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Expression, characterization and immunolocalization of translation termination factor eRF3 in the ciliate *Euplotes octocarinatus*

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Abstract

Class II polypeptide release factor eRF3 stimulates translation termination in a GTP-dependent manner. Recent data show that eRF3 is also involved in cell cycle regulation apart from its function in translation. Here we show that recombinant *Eo-eRF3* from the ciliate *Euplotes octocarinatus* expressed in *Escherichia coli* can be tested for its ribosome-dependent GTPase activity using an in vitro system. Moreover, polyclonal antibodies against recombinant Eo-eRF3 were used to determine the localization of Eo-eRF3 using immunofluorescence and immunoelectron microscopy, which showed that the Eo-eRF3 is distributed not only around the macronucleus, but also in basal bodies in the cortex of *E. octocarinatus* cells. The results suggest that Eo-eRF3 may be involved in cytoskeleton organization in addition to its function in translation termination.
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Keywords: *Euplotes octocarinatus*; Polypeptide release factor 3; GTPase activity; Immunolocalization; Cytoskeleton

1. Introduction

In eukaryotes, two factors responsible for translation termination have been characterized: class I polypeptide release factor eRF1, which recognizes stop codons UAA, UAG and UGA at the A site of the ribosome, inducing peptidyl-tRNA hydrolysis at the peptidyl transferase center; and class II polypeptide release factor, eRF3, which stimulates translation termination in a GTP-dependent manner [6,19].

eRF3 has a complex domain structure in which only its C-terminal part is responsible for termination of translation. This domain is evolutionarily conserved and shows considerable sequence similarity to the translation elongation factor eEF1A, which transports aminoacyl-tRNAs to the ribosomal A site [11]. Recently, some evidence indicates that eRF1 and eRF3 are multifunctional proteins possibly involved in cytoskeleton organization in addition to translation termination in cells [9]. In the yeast, depletion of eRF3 may cause sensitivity of cells to the microtubule-poisoning drug benomyl and a defect in chromosome segregation at anaphase [3]. It has been shown that mutation of eRF3 has various effects on yeast cell morphology, such as the accumulation of large-budded cells with defects in the actin cytoskeleton, mitotic spindle fibers and nuclear division and cell segregation [4,18].

In contrast to other higher eukaryotes, ciliates, among unicellular protozoa, have reassigned stop codons as sense codons in the course of evolution. For example, in *Euplotes,* UGA encodes cysteine in addition to the conventional cysteine codons [13], while UAA is used as the main stop codon, whereas UAG is only rarely found. To understand the origins of release factors, class II release factor eRF3 was cloned from ciliates *Euplotes aediculatus* by Inagaki (2000) [8]. The relation and origins of the factors related to protein synthesis were elucidated. ERF3 is related to elongation factor eEF1A. To understand the various functions of eRF3 in evolution, we cloned the *Eo-eRF3* gene from the ciliate *E. octocarinatus*, which has been assigned to a special phylogenic position similar to *E. aediculatus* [14]. Here we report the function of Eo-eRF3, which was assayed by establishing an in vitro system for GTPase activity. Immunofluorescent staining of Eo-eRF3 in *E. octocarinatus* cells was performed using polyclonal antibodies against recombinant Eo-eRF3.

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Table 1
PCR primers used to generate mutant *Eo-eRF3*

Name	Primer with mutant base	Mutant site
PeRF3M1.1	5′-CATTTTTGATTGATCAATG[T]GGA-3′	1440
PeRF3M1.2	5′-GAGTTACTCCGCTGAGAATGTC-3′	
PeRF3M2.1	5′-GCATCTTGTTG[T]CCATGGTATA-3′	1521
PeRF3M2.2	5′-CAATTTATCAATGTTATGCCCA-3′	
PeRF3M3.1	5′-TACTAAATG[T]CAAGTAGTTGGAATA-3′	1716
PeRF3M3.2	5′-CATAACGGCAAGTTTAGGTCCAATT-3′	

Note. Letters in boxes indicated the transition from TGA to TGT (detailed in the text).

