A single amino acid change of translation termination factor eRF1 switches between bipotent and omnipotent stop-codon specificity†

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ABSTRACT

In eukaryotes a single class-1 translation termination factor eRF1 decodes the three stop codons: UAA, UAG and UGA. Some ciliates, like Euplotes, have a variant code, and here eRF1s exhibit UAR-only specificity, whereas UGA is reassigned as a sense codon. Since eukaryote eRF1 stop-codon recognition is associated with its N-terminal domain, structural features should exist in the N domain of ciliate eRF1s that restrict their stop-codon specificity. Using an in vitro reconstituted eukaryotic translation system we demonstrate here that a chimeric eRF1 composed of the N domain of Euplotes aediculatus eRF1 fused to the MC domains of human eRF1 exhibits UAR-only specificity. Functional analysis of eRF1 chimeras constructed by swapping Euplotes N domain sequences with the cognate regions from human eRF1 as well as site-directed mutagenesis of human eRF1 highlighted the crucial role of the alanine residue in position 70 of E. aediculatus eRF1 in restricting UGA decoding. Switching the UAR-only specificity of E. aediculatus eRF1 to omnipotent mode is due to a single point mutation. Furthermore, we examined the influence of eRF3 on the ability of chimeric and mutant eRF1s to induce peptide release in response to different stop codons.

INTRODUCTION

The termination of protein synthesis in eukaryotes is governed by a single polypeptide chain class-1 release factor eRF1, which recognizes all three stop codons, UAA, UAG and UGA at the ribosomal A-site when the P-site is occupied by peptidyl-tRNA (1–3). In eukaryotes, the highly conserved eRF1 is composed of three well-defined domains (4,5). The N-terminal domain is responsible for stop codons decoding (6–16), the M (middle) domain participates in peptidyl-tRNA hydrolysis (4,17,18) and the C-terminal domain binds to eRF3 (19–24). Class-2 release factor, eRF3 encoded by an essential gene, is a ribosome- and eRF1-dependent GTPase (19), which enhances termination efficiency by stimulating the activity of eRF1 (19,25,26).

Many approaches have been used to identify the key amino acid residues within the N domain of eRF1 that mediate stop-codon recognition (4,7–16). However, the data are contradictory.

One possible approach for solving this problem was to design chimeric eRF1s, which contain different parts of the N domain of eRF1s from organisms with universal and variant genetic codes. Exceptions from the standard genetic code exist in mitochondria, ciliates, Candida and other species (14). Among the ciliates, Tetrahymena, Stylonychia and Paramecium species recognize UGA as a stop codon, while UAA and UAG are reassigned to glutamine codons (27). Euplotes and Blepharisma species decode UAA and UAG as stop codons but use UGA as cysteine or tryptophan codons, respectively (28). A chimeric eRF1 with N domain derived from Tetrahymena thermophila eRF1 and the MC domains from Schizosaccharomyces pombe was already reported and the amino acid determinants responsible for the UGA-only specificity of T. thermophila eRF1 were localized in the N domain using in an in vitro RF assay (29). In contrast, a chimeric eRF1 consisting of the N domain of T. thermophila and the MC domains from Saccharomyces cerevisiae efficiently terminated at all three stop codons when expressed in yeast cells (30). Thus, the localization of stop-codon determinants in T. thermophila eRF1 requires additional studies. Furthermore, regions sufficient for the UGA-only specificity of T. thermophila eRF1 were localized in the N domain using in an in vitro RF assay (29). In contrast, a chimeric eRF1 consisting of the N domain of T. thermophila and the MC domains of Saccharomyces cerevisiae efficiently terminated at all three stop codons when expressed in yeast cells (30). Thus, the localization of stop-codon determinants in T. thermophila eRF1 requires additional studies. Furthermore, regions sufficient for the UGA-only

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†This paper is dedicated in memory of Lev Kisselev.

