T. Muramatsu, Y. Kuchino, H. Beier, and K. Heckmann. 2001. The ciliate *Euplotes octocarinatus* expresses two polypeptide release factors of the type eRF1. *Gene* **262:** 161–168.
16. Lozupone, C. A., R. D. Knight, and L. F. Landweber. 2001. The molecular basis of nuclear genetic code change in ciliates. *Curr. Biol*. **23:** 65–74.
17. Meyer, F., H. J. Schmidt, E. Plumper, A. Hasilik, G. Mersmann, H. E. Meyer, A. Engstrom, and K. Hechmann. 1991. UGA is translated as cysteine in pheromone 3 of *Euplotes octocarinatus*. *Proc. Natl. Acad. Sci. U.S.A.* **88:** 3758–3761.
18. Mitkevich, V. A., A. V. Kononenko, I. Y. Petrushanko, D. V. Yanvarev, A. A. Makarov, and L. Kisselev. 2006. Termination of translation in eukaryotes is mediated by the quaternary eRF1·eRF3·GTP·Mg^{2+} complex. The biological roles of eRF3 and prokaryotic RF3 are profoundly distinct. *Nucleic Acids Res.* **34:** 3947–3954.
19. Nakamura, Y. and K. Ito. 2003. Making sense of mimic in translation termination. *Trends Biochem. Sci.* **28:** 99–105.
20. Pace, C. N., F. Vajdos, L. Fee, G. Grimsley, and T. Gray. 1995. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4:** 2411–2423.
21. Pisareva, V. P., A. V. Pisarev, C. U. Hellen, M. V. Rodnina, and T. V. Pestova. 2006. Kinetic analysis of interaction of eukaryotic release factor 3 with guanine nucleotides. *J. Biol. Chem.* **281:** 40224–40235.
22. Salas-Marco, J. and D. M. Bedwell. 2004. GTP hydrolysis by eRF3 facilitates stop codon decoding during eukaryotic translation termination. *Mol. Cell Biol*. **24:** 7769–7778.
23. Schwede, T., J. Kopp, N. Guex, and M. C. Peitsch. 2003. SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res*. **31:** 3381–3385.
24. Song, L., B. F. Chai, W. Wang, and A. H. Liang. 2006. Identification of translational release factor eRF1a binding sites on eRF3 in *Euplotes octocarinatus*. *Res. Microbiol.* **157:** 842–850.
25. Song, L., Y. Y. Wang, B. F. Chai, W. Wang, and A. H. Liang. 2007. C-Terminal 76 amino acids of eRF3 are not required for the binding of release factor eRF1a from *Euplotes octocarinatus*. *J. Genet. Genomics* **34:** 1–5.
26. Sun, Q. H., B. F. Chai, and A. H. Liang. 2002. Cloning and sequence of eRF3 gene from *Euplotes octocarinatus*. *Chin. J. Biochem. Mol. Biol.* **18:** 347–351.
27. Zhouravleva, G., L. Frolova, L. X. Goff, L. R. Guellec, S. Inge-Vechtomov, L. Kisselev, and M. Philippe. 1995. Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. *EMBO J.* **14:** 4065–4072.