2. Materials and methods

2.1. Amplification of the Eo-eRF3 *gene from macronuclear DNA and mutagenesis of in-frame nonsense codons*

Euplotes macronuclear DNA was isolated as described previously [12]. The *Eo-eRF3* gene was amplified from macronuclear DNA using primers PeRF3Bam 5′-GTCAC*GGATCC*-GAATGGACAAGAACTCAGAT-3′ and PeRF3Xho 5′-CTG-CA*CTCGAG*TTACTCTGCGTTTTCACTTT-3′. The *Bam*HI site and the *Xho*I site created for inserting the *Eo-eRF3* gene into the expression vector pET-28c (Novagen) are underlined. Amplified products were cloned into pGEM-T Easy (Promega) to generate the plasmid pGEM-eRF3 for mutagenesis of the in-frame nonsense codons. Mutagenesis was mediated by site-directed PCR procedures described in the manual of the MutanBEST Kit (TaKaRa). For mutagenesis of the in-frame nonsense codons, the following primers were synthesized as shown in Table 1.

2.2. Expression and purification of the Eo-eRF3 protein in E. coli

Expression plasmid pET-eRF3 was generated by subcloning the *Eo-eRF3* gene from pGEM-eRF3 into the expression plasmid pET-28c using the restriction enzyme sites *Bam*HI and *Xho*I within the end of the target gene. Eo-eRF3 was overexpressed using the *E. coli* expression strain BL21(DE3). After induction with isopropyl-β-thiogalatopyranoside (IPTG) (0.4 mM final concentration), cells were grown for an additional 6 h at 25 °C, harvested by centrifugation and resuspended in 20 ml lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonylfluoride (PMSF), 2 mM β-mercaptoethanol 0.1% NP-40, pepstatin (0.0073 mg/ml), leupeptin (0.031 mM), benzamidine (146 mM), and aprotinin (0.004 mM), and the cell lysates prepared by low speed centrifugation. The fusion protein His_6-Eo-eRF3 was purified by affinity chromatography using the Ni-NTA agarose (Qiagen) column according to the manufacturer's instruction with modifications. Washing buffer and elution buffer were lysis buffer complemented with imidazole 100 mM and 1 M final concentrations, respectively. The SDS–PAGE of protein analysis was stained with Coomassie brilliant blue G250.

2.3. Assay for GTPase activity of Eo-eRF3

GTPase activity of Eo-eRF3 was assayed by accumulation of [^{32}P]Pi on polyethyleneimine-cellulose coated plates (Macherey-Nagel) after hydrolysis of [γ^{-32}P]GTP (Fu rei Bio-Eng. Beijing) using thin-layer chromatography as described [7]. Rabbit reticulocyte ribosome subunits were isolated using New Zealand rabbits as described [5]. The incubation mixture of 15 μl contained 7.5 μM [γ^{-32}P]GTP (5000 cpm/pmol) and 0.1 μM rabbit ribosomes, 0.4 μg eRF1 (from *Blepharisma japonicum* provided by Dr. Wang), 0.4 μg Eo-eRF3, and 0.4 μg Eo-eRF3C (provided by Dr. Sun), which were a GST fusion C-terminal domain (from 565aa to 799aa) of Eo-eRF3. Eo-eRF3C was expressed in *E. coli* and purified using affinity chromatography. The reaction was stopped with 1 μl 20 mM EDTA with 5% SDS. 5-μl aliquots were spotted onto polyethyleneimine-cellulose coated plates and resolved in 1 M acetic acid and 1 M LiCl for 45 min. The plates were dried and exposed to Koda X-Omat K film (Koda, Guangdong).