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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specificity of eRF1s from Paramecium tetraurelia and Stylonychia mytilus were identified in the N-terminal domain by creating chimeric constructs and using site-directed mutagenesis (8,16). The importance of the N domain of E. aediculatus eRF1 and E. octocarinatus eRF1 in determining UAR-only specificity was confirmed for interdomain eRF1 chimeras with MC domains from human and S. cerevisiae eRF1s, respectively (11,30). Human and Euplotes eRF1s share significant sequence homology, but at least some of the existing differences between the amino acid sequences can be responsible for their different stop-codon specificities (Figure 1). In particular, suppressor mutations which change the stop-codon recognition pattern of eRF1 were identified in chimeric proteins carrying the E. octocarinatus N domain fused to S. cerevisiae MC domains (31). Nevertheless, the mechanism of stop-codon reassignment in the organisms with variant genetic code is still poorly understood.

In the current study, we used both a molecular chimera approach and site-directed mutagenesis followed by in vitro functional analysis in a reconstituted eukaryotic translation system; and we have identified a single amino acid residue that confers UAR-only specificity to Euplotes eRF1 and switches the omnipotent specificity of human eRF to UAR-only specificity. Moreover, we studied the effect of class-2 eukaryotic translation termination factor eRF3 on stop-codon decoding of chimeric and mutant eRF1s as a function of the length of the Euplotes region in the eRF1 chimera.

MATERIALS AND METHODS

Amplification of the sequence encoding the N domain of the E. aediculatus eRF1b gene

The eRF1b gene sequence encoding the N domain of E. aediculatus eRF1b was amplified by PCR from E. aediculatus cell culture harvested by centrifugation at 11,000g for 10 min. The cells were slurred in 50 μl of PCR-mix containing 70 mM Tris–HCl, pH 8.6, 16 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mM of each dNTP, 2 μM of each oligonucleotide primer containing NdeI or XhoI restriction site at the 5′-end (see Supplementary Data) and 2.5 U of HiFi DNA-polymerase (Fermentas). The resulting PCR product corresponding to the expected length (433 bp) of the N domain of E. aediculatus eRF1b was purified using a GFX PCR DNA purification kit (GE Healthcare), hydrolyzed with NdeI and XhoI and ligated in pERF4b-Sal plasmid treated with NdeI and SalI. pERF4b-Sal plasmid contained the human eRF1 gene with an inserted SalI restriction site at amino acid positions 144–145 (20). The coding sequence of the N domain of E. aediculatus eRF1b contained one UGA codon at amino acid position 132 (numeration of amino acid position according to human eRF1). This codon was replaced by a UGC codon for cysteine, because Euplotes used UGA to encode this amino acid. The resulting construct was named Eu(1–145) (for details see Supplementary Data).

Swapping of the N domain sequences between human and E. aediculatus eRF1s

All constructs were prepared by site-directed mutagenesis with appropriate primers in two rounds of PCR using different templates (see Supplementary Data). The resulting PCR product was treated with NdeI and SalI endonucleases and inserted into pERF4b-Sal treated with the same endonucleases.

Point mutations in the N domains of the human and E. aediculatus eRF1s

The N domains of Hs-eRF1 with substitutions S70A, G73S, S70A+G73S, V78A, Q79K, Q80E and E. aediculatus eRF1 with substitutions A70S and A70S+S73G (residue numbering as in Hs-eRF1) were obtained by site-directed mutagenesis using the PCR-based 'megaprimer' method as described (12). The resulting PCR products were inserted into the plasmid pERF4b. For cloning details of eRF1 mutants see Supplementary Data.

Figure 1. Alignment of amino acid sequences from the N-terminal domains of human and Euplotes eRF1s. Residue numbering is that of the human eRF1. Identical, conserved and semiconserved amino acid residues are black, dark gray and light gray, respectively. The arrow indicates position 70.
Proteins and ribosomal subunits

The 40S and 60S ribosomal subunits, as well as eukaryotic translation factors eIF2, eIF3, eIF4F, eEF1H and eEF2, were purified from a rabbit reticulocyte lysate as described (25). The eukaryotic translation factors eIF1, eIF1A, eIF4A, eIF4B, eIF5B, eIF5, eRF1, wild-type (wt) eRF1, mutant and chimeric eRF1s and eRF3 were produced as recombinant proteins in *Escherichia coli* strain BL21 with subsequent protein purification on Ni-NTA agarose and ion-exchange chromatography (17,25).

