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Stop codon recognition in ciliates: *Euplotes* release factor does not respond to reassigned UGA codon

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In eukaryotes, the polypeptide release factor 1 (eRF1) is involved in translation termination at all three stop codons. However, the mechanism for decoding stop codons remains unknown. A direct interaction of eRF1 with the stop codons has been postulated. Recent studies focus on eRF1 from ciliates in which some stop codons are reassigned to sense codons. Using an *in vitro* assay based on mammalian ribosomes, we show that eRF1 from the ciliate *Euplotes aediculatus* responds to UAA and UAG as stop codons and lacks the capacity to decipher the UGA codon, which encodes cysteine in this organism. This result strongly suggests that in ciliates with variant genetic codes eRF1 does not recognize the reassigned codons. Recent hypotheses describing stop codon discrimination by eRF1 are not fully consistent with the set of eRF1 sequences available so far and require direct experimental testing.

INTRODUCTION

Termination of protein synthesis is governed by the presence of a stop codon in the ribosomal A site and by polypeptide chain release factors (RFs) (reviewed by Kisselev and Buckingham, 2000). In eukaryotes, a single factor, eRF1, decodes all three stop codons, UAA, UAG and UGA, whereas in prokaryotes, RF1 responds to UAA and UAG while RF2 responds to UAA and UGA.

None of the hypotheses postulating the mechanism of decoding the termination codons has been proved directly. It is assumed that stop codons within the ribosome are recognized by class-1 termination factors RF1, RF2 and eRF1 (see Nakamura *et al.*, 2000). The main argument is the very tight contact between class-1 RFs and stop codons within the ribosome, revealed by photocrosslinking both in prokaryotes (Brown and Tate, 1994; Poole *et al.*, 1997) and eukaryotes (Chavatte *et al.*, 2001). Another argument came from experiments showing that mutagenesis of class-1 RF sequences resulted in the modification of their stop codon recognition pattern (Bertram *et al.*, 2000; Ito *et al.*, 2000). Alternatively, it was proposed that stop codons can be recognized by specific sequences in ribosomal RNAs (see Arkov and Murgola, 1999; Ivanov *et al.*, 2001).

A remarkable feature of some ciliate species is their use of alternative nuclear genetic codes, which have possibly arisen independently, even within a single class of ciliates (Baroin-Tourancheau *et al.*, 1995). The known changes concern the reassignment of stop codons to sense codons. For example, *Tetrahymena* and *Paramecium*, and the hypotrichs *Stylonychia* and *Oxytricha*, translate UAA and UAG as glutamine, UGA being the only stop codon, whereas the hypotrich *Euplotes* translates UGA as cysteine and uses UAA and UAG as stop codons (for review see Lozupone *et al.*, 2001). It has been postulated that in addition to changes in tRNAs, stop codon reassignment has to involve alterations of eRF1 structure. Substantial efforts were undertaken to sequence *eRF1* genes from ciliate species with variant genetic codes (Karamyshev *et al.*, 1999; Inagaki and Doolittle, 2001; Liang *et al.*, 2001; Lozupone *et al.*, 2001). In conjunction with the hypothesis that the N-terminal domain of eRF1 was implicated in stop codon recognition (Bertram *et al.*, 2000), multiple sequence alignments were analyzed in an attempt to predict which amino acids of eRF1 were involved in stop codon recognition. However, depending on the number of eRF1 sequences used, different sets of amino acid residues of the N-terminal domain were chosen (Lehman, 2001; Lozupone *et al.*, 2001; Muramatsu *et al.*, 2001).

In this study, we verified the assumption that eRF1 from a ciliate with variant genetic code does not recognize the reassigned stop codon. Our results show that *Euplotes* eRF1 does

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not respond to UGA, which is used as a cysteine codon in this organism. We also show that introduction of new eRF1 sequences into eRF1 alignment questioned most of the recent hypotheses concerning stop codon recognition in eukaryotes.