2.4. Preparation and analysis of rat anti-Eo-eRF3 antibodies

Rat anti-Eo-eRF3 polyclonal antibodies were generated against His_6-Eo-eRF3 expressed in *E. coli* and affinity-purified as described [1]. Titer analysis of polyclonal antibodies was performed by ELISA as described [1]. The antiserum obtained was used to perform the ELISA assay, which indicated that the titer of polyclonal antibodies was higher than 6000 ($P/N \geqslant 2.5$). Antibodies were purified by the PVDF membrane (Millipor, Germany) binding to the antigen Eo-eRF3. To characterize this antibody preparation, western blots were performed using standard procedures [16] with a 1:500 dilution of the affinity-purified antiserum. Bound secondary antibodies conjugated with HRP (horseradish peroxidase) were detected by DAB (diaminobenzidine) following the instructions of the manufacturer (Zhongshan, Beijing).

2.5. Immunofluorescent staining of Eo-eRF3 in E. octocarinatus *cells*

Samples of 50–80 cells were handled in depression slides with a micropipette, under a dissecting microscope. The cells were permeabilized by treating the living cells with PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM ethylene glycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA),

2 mM $MgCl_2$) containing 0.5% Triton X-100 for 7 min. After 1 h fixation at room temperature in 2% paraformaldehyde in PHEM, cells were washed three times in a modified TBST buffer (10 mM Tris–HCl (pH 7.4), 0.15 mM NaCl, 0.01% Tween-20, 10 mM EGTA, 2 mM $MgCl_2$, 3% bovine serum albumin). Then the cells were incubated for 1 h at 28 °C or overnight at 4 °C with the anti-Eo-eRF3 antibodies (diluted 1:200 to 1:400 in TBST), washed three times in TBST, and incubated for 1 h with anti-rat immunoglobulins labeled with fluorescein isothiocyanate (FITC). After three additional washes in TBST (the last one containing 0.1 μg/ml Hoechst 33258 (Sigma)), cells were mounted in a mounting agent (4% *n*-propylgalate, 70% glycerol in PBS), and observed under an Olympus epifluorescence photomicroscope with photographs using Konoca Monochrome VX400 film.

2.6. *Preparation of* Euplotes *cells, ultrathin sectioning and immunoelectron microscopy*

Cells were enriched in a glass groove with a micropipette under a dissecting microscope. The enriched cell solutions were washed with PBS to remove impurity. Cells were fixed in 2% (w/v) paraformaldehyde containing 0.5% (v/v) glutaraldehyde for 1 h at 4 °C. The fixed samples were then rinsed with PBS buffer (pH 7.4) and gradually dehydrated in mixtures of distilled water and ethanol in a series of steps of increasing ethanol concentration. Before dehydration in 100% acetone, cells were dehydrated in a mixture of 90% ethanol and 90% acetone at a proportion of 1:1 for 10 min. Then, cells were transferred into 100% acetone for 10 min and washed three times. The dehydrated cells were prepenetrated in a mixture of resin and acetone at a proportion of 1:1 at 35 °C for 24 h and finally transferred into resin to penetrate at 35 °C for 24 h. The penetrated cells were transferred into a capsule to be embedded in resin at 55 °C for 48 h. After polymerization, the samples were cut with a diamond knife on an ultramicrotome and the ultrathin sections (about 500 nm in thickness) were collected on gold grids. Section staining was performed using floating grids, sections down, on droplets of the immunolabeling and washing solutions placed on strips of parafilm, taking care not to wet the reverse side of the grid or to let the sections dry. After each incubation, grids were washed by successive flotations on fresh droplets of buffer, and the excess reagent or buffer was removed with pieces of filter paper. All steps of the immunolabeling procedure were carried out at temperature as follows. The sections were etched in 10% H^2O^2 for 15 min, then thoroughly washed with PBS. Blocking was performed in 1% (w/v) BSA in PBS for 5 min. The sections were then incubated with 1:100 rat anti-Eo-eRF3 IgG for 2 h at room temperature. The samples were then washed three times in blocking buffer and incubated for 1 h with 1:30 dilution of anti-rat IgG gold conjugate in blocking buffer. The final immunolabeling step was followed by three washes of 5 min each of PBS. At the end, the grids were washed in distilled water, air-dried, and stained with lead citrate and uranyl acetate, and observed under a JEOL-1230 electron microscope.