mRNA transcripts

mRNA was transcribed by T7 RNA polymerase on MVHL-stop plasmids, contained T7 promoter, four mRNA transcripts ion-exchange chromatography (17,25). Subsequent protein purification on Ni-NTA agarose and 5 mM MgCl₂. Fractions corresponding to pre-termination linear sucrose density gradient prepared in buffer A with recombinant proteins in eIF4A, eIF4B, eIF5B, eIF5, eRF1, wild-type (wt) eRF1, (25). The eukaryotic translation factors eIF1, eIF1A, eIF2, eIF3, eIF4F, eEF1H and eEF2, The 40S and 60S ribosomal subunits, as well as eukaryotic translation factors eIF2, eIF3, eIF4F, eIF4A, eIF4B, eIF5B, eIF5, eRF1, wild-type (wt) eRF1, mutant and chimeric eRF1s and eRF3 were produced as recombinant proteins in *Escherichia coli* strain BL21 with subsequent protein purification on Ni-NTA agarose and ion-exchange chromatography (17,25).

Pre-termination complex assembly and purification

Pre-termination complexes were assembled as described (25). Briefly, 37 pmol of MVHL-stop mRNAs were incubated for 30 min in buffer A (20 mM Tris acetate, pH 7.5, 100 mM KAc, 2.5 mM MgCl₂, 2 mM dithiothreitol) supplemented with 400 U RNAse inhibitor (RiboLock, Fermentas), 1 mM ATP, 0.25 mM spermidine, 0.2 mM GTP, 75 μg total tRNA (acylated with Val, His, Leu and [³⁵S]Met), 75 pmol 40S and 60S purified ribosomal subunits, 125 pmol eIF2, eIF3, eIF4F, eIF4A, eIF4B, eIF1, eIF1A, eIF5, eIF5B each, 200 pmol eEF1H and 50 pmol eEF2, and then centrifuged in a Beckman SW55 rotor for 95 min at 4°C and 50,000 r.p.m. in a 10–30% linear sucrose density gradient prepared in buffer A with 5 mM MgCl₂. Fractions corresponding to pre-termination complexes according to optical density and the presence of [³⁵S]Met were combined, diluted 3-fold with buffer A containing 1.25 mM MgCl₂ (to a final concentration of 2.5 mM Mg²⁺) and used for peptide release assays.

Termination efficiency determination

Termination efficiency was determined as described (32) with some modifications. Aliquots containing 0.0125 pmol of pre-termination complexes assembled in the presence of [³⁵S]Met-tRNA were incubated at 37°C with 2.5 pmol of eRF1 for 0–15 min or with 0.125 pmol eRF1 in presence of 0.125 pmol eRF3 and 0.2 mM GTP, 0.2 mM MgCl₂ for 0–3 min. Ribosomes and tRNA were pelleted with ice-cold 5% trichloroacetic acid supplemented with 0.75% casamino acids and centrifuged at 4°C and 14,000g. The amount of released [³⁵S]Met-containing tetrapeptide, which indicated the efficiency of peptideyl-tRNA hydrolysis, was determined by scintillation counting of supernatants on an Intertechnique SL-30 liquid scintillation spectrometer.

Calculation of $k_{\text{cat}}/K_M$ values

Values of the $k_{\text{cat}}/K_M$ ratio were determined from $k_{\text{obs}}$. Under first-order conditions at a substrate concentration far below the estimated $K_M$, the Michaelis–Menten equation $v = (V_m S)/(K_M + S)$ became $v = (V_m S)/K_M$ since $S << K_M$ or $v = (V_m S)/K_M = k_{\text{obs}} S$. This was integrated as $\ln S = -k_{\text{obs}} t + \ln S_0$, where $S_0$ is the starting substrate concentration and $S$ the substrate concentration at a given time. Continuous recording of substrate hydrolysis allows determination of $k_{\text{obs}}$ from the graph of lnS versus time. The velocity was proportional to the substrate concentration. $k_{\text{cat}} = V_m/K_M = (k_{\text{cat}} E_1)/K_M$ since $V_m = k_{\text{cat}} E_1$, therefore $k_{\text{cat}}/K_M = k_{\text{obs}}/E_1$.