RESULTS

Isolation of the *Euplotes aediculatus* gene encoding eRF1

To isolate the gene encoding eRF1, we have screened a pUC18 library of *E. aediculatus* macronuclear DNA using *Paramecium tetraurelia eRF1* gene as a probe. Four positive colonies were selected after three rounds of isolation and purification. Plasmid DNA of these clones was analyzed by Southern blotting and two of the inserts giving a strong positive signal were sequenced. The two sequences were identical and contained 5′ and 3′ telomeric 5′-dC_4dA_4-3′ repeats, confirming the presence of an entire transcription unit. The sequence was identified through BLAST searches (Altschul and Koonin, 1998). The results of BLASTX (e-value >10^{-100} with eRF1s) suggested that the selected plasmids contained a gene encoding eRF1. The sequence was analyzed using the results of BLASTX. A variant genetic code in which UGA encodes cysteine was used. The positions of ATG start and TAA stop codons were assigned based on the optimal amino acid sequence alignment and the maintenance of open reading frame (ORF); the sequence was free of introns. The coding sequence was 1314 bp long and encoded a 437-amino-acid protein with a calculated molecular mass of 49 109 Da. Four UGAs encoding cysteines presented in the ORF were mutagenized to UGC and the resulting DNA was cloned into pET21. When this work was completed, four *Euplotes* eRF1 genes were sequenced. The two eRF1s from *E. aediculatus* (Inagaki and Doolittle, 2001) shared 100 and 83.5% of amino acid identity with our sequence, and the two eRF1s from *Euplotes octocarinutus* (Liang *et al.*, 2001) shared 90.6 and 78.7% of identity with our sequence. Conversely to *E. octocarinatus*, two *eRF1* genes from *E. aediculatus* contain UGA encoding cysteine in their ORFs.

Release activity of *Euplotes* eRF1 *in vitro*

The release activity of the purified human and *Euplotes* eRF1 (*Eu*-eRF1) was measured with the three stop codons and the near-cognate tryptophan UGG codon in an *in vitro* RF assay. As known from a previous study (Frolova *et al.*, 1994), human eRF1 in the given assay system responded to the three stop codons (Table I). However, under the same conditions, *Eu*-eRF1 responded only to UAA and UAG, but not to UGA, which encodes cysteine in *Euplotes*. No activity was observed with the sense UGG codon, with both factors (Table I) showing the maintenance of discriminating capacity of *Eu*-eRF1 and human eRF1 toward near-cognate codon. As in the case of vertebrate eRF1s (Frolova *et al.*, 1994; Zhouravleva *et al.*, 1995), *Eu*-eRF1 was active without eRF3 and GTP, confirming the conclusion that these components are not implicated in the hydrolysis of peptidyl-tRNA. With rabbit ribosomes, the release activity of *Eu*-eRF1 was slightly lower than that of human eRF1 (Table I). This insignificant difference could be associated with the use of a heterologous system in which the fitness of *Eu*-eRF1 for the mammalian ribosome could be not entirely perfect.

Table I. Release activity of *Eu*-eRF1 and human eRF1 in an *in vitro* RF assay

eRF1	f[^{35}S]Met released, c.p.m.			
	UGAA	UAGA	UAAA	UGGA
Exp.1 Human	5590	4640	5120	0
Euplotes	0	3050	4030	0
Exp.2 Human	9440	6420	7920	0
Euplotes	0	3200	4580	0

The amount of f[^{35}S]Met released in the absence of tetraplet (background 500–800 c.p.m.) was subtracted from all values. Experiments 1 and 2 were performed with different f[^{35}S]Met-tRNA preparations. In each of these experiments an average from three independent measurements is presented. The standard deviation of the measurements was 11%.

DISCUSSION

The human and frog eRF1s recognize all three stop codons and discriminate them from the near-cognate UGG codon without other factors and GTP in an *in vitro* translation termination system with rabbit reassociated ribosomal subunits (Frolova *et al.*, 1994). However, there are no data available so far concerning the stop codon specificity of eRF1s from organisms with variant genetic codes. In these organisms, reassignment of a stop codon to a sense codon is governed either solely by a suppressor-like tRNA harboring the cognate anticodon, or by the concomitant presence of a cognate tRNA and a modified eRF1 that has lost its ability to recognize the reassigned stop codon, or even by the ribosome on its own. In the former case, due to the competition between suppressor tRNAs able to decipher stop codon and eRF1 (Drugeon *et al.*, 1997; Le Goff *et al.*, 1997), the synthesis of full-length proteins requires a high abundance of the suppressor tRNA and reduced amounts of eRF1. In the latter, one of the components of the ribosome must be involved in stop codon recognition.