3. Results and discussion

3.1. *Amplification of the* Eo-eRF3 *gene from macronuclear DNA and mutagenesis of the in-frame nonsense codons*

Using primers PeRF3Bam and PeRF3Xho (see experimental procedures), we amplified the Eo-eRF3 coding region from the macronuclear DNA of *E. octocarinatus*. The gene fragment was cloned into pGEM-T Easy vector resulting in plasmid pGEM-eRF3. Sequence analysis of the construct indicated that the *Eo-eRF3* gene contains a 2400-bp open reading frame (ORF), which encodes a deduced polypeptide of 799 amino acids [AF440195]. Three UGA-encoded cysteines exist in the ORF of Eo-eRF3 (positions 480, 507, 572). This is consistent with earlier reports that UGA codes for cysteine in *Euplotes* [3]. To express the Eo-eRF3 in *E. coli*, the three UGA codons were mutated into UGU encoding cysteines in *E. coli* using PCR-mediated site-directed mutagenesis procedures. Using primer pairs with altered sequences and the plasmid pGEM-eRF3 as templates, mutant DNA fragments were blunted and ligated into circles. Results of sequence analysis showed that the TGA codon in *Eo-eRF3* was mutated into TGT at position no. 480 in the deduced amino acid sequence in mutant products. Likewise, the other two in-frame TGA codons at amino acid positions of 507 and 572 were also mutated to TGT.

3.2. *Expression and purification of Eo-eRF3*

The mutant gene in plasmid pGEM-eRF3 was digested with *Bam*HI and *Xho*I restriction endonucleases and subcloned into expression vector pET-28c to generate plasmid pET-eRF3. The fusion protein His_6-Eo-eRF3 was expressed in *E. coli* BL21(DE3) and purified by affinity chromatography using an Ni-NTA agarose column (Fig. 4). The molecular mass of the recombinant protein product was close to 97 kDa, as expected from its deduced amino acid sequence. The percentage of recombinant protein as a fraction of total *E. coli* protein extracts reached 35% and the recombinant protein was more than 90% pure, as assessed by SDS–PAGE analysis (Fig. 1). 3.0–5.0 mg purified recombinant protein was isolated from bacterial cells grown in 200 ml. Recombinant Eo-eRF3 displayed limited proteolysis during cell lysis and purification as shown by western blot analysis. In an attempt to eliminate or reduce the proteolysis of Eo-eRF3, culture conditions were changed and a mixture of protease inhibitors was used. However, none of these alterations reduced the degradation products of Eo-eRF3 as visualized by western blotting (Fig. 2).

Purified Eo-eRF3 exhibited GTPase activity, which was eRF1- and ribosome-dependent (Fig. 3). The domain of GTPase activity, which is an eEF-1a-like domain, is located in the central region of Eo-eRF3, at position 342–556 on the deduced protein sequence [17]. The Eo-eRF3C, which is the C-terminal domain of Eo-eRF3, did not exhibit GTPase activity. This domain is responsible for the interaction with eRF1 to form a functional complex in the process of translation termination.

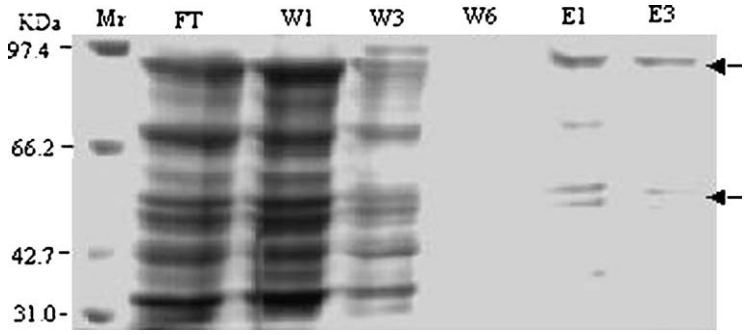

Fig. 1. Analysis of affinity purification of Eo-eRF3 by Ni-NTA agarose. SDS–PAGE analysis of flow-through (FT) protein extracts after six washes with 10 ml washing buffer (W1–W6) and three fractions eluted with 5 ml elution buffer (E1–E3). Protein markers are given in kDa. Gel was stained with Coomassie brilliant blue.