RESULTS

Functional analysis of chimeric eRF1s in various assay systems

Earlier, using the dual-gene reporter system, we have shown that two regions in the N domain of *E. aediculatus* eRF1 (Eu-eRF1) 38–50 and 123–145 are critical for its UAR-specificity [construct Eu-11 (33)]. To confirm these results, we have tested the chimeric protein Eu(38–50/123–145) containing the same regions of Eu-eRF1 in a fully reconstituted *in vitro* eukaryotic translation system (25) and in an *in vitro* RF assay (16,34). Surprisingly, Eu(38–50/123–145) effectively recognized all three stop codons in these systems (Supplementary Table S2). Moreover, we examined the functional activity of this construct in the dual-gene reporter system described in Ref. (16) but with another stop-codon context. A leaky termination signal derived from the dystrophin context—GCC CGG TGT stop GAT AAT TTA from human mRNA dystrophin transcript variant Dp4271 (35)—was replaced by the other leaky termination signal—the tobacco mosaic virus (TMV) context—CAA stop CAA UUA (36). In the dual-gene reporter system with the TMV context, the Eu(38–50/123–145) showed no detectable UGA readthrough increase (Supplementary Table S2).

We measured the functional activity of another chimeric eRF1 with the N domain of *E. aediculatus* eRF1 and the MC domains of the human eRF1 (Hs-eRF1) [Eu(1–145) construct] *in vitro* and *in vivo*. Insignificant differences in stop-codon readthrough for this construct have been revealed in an *in vivo* dual-gene reporter system with TMV context (Supplementary Table S2), while Eu(1–145) with the dystrophin context shows strict UAR-only specificity in the dual-gene reporter system with TMV context, while for the dystrophin context practically no increase of readthrough efficiency, i.e. omnipotent specificity, is observed in the
presence of all three stop codons (Supplementary Table S2). It follows that the different contexts used in the dual-gene reporter system lead to ambiguous and controversial results for the same eRF1. Therefore, the dual-gene reporter system with the dystrophin context should not be used for functional analysis of eRF1; earlier results with the dystrophin context pointing to the importance of regions 38–50 and 123–145 of *E. aediculatus* for UAR-only stop-codon specificity (33) are therefore questionable.

In the current study, we have used the recently developed fully reconstituted *in vitro* eukaryotic translation system (25) for determining the regions in *E. aediculatus* eRF1 crucial for its UAR-only specificity.

**A chimeric protein containing the N-terminal domain of Eu-eRF1 exhibits UAR-only specificity**

In *Euplotes* two forms of eRF1 with the same specificity (37) are present, viz. eRF1a and eRF1b. Here, we used the constructs with the N domain of *E. aediculatus* eRF1b, because it was shown that eRF1b is more abundant in *Euplotes* cells.

The RF activity of chimeric protein (Figure 2A), containing the N-terminal domain of Eu-eRF1 and the MC domains of Hs-eRF1 [Eu(1–145)] was determined using a reconstituted eukaryotic translation system consisting of purified individual eukaryotic translation factors, 40S and 60S rabbit ribosomal subunits, aminoacylated tRNAs and synthetic mRNA (25). The rate of peptidyl-tRNA hydrolysis for different eRF1s was determined as the amount of 35S-labelled tetrapeptide (MVHL) released as a function of time from termination complexes in the presence of UAA (Figure 3A), UAG (Figure 3B) and UGA (Figure 3C) stop codons. It can be seen from Figure 3 that Eu(1–145) is active with UAA and UAG stop codons but that the RF activity towards UGA is significantly decreased, i.e. this protein exhibits mainly UAR-only specificity.