To distinguish between these possibilities we have combined *in vitro* mammalian ribosomes with eRF1 from *E. aediculatus* in which UGA encodes cysteine and only UAA and UAG remain as termination signals. Thus, we addressed the question of whether two or three codons will be decoded in this heterologous system. The results of Table I show that UGA is not decoded as a stop codon by *Eu*-eRF1 in this system, demonstrating that *Euplotes* eRF1 has lost its ability to respond to the reassigned UGA and that reassignment of UGA is not mediated by tRNA competing with eRF1 within the ribosome. Moreover, these results also prove that the stop codon specificity is disclosed by the termination factor and not by the ribosome itself or ribosomal components. The ability of *Eu*-eRF1 to function in mammalian ribosome implies that in spite of large sequence divergence between eRF1s from organisms with canonical and variant genetic codes, the ribosome binding sites of eRF1s are well conserved and allow cross-reactivity of ribosomes and factors from evolutionarily distant species.

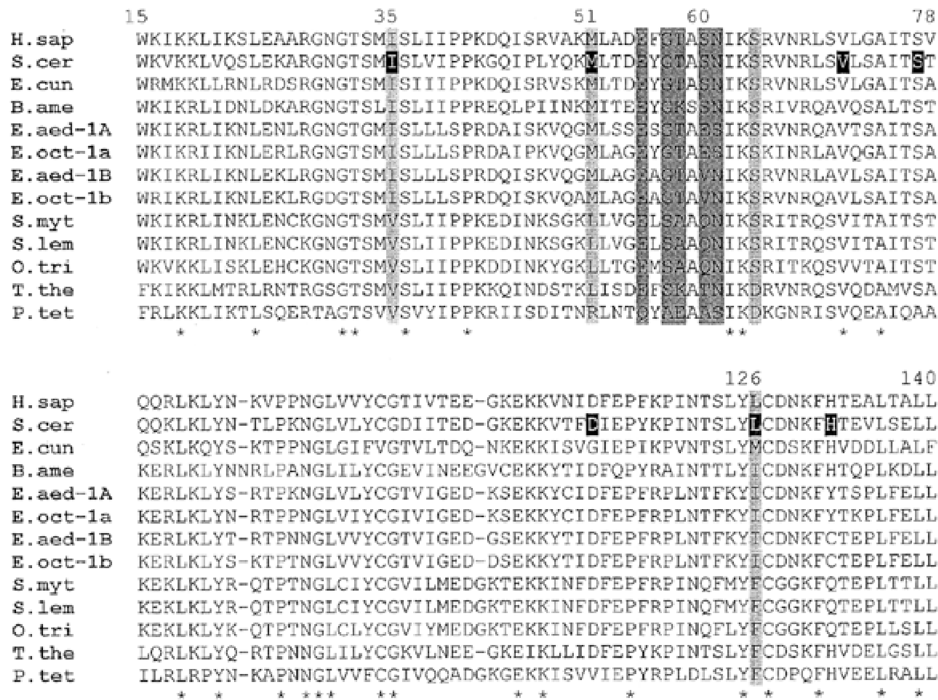

Fig. 1. Alignment of the N-terminal domain of eRF1 from human, *S. cerevisiae*, *Encephalitozoon cuniculi* and various ciliate species. Alignment has been performed using the CLUSTAL_W program (Thompson *et al.*, 1994). Numbering is according to the human eRF1 sequence. Sequences were extracted from DDBJ/EMBL/GenBank [H.sap, *Homo sapiens*, P46055; S.cer, *Saccharomyces cerevisiae*, CAA51935; E.cun, *Encephalitozoon cuniculi*, 13560073; B.ame, *Blepharisma americanum*, AF317831; E.aed-1A, *Euplotes aediculatus*-1A, AF298831; E.oct-1a, *Euplotes octocarinatus*-1a, AJ272501; E.aed-1B, *Euplotes aediculatus*-1B, AF298832, AF334757 (this work); E.oct-1b, *Euplotes octocarinatus*-1b, AF245454; S.myt, *Stylonychia mytilus*, AF317833; S.lem, *Stylonychia lemnae*, AF317834; O.tri, *Oxytricha trifallax*, AF298830; T.the, *Tetrahymena thermophila*, AF298833; P.tet, *Paramecium tetraurelia*, AF149035 and AF149036]. The bottom line indicates the residues conserved in all sequences presented. Residues in black were identified by Bertram *et al.* (2000) in yeast mutants exhibiting either a UGA suppressor phenotype (mutations I35F, V71I, L126V and H131R) or a UAG suppressor phenotype (mutations M51I, S77F and D113G). Residues shaded in gray were supposed to be involved in stop codon recognition: dark gray (Liang *et al.*, 2001; Muramatsu *et al.*, 2001), light gray (Knight and Landweber, 2000; Lehman, 2001; Lozupone *et al.*, 2001).