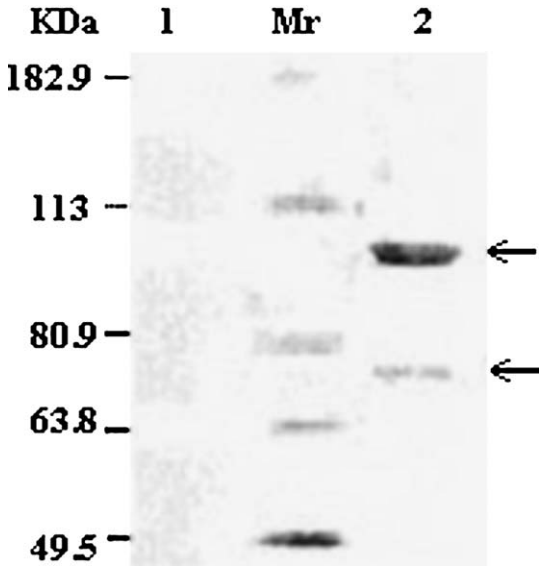

Fig. 2. Western blot analysis of purified His_6-Eo-eRF3 probed by goat anti-His antibodies. Lane 1: cell lysate of *E. coli* BL21(DE3) as negative control; lane 2: putative fusion protein His_6-Eo-eRF3. Prestained protein marker is shown in kDa.

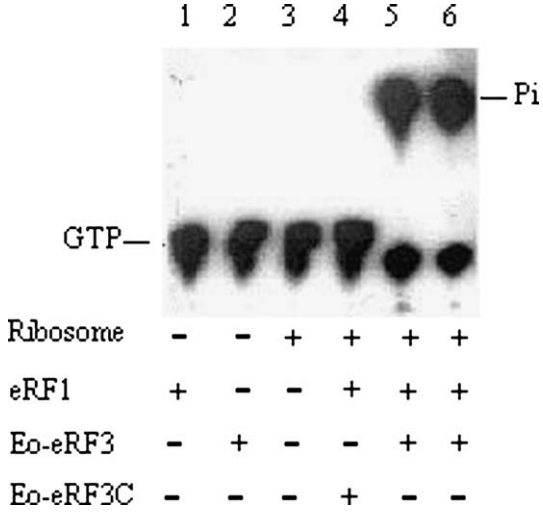

Fig. 3. GTPase activity analysis of Eo-eRF3 and Eo-eRF3C by thin-layer chromatography on polyethleneimine-cellulose. Lanes 1–4 show that the C-terminus of Eo-eRF3 has no GTPase activity. Lanes 5–6 show that the full length of Eo-eRF3 possesses eRF1 and ribosome-dependent GTPase activity.