The termination efficiencies for Hs-eRF1 and *Euplotes*-human interdomain chimera were calculated as $k_{cat}/K_M$ values (Table 1, Figure 2B). While Hs-eRF1 shows $k_{cat}/K_M$ values of $2.88 \times 10^4 \text{ s}^{-1} \text{M}^{-1}$ for UAA, $2.57 \times 10^4 \text{ s}^{-1} \text{M}^{-1}$ for UAG and $2.81 \times 10^4 \text{ s}^{-1} \text{M}^{-1}$ for UGA, Eu(1–145) demonstrates a >5-fold relative decrease of kinetic efficiency only on UGA stop codon compared to Hs-eRF1.

**Region 70–80 of Eu-eRF1 is responsible for UAR-only specificity**

To find the region in the N domain of *E. aediculatus* eRF1 responsible for UAR-only specificity, we used a strategy of isolation of intra-N-domain chimeras (16) containing swapped fragments from Eu-eRF1 and Hs-eRF1 with subsequent functional analysis of these proteins in the reconstituted *in vitro* eukaryotic translation system.

We selected two points in the *Euplotes* N domain for swapping: amino acids 50 and 122 (the numeration of amino acid positions in all constructs is as in Hs-eRF1, Figure 2A). In chimera Eu(1–50) the first (positions 1–50) and second (positions 51–145) parts are derived from Eu-eRF1 and Hs-eRF1, respectively. Eu(1–50) possesses omnipotent specificity, its kinetic efficiencies at all three stop codons are comparable with Hs-eRF1. The reciprocal construct Eu(51–145) demonstrates another pattern of stop-codon recognition: a relative increase of kinetic efficiency on UAA and UAG is 1.6 and 2.4, respectively; however, some decrease of efficiency is observed on the UGA stop codon (Table 1, Figure 2B). Thus, the functional pattern for Eu(51–145) corresponds to Eu-eRF1 stop-codon specificity—the UAA and UAG stop codons are recognized much more efficiently than the UGA codon. However, the $k_{cat}/K_M$ values for this chimera are higher compared to the $k_{cat}/K_M$ values for Hs-eRF1 (Table 1). We suggest that this effect is probably caused by an increase of the eRF1 affinity towards the ribosomes as a consequence of amino acid substitutions in Hs-eRF1 important for ribosome binding.

Another pair of inversed chimeras is Eu(1–122) and Eu(123–145) (Figure 2A). Since the Eu(1–122) possesses UAR-only specificity (Table 1, Figure 2B), the determinants preventing the UGA decoding are located in the region 1–122. Eu(123–145) recognizes all three stop codons, although a near equal reduction in RF activity is observed (Table 1, Figure 2B).

The data obtained so far indicate that amino acids responsible for *Euplotes* eRF1 stop-codon specificity lie between positions 51 and 122. To verify this suggestion, we used the Eu(51–125) chimera (Figure 2A). The functional activity of Eu(51–125) is similar to that of Eu(51–145) and confirms the importance of this region for UGA non-decoding (Table 1, Figure 2B). For the chimera Eu(51–80), we again observe a significant increase of RF activity in response to UAA and UAG but not to the UGA stop codon as in the case of Eu(51–145) and Eu(51–125). In contrast, Eu(81–122) exhibits omnipotent specificity typical of Hs-eRF1. Chimera Eu(51–69) reveals an almost equal increase of $k_{cat}/K_M$ values at all three stop codons (Table 1, Figure 2B). Consequently, the region corresponding to positions 51–69 of Eu-eRF1 might be responsible for enhancing eRF1-ribosome binding, and these amino acids apparently are not involved in UGA recognition.

Eu(70–80) contains the smallest fragment of Eu-eRF1 sequence and remains still active on UAA and UAG stop codons, like Hs-eRF1, whereas a significant decreased RF activity on UGA stop codon is observed (Table 1, Figure 3B). Taken together, these results imply that amino acid(s) preventing UGA recognition in Eu-eRF1 are located between positions 70–80.