The N-terminal domain of eRF1 probably mimics the anticodon arm of tRNA (Song *et al.*, 2000). If so, a 'protein anticodon' recognition model might be postulated as a way to decode stop codons (Ito *et al.*, 2000; Nakamura *et al.*, 2000). Bertram *et al.* (2000) identified mutations in yeast eRF1 that increased UAG or UGA suppression. All mutations were located in the N-terminal domain of eRF1, confirming that this domain is responsible for stop codon discrimination. Then, the challenge was to identify the amino acids of eRF1 interacting with the stop codon similarly to what was demonstrated for bacterial class-1 RFs (Ito *et al.*, 2000). The strategy was based on the data of the eRF1 crystal structure and on the comparison of numerous eRF1 sequences from highly divergent eukaryotic species including those from ciliates (Figure 1). It was assumed that the changes in stop codon usage were mediated by the amino acids of eRF1 interacting with stop codons. The conserved NIKS motif (positions 61–64) was thought to be involved in stop codon recognition (Knight and Landweber, 2000). This assumption was abandoned when in eRF1 sequences from *Stylonichia* and *Oxytricha*, two ciliates using the same variant genetic code as *Tetrahymena thermophila*, the NIKS (not NIKD as in *T. thermophila*) motif was identified (Lozupone *et al.*, 2001).

In the presumed 'protein anticodon' region, some residues in eRFs are conserved in all eukaryotes except ciliates, where UAR codons encode glutamine, i.e. I35V, M51L and L126F (Lozupone *et al.*, 2001), and L126I in *Euplotes*, where UGA encodes cysteine (Figure 1). The fact that residues 35 and 126 are close to each other in the spatial structure (Song *et al.*, 2000) is consistent with their potential involvement in stop codon recognition (Lehman, 2001). However, the eRF1 sequence of the microsporidia *Encephalitozoon cuniculi*, which uses a canonical genetic code, has methionine at position 126 (Figure 1). How can this change be accommodated with the model described above?

Muramatsu *et al.* (2001) proposed that E55, G57/T58 and S60/N61 recognize the first, second and third base of the stop codons, respectively. We have sequenced the *eRF1* gene from *P. tetraurelia* (S. Kervestin, unpublished), and alignment of this sequence with the other eRF1s (Figure 1) shows that the divergences observed at positions 55, 57, 58 and 60 do not fit well with this model. For instance, in *Tetrahymena*, *Stylonichia* and *Oxytricha*, position 57 is occupied by serine, whereas *Paramecium* eRF1 has alanine in this position. These substitutions are not correlated with codon reassignments in ciliate eRF1s in

Fig. 2. *Euplotes aediculatus* stained with an anti-tubulin antibody. Photograph courtesy of Anne Fleury, University of Paris, France.

comparison with eRF1s from organisms with universal genetic code. Ciliate eRF1s exhibit a high evolutionary rate, which is reflected in an increased number of variable positions (Inagaki and Doolittle, 2001; David Moreira, personal communication). Thus, it can not be ruled out that either residues of eRF1 involved in stop codon recognition may occupy different variable positions in ciliate sequences, or variable positions modify the functional constraints at a few fixed positions. Additional eRF1 sequences, particularly from ciliate species, could help to select amino acids implicated in stop codon recognition.