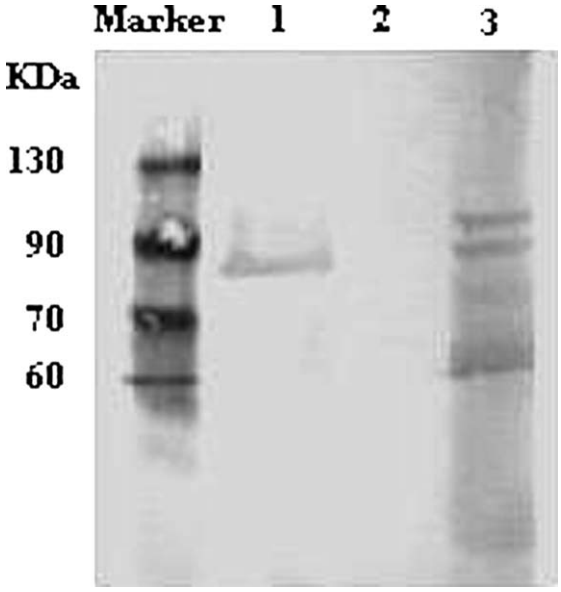

Fig. 4. Western blotting analysis of Eo-eRF3 from an extract of *Euplotes* probed with antibodies of Eo-eRF3. Lane 1: cell extract from *Euplotes*; lane 2: cell lysate of *E. coli* BL21 as negative control; lane 3: purified Eo-eRF3.

3.3. Preparation and analysis of anti-Eo-eRF3 antibodies

Rat polyclonal antibodies were prepared using purified recombinant Eo-eRF3. The purified anti-Eo-eRF3 antibodies were used to probe western blots of *Euplotes* protein extracts shown in Fig. 4. These antibodies detected only a single band with an apparent molecular weight of approximately 90 kDa. This is close to the molecular weight expected from the deduced amino acid sequence, which predicts a size of 91.4 kDa. The protein extracts utilized in this western blot were from *E. octocarinatus*, which were starved for 10 days to exclude the effect of food material inside the cell.

3.4. Cellular localization of Eo-eRF3

The results of ELISA and western blot analysis showed that purified antibodies are specific for recognizing Eo-eRF3 epitopes. On western blots containing bacterial and *Euplotes* protein extracts, the antibodies recognize exclusively recombinant

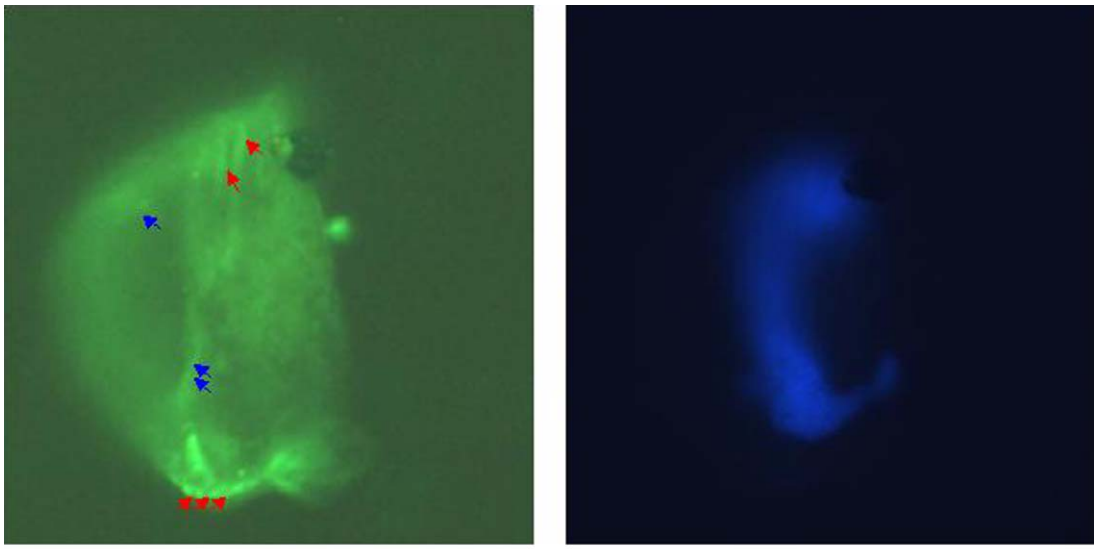

Fig. 5. Immunofluorescent localization of Eo-eRF3 in *Euplotes*. A shows immunofluorescent staining of Eo-eRF3 around the macronucleus (blue arrows) and basal bodies (red arrows); B shows the corresponding macronucleus stained with Hoechst 33258. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