**Position 70 in eRF1 is critical for changing stop-codon specificity**

As is evident from alignment of the N domain amino acid sequences of Hs-eRF1 and Eu-eRF1, five variable amino acid residues are located in the region 70–80 (Figure 1). To examine the influence of a single amino acid substitution in the region 70–80 on RF activity, S70A, G73S, V78A, Q79K and Q80E Hs-eRF1 mutants were constructed by site-directed mutagenesis and their activities were assayed in the reconstituted eukaryotic translation system.
Substitutions of one out of the two amino acids, G73S or V78A in Hs-eRF1, did not affect the kinetic efficiency of the mutant proteins (Table 1, Figure 2B). Replacement of the glutamine in position 79 by a positively charged lysine led to an equal increase of \( k_{cat}/K_{M} \) values in response to all three stop codons. In contrast, mutation Q80E in Hs-eRF1, which eliminates the partial positive charge, results in a significant decrease of termination efficiency at all stop codons (Table 1, Figure 2B). These effects might be indirect by effecting eRF1 binding to ribosomes probably involving rRNA and thus being sensitive to the charge changes of corresponding amino acid residues. In any case, the substitutions G73S, V78A, Q79K and Q80E in Hs-eRF1 are not essential for stop codon discrimination.

However, the Hs-eRF1 S70A mutant shows a 2.4-fold reduction of the kinetic efficiency in the presence of the UGA as compared to the wild-type Hs-eRF1 (Table 1, Figure 3). Thus we suggest that alanine in position 70 of Eu-eRF1 plays an important role in discrimination against UGA. To prove this hypothesis, we introduced an A70S mutation into the chimera Eu(70–80). This construct, designated Eu(71–80), recognizes all three stop codons equally well as does Hs-eRF1 (Table 1, Figure 2B). The fact that the reverse mutation A70S in Eu(70–80) restores the omnipotent specificity of the factor confirms the crucial role of serine in position 70 of Hs-eRF1 for UGA recognition as stop codon.

Eu(1–145) A70S/S73G mutant exhibits omnipotent specificity

To verify the importance of serine in position 70 for UGA decoding by Hs-eRF1, we substituted alanine for serine in position 70 in Eu(1–145). Unfortunately, we could not isolate recombinant protein for mutant Eu(1–145)A70S, apparently due to the appearance of an \( E. \) coli protease site after A70S substitution (Supplementary Figure S1). Notably, the chimeric protein Eu(1–145) was not...
degraded during its expression in *E. coli*. Taking into account that the region 61–77 of Hs-eRF1 and Eu-eRF1 includes two variable amino acid residues in positions 70 and 73 (Figure 1) and that the mutation G73S in Hs-eRF1 does not affect the stop-codon specificity of the factor (Table 1, Figure 2B), we have designed a new chimeric construct with the double mutation A70S/S73G in Eu(1–145). The termination efficiencies of the Eu(1–145) A70S/S73G mutant were nearly identical to Hs-eRF1 at all three stop codons (Table 1, Figure 2B), whereas the reverse double mutant S70A/G73S of Hs-eRF1 revealed UAR-only specificity clearly demonstrating the significance of the position 70 for UGA decoding.

**Discrimination of the eRF1 stop-codon recognition is augmented by eRF3**

Translation termination in eukaryotes is governed by the cooperative action of two interacting polypeptide chain factors, eRF1 and eRF3. It is still unknown which step of the translation termination process is enhanced by the eRF1–eRF3 interaction. Here, we have examined the effect of eRF3 on the activities of the chimeric and mutant eRF1s investigated above.

The termination efficiencies \( (k_{cat}/K_M) \) of six intra-N-domain chimera eRF1s (Euplotes–human) with pronounced tendency towards UAR-only specificity were monitored in the presence of eRF3-GTP, as well as the S70A mutant of Hs-eRF1 (UAR-only specificity) and Eu(1–145) A70S/S73G mutant (omnipotent stop-codon specificity) (Table 2, Figure 4).

The addition of eRF3-GTP increased the termination efficiency of Hs-eRF1 by more than two orders of magnitude. In contrast, \( k_{cat}/K_M \) values of prokaryotic release factors RF1/RF2 did not change upon RF3-GTP addition (38). This observation confirms functional differences between prokaryotic and eukaryotic class-2 release factors.