Our data on inability of *Eu*-RF1 to respond to UGA strongly support the assumptions that (i) in all ciliates with variant genetic codes, eRF1 does not respond to reassigned stop codons; (ii) in these organisms, modifications of eRF1 amino acid sequences are responsible for the pattern of stop codon recognition; and (iii) presumably, ribosomes from ciliates possess the same ability to support termination of the three stop codons as ribosomes from organisms with conventional genetic code.

METHODS

Plasmids, library screening, gene manipulations, DNA sequencing and PCR amplification. The *E. aediculatus eRF1* gene (Figure 2) was isolated from a macronuclear DNA pUC18 library provided by A. Baroin-Tourancheau (Université Paris-Sud). Because eRF1s of ciliates are highly divergent from other eukaryotes, the coding sequence of *eRF1* from *P. tetraurelia* (S. Kervestin, unpublished) was used as a probe for library screening. Library screening and gene manipulations were carried out by the standard procedures (Sambrook *et al.*, 1989). Bacterial colonies transferred on Hybond N+ filters (Amersham-Pharmacia Biotech.) were detected by hybridization at non-stringent conditions (1 h at 60°C followed by slow cooling to 30°C) and washing in 2× SSC (0.3 M NaCl, 30 mM sodium citrate) plus 0.1% SDS at 35°C. The entire nucleotide sequence of inserts was determined on both strands. PCR amplifications of DNA were carried out in 25 μl reaction mixtures containing 1 ng of plasmid DNA, 100 pmol of each primer, 200 μM each deoxynucleoside triphosphate, 1× commercial PCR buffer and 2.5 U of Pwo DNA polymerase (Roche). Amplifications were run for 20 cycles (94°C, 30 s; 50°C, 30 s; 72°C, 1 min) in a thermocycler.

Site-directed mutagenesis. This was performed to transform four in-frame UGA codons of the *E. aediculatus eRF1* gene into the canonical cysteine UGC codon using the Transformer site-directed mutagenesis kit (Clontech). The resulting modified *eRF1* DNA (from the AUG initiation codon to the last codon) was then amplified by PCR with appropriate oligonucleotides containing restriction sites (*Nde*I and *Hind*III) for direct cloning into pET21b (Novagen). The final construct, named pET-*Eu*-RF1-His_6, contained *E. aediculatus* eRF1 ORF under control of T7 promotor.

Expression and purification of eRF1s. cDNA encoding the full-length human eRF1 was inserted into *Nde*I–*Xho*I sites of pET23b(+) (Novagen). *Eu*-eRF1 and human eRF1 containing a His-tag at the C-terminus were expressed in *Escherichia coli* strain BL21(DE3), and purified using Ni-NTA resin, Superflow (Qiagen), as described (Frolova *et al.*, 1994, 2000).

Ribosomes. Rabbit reticulocyte ribosomal subunits were kindly provided by P. Simonenko (Institute of Protein Research, Pushchino). 80S ribosomes washed with 0.5 M KCl were treated with puromycin and GTP for dissociation into subunits, which were subsequently resolved by centrifugation in a 10–25% (w/v) sucrose gradient containing 0.3 M KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol and 20 mM Tris–HCl pH 7.6. Before addition to the incubation mixtures, the subunits were combined in an equimolar ratio.

***In vitro* RF assay.** The eRF1 activity was measured as described (Caskey *et al.*, 1974; Frolova *et al.*, 1994) at saturation levels (50 μM) of one out of the three stop-codon-containing tetraplets or UGGA tetraplet containing codon for tryptophan. The incubation mixture (25 μl) contained 20 mM Tris–HCl pH 7.5, 15 mM $MgCl_2$, 8 mM NH_4Cl, 1.5 pmol f[^{35}S]-Met-$tRNA_f^{Met}$–AUG–ribosome complex and eRF1 (0.2–0.3 μg). AUG and ribotetraplets were synthesized by A. Veniaminova and M. Ryabkova (Institute of Biorganic Chemistry, Novosibirsk).

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