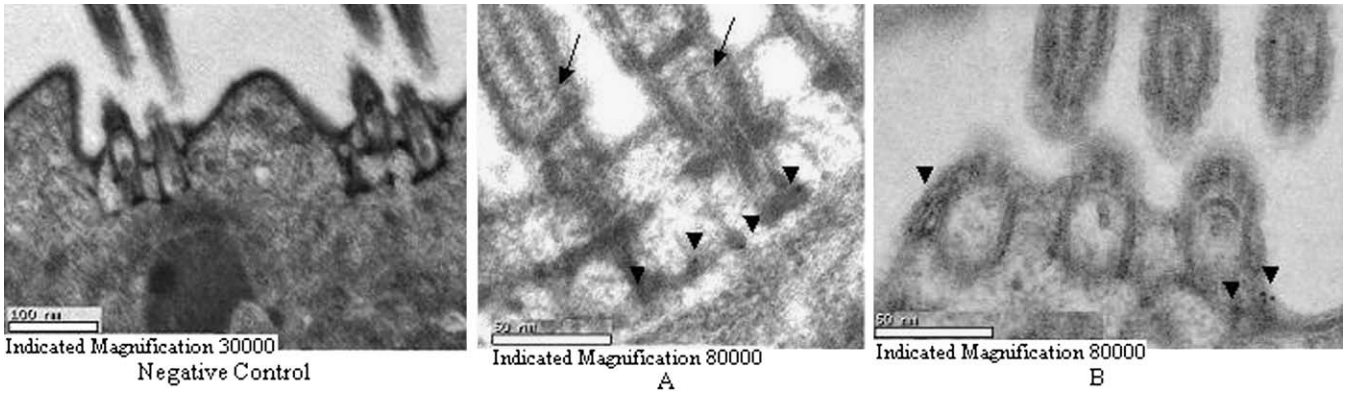

Fig. 6. Colloidal gold immunoelectron microscopy of ultrathin sections in the cortex of *E. octocarinatus*. PBS was used for first antibodies to substitute for eRF3 antibodies in control experiments. Micrographs A and B show the localization of eRF3 in basal bodies (arrows heads). Arrow shows cilia of *Euplotes*.

fusion proteins containing His_6-Eo-eRF3 (Fig. 4), and a single band in *Euplotes* cell extracts.

Immunolocalization of Eo-eRF3 revealed intense staining around the macronuclear chromosome and in the basal bodies of *Euplotes* (Fig. 5). The control using pre-serum as first antibodies did not reveal staining in cells (not shown). Like other ciliates, *E. octocarinatus* possesses two types of nuclei that differ in structure and function. The micronucleus is transcriptionally silent during growth and asexual reproduction, but gives rise to a macronucleus in sexual reproduction, which is transcriptionally active. Expressed mRNA is used for protein translation immediately around the macronuclear chromosome [10,15]. Considering the function of eRF3, we can presume that the observed foci around the macronuclear chromosome were representative of Eo-eRF3 distribution. It is expected that a protein that functions as a translation factor is widely dispersed in the cell, especially around the macronucleus.

In order to confirm the localization of eRF3 in basal bodies of *E. octocarinatus*, we performed a subcellular localization using immunoelectron microscopy as shown in Fig. 6. In accordance with our results of immunofluorescence localization, eRF3 was also localized specifically within basal bodies.

We sought to determine the significance of anti-Eo-eRF3-stained foci in the cortex (basal bodies). Recently, some observations indicated that release factors (eRF1 and eRF3 in eukaryotes) are related to the formation and organization of the cytoskeleton [2,4,13]. Basal bodies are located in the cortex of ciliates, where α- and β-tubulin assemble in basal bodies to form the cilium. Cilia are an important "organ" responsible for the movement and prey behavior of ciliates. At present, we cannot identify the specific function of Eo-eRF3 in basal bodies. Co-localization of a release factor with basal bodies could indicate involvement in the organization and activity of the cilium. This association may provide important clues to the functional role of this protein during organization of the cytoskeleton.

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