Similarly, all examined mutant factors also showed \( k_{cat}/K_M \) values about two orders of magnitude larger than those in the absence of eRF3-GTP (Table 2, Figure 2C). Interdomain chimera Eu(1–145) exhibited UAR-only specificity (Figure 4) with a 10-fold decrease in kinetic efficiency in response to the UGA. However, reducing the sequence swapped from Eu-eRF1 within the N domain of chimeric constructs led to the restoration of RF activity towards UGA up to the omnipotent specificities of Eu(70–80) and Hs-eRF1 S70A mutant (Table 2, Figure 4). We suppose that eRF1 stop-codon recognition is improved by eRF3. Interestingly, there was no relative increase of the \( k_{cat}/K_M \) values for Eu(51–145) and Eu(51–80) in response to UAA and UAG stop codons, as was observed earlier without eRF3-GTP. The increase in termination efficiencies after the addition of the eRF3-GTP by more than two orders of magnitude probably compensates for different eRF1 affinities to the ribosome revealed in the absence of class-2 release factor.

The mutant Eu(1–145) A70S/S73G recognized all three stop codons when eRF3-GTP was added to the termination reaction confirming the crucial importance of the amino acid residue in position 70 of eRF1 for UGA recognition.

**DISCUSSION**

Previously, we evaluated the stop-codon-dependent translation termination activity of eRF1s by two assays: an *in vivo* dual-gene reporter system and an *in vitro* RF activity test (16). The relative simplicity of these two methods is a significant advantage but both have
The error in kinetic efficiency values varied from 0.12 \times 10^4 to 0.35 \times 10^4 for different release factors.

The error in kinetic efficiency values varied from 0.45 \times 10^6 to 0.57 \times 10^6 for different release factors.

limitations—the absence of natural mRNA, eRF3 and GTP in the in vitro assay and the presence of endogenous eRF1/eRF3 in the cell culture used in the in vivo assay. Moreover, it appeared that the dual-gene reporter system in some cases was not suitable for determining the functional activity of chimeric eRF1s. For example, the *E. aediculatus*–human hybrid, construct Eu(1–145) showed contradictory results in the dual reporter system in vivo–human hybrid, construct Eu(1–145) showed contradictory results in the dual reporter system.

Another approach for the stop-codon specificity determination of eRF1 consisted in an in vitro photocrosslinking of *Euplotes*–human eRF1 hybrids with in-frame stop codons in 42-mer mRNA analog (11). However, only the whole N domain of *E. aediculatus* appeared to be UAR-specific and the authors could not localize determinants preventing UGA recognition via UTRs and all necessary translation components.

With these improvements, we examined the role of individual amino acids of eRF1 in governing stop-codon specificity. The comparison of the eRF1 sequence from organisms with universal and variant genetic code revealed variable residues in the N domains, which is responsible for the stop-codon specificity. Two approaches were used: (i) various regions of the eRF1 N domain were swapped between ciliate (*E. aediculatus*) and human factors, and (ii) amino acid residues within the N domain were mutated and the activity of the resulting eRF1s were assessed.

For the first time, we determined the $k_{cat}/K_M$ values of the eukaryotic translation termination reaction for Hs-eRF1 in the presence or absence of eRF3-GTP at different stop codons (Tables 1 and 2). Hs-eRF1 without
eRF3-GTP displayed practically equal $k_{cat}/K_M$ values for all stop codons and the addition of eRF3 increased all values more than two orders of magnitude. It should be noted that the termination efficiencies in bacterial system do not depend upon the presence of RF3-GTP (24) indicating the functional difference between the eukaryotic and bacterial termination. We suppose that eRF3 directly stimulates eRF1 activity and does not recycle eRF1 by displacing it from the ribosome after peptide release as described also for S. cerevisiae termination factors (26).

Chimeric protein Eu(1–145) exhibited UAR-only specificity in the reconstituted translation system with ~5-fold relative decrease of the termination efficiency towards the UGA stop codon compared to Hs-eRF1 (Table 1, Figure 3). The addition of eRF3-GTP improved discrimination against the UGA stop codon (Table 2, Figure 4) and the corresponding $k_{cat}/K_M$ value decreased 10-fold with respect to Hs-eRF1. However, we did not observe a complete discrimination against the UGA stop codon for Eu(1–145). Possible reasons are: (i) system heterogeneity (the N domain was derived from E. aediculatus eRF1, while the MC domains were from the human eRF1, and the ribosomes were isolated from rabbit reticulocyte lysate), and (ii) additional amino acid residues of eRF1 might be involved in preventing UGA decoding completely.

We have localized amino acid residues prohibiting UGA recognition in the region 70–80 of E. aediculatus eRF1 (Table 1, Figure 3). This area was not considered earlier to play a decisive role in this function. In different studies, the following positions were suggested in the N domain of Hs-eRF1 being important for UGA decoding: 31, 32 (37); 60, 61, 127 (31); regions 38–50 and 123–145 (33). Here, we have unambiguously shown the exceptional importance of the region 70–80 of E. aediculatus eRF1 in UGA decoding. The importance of region 51–69 for ribosome binding might be suggested. The sequences 51–69 of Eu-eRF1 and Hs-eRF1 differ by three amino acids and include the NIKS-motif (residues 61–64 in Hu-eRF1) (Figure 1). Earlier, it was shown that region 51–69 effectively cross-linked with the mRNA inside the ribosome (11). Moreover, the importance of amino acids 60 and 61 for ribosome binding was reported (31).

Site-directed mutagenesis of amino acids from region 70–80 of Hs-eRF1 has revealed that serine substitution in position 70 is sufficient to turn off the UGA recognition by the omnipotent factor (Table 1, Figure 3). It probably occurs due to the loss of the Ser hydroxyl group, which might be directly involved in UGA recognition of Hs-eRF1. The insignificance of the other amino acid residues in the region 70–80 of Eu-eRF1 for UGA non-decoding was demonstrated by the ability of Eu-eRF1 supporting our finding of the importance of this amino acid for UGA non-decoding.

Earlier Fan-Minogue et al. (31) demonstrated that some mutations in the chimeric eRF1, which contained the N domain of E. octocarinatus and the MC domains of S. cerevisiae and exhibited UAR-only specificity, switched the specificity from bipotent to omnipotent. One of the critical mutations was the C124S (numeration according to S. cerevisiae eRF1), which successfully
restored efficient recognition of the UGA stop codon without significant effect on UAA and UAG. However, this amino acid residue is located inside of the YxCxxxF motif, which is universally conserved among all known eukaryotic class-I release factors and therefore cannot take part in switching the stop-codon recognition pattern. To verify the importance of C127 (numeration according to Hs-eRF1) in determining of the UAR-specificity of *Euplopes*, two point mutations, C127S and C127A, were introduced into the chimeric Eu(1–145) construct described above, and functional activity was measured in the reconstituted eukaryotic translational system. Both mutant eRF1s exhibited negligible RF activity on all three stop codons (Supplementary Table S2). It is thus likely that C127 in eRF1 is necessary for supporting the N domain overall structure or, alternatively, it is important for UAR decoding since Eu(1–145) does not recognize the UGA stop codon.

Though in the absence of eRF3-GTP the Hs-eRF1 S70A mutant exhibits UAR-only specificity (Table 1, Figure 3), the presence of eRF3-GTP, the mutant factor recognizes all three stop codons (Table 2, Figure 4). The same influence of eRF3-GTP on stop-codon specificity was revealed for Eu(70–80). Extending the sequence from Eu-eRF1 in chimeric constructs reduced the influence of eRF3-GTP on the RF activity in the presence of the UGA stop codon (Table 2, Figure 4). Eu(51–80) is the minimal construct in our chimera collection, which exhibits UAR-only specificity in the presence of eRF3-GTP. Thus, eRF3 improves the eRF1 stop codon decoding during eukaryotic translation termination.

Taken together, our results demonstrate that a single amino acid mutation can restore UGA recognition by *Euplopes* eRF1. The amino acid in position 70 is decisive for the stop-codon-recognition specificity of eRF1: S70 mediates omnipotent and A70 bipotent specificity.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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