RNA (2000), *6*:381–390. Cambridge University Press. Printed in the USA.

Translation termination in eukaryotes: Polypeptide release factor eRF1 is composed of functionally and structurally distinct domains

LUDMILA YU. FROLOVA,[1,2,3,*] TATYANA I. MERKULOVA,[2,*] and LEV L. KISSELEV[1,2]

[1]Engelhardt Institute of Molecular Biology, 117984 Moscow, Russia
[2]U248 Institut Nationale de la Santé et de la Recherche Médicale, Institut Curie, Paris 70005, France
[3]UPR41 Centre National de la Recherche Scientifique, Universite de Rennes 1, 350042 Rennes Cedex, France

ABSTRACT

Class-1 polypeptide chain release factors (RFs) trigger hydrolysis of peptidyl-tRNA at the ribosomal peptidyl transferase center mediated by one of the three termination codons. In eukaryotes, apart from catalyzing the translation termination reaction, eRF1 binds to and activates another factor, eRF3, which is a ribosome-dependent and eRF1-dependent GTPase. Because peptidyl-tRNA hydrolysis and GTP hydrolysis could be uncoupled in vitro, we suggest that the two main functions of eRF1 are associated with different domains of the eRF1 protein. We show here by deletion analysis that human eRF1 is composed of two physically separated and functionally distinct domains. The "core" domain is fully competent in ribosome binding and termination-codon-dependent peptidyl-tRNA hydrolysis, and encompasses the N-terminal and middle parts of the polypeptide chain. The C-terminal one-third of eRF1 binds to eRF3 in vivo in the absence of the core domain, but both domains are required to activate eRF3 GTPase in the ribosome. The calculated isoelectric points of the core and C domains are 9.74 and 4.23, respectively. This highly uneven charge distribution between the two domains implies that electrostatic interdomain interaction may affect the eRF1 binding to the ribosome and eRF3, its activity in the termination reaction and activation of eRF3 GTPase. The positively charged core of eRF1 may interact with negatively charged rRNA and peptidyl-tRNA phosphate backbones at the ribosomal eRF1 binding site and exhibit RNA-binding ability. The structural and functional dissimilarity of the core and eRF3-binding domains implies that evolutionarily eRF1 originated as a product of gene fusion.

Keywords: domain structure; eRF1; eRF3; eukaryotes; polypeptide release factors; translation termination

INTRODUCTION

Termination of protein synthesis requires two classes of polypeptide chain release factors (RFs). Class-1 RFs (RF1 and RF2 in prokaryotes, and eRF1 in eukaryotes) are termination-codon specific, and trigger hydrolysis of peptidyl-tRNA at the ribosomal peptidyl transferase center (reviewed by Tate & Brown, 1992; Buckingham et al., 1997; Nakamura & Ito, 1998). Class-2 RFs (RF3 and eRF3 in prokaryotes and eukaryotes, respectively) are guanine nucleotide-binding proteins possessing GTPase activity (Grentzmann et al., 1994, 1998; Mikuni et al., 1994; Zhouravleva et al., 1995; Frolova et al., 1996; Freistroffer et al., 1997; Pel et al., 1998).

Although the basic biological function of class-1 RFs is similar in prokaryotes and eukaryotes, they exhibit distinct structural and functional features. Thus eRF1 responds to all three termination codons (Konecki et al., 1977; Frolova et al., 1994), whereas each prokaryotic factor responds to two out of the three termination codons (Scolnick et al., 1968). Moreover, eRF1 binds to eRF3 in vivo and in vitro (Stansfield et al., 1995a; Zhouravleva et al., 1995; Paushkin et al., 1997; Frolova et al., 1998), whereas there is no evidence for the binding of RF1 or RF2 to RF3 in vivo or in vitro (Nakamura et al., 1996; Pel et al., 1998). The prokaryotic and eukaryotic amino acid sequences of class-1 RFs are significantly different (Frolova et al., 1994), although a limited similarity between certain eRF1 and RF1/2 sequences was noticed (Nakamura et al., 1995; Ito et al., 1996). Only one short, unique, and extremely conserved motif is universal for all class-1 RFs known so far (Frolova et al., 1999); it has been missed in earlier alignment (Ito et al., 1996). For all these reasons, the

Reprint requests to: Lev Kisselev, Engelhardt Institute of Molecular Biology, Vavilov str. 32, 117984 Moscow, Russia; e-mail: kissel@imb.imb.ac.ru.

*These authors equally contributed to this work.

functional anatomy of class-1 RFs is probably distinct for prokaryotes and eukaryotes and cannot be simply extrapolated from one group of factors to the other.

Recently, examination of the eRF1 functional anatomy has been initiated for yeast *Schizosaccharomyces pombe* (Ito et al., 1998a; Ebihara & Nakamura, 1999) and *Saccharomyces cerevisiae* (Eurwilaichitr et al., 1999) as well as for human (Merkulova et al., 1999). Surprisingly, although the overall structural homology between these eRF1s is rather high (Frolova et al., 1994), the results proved to be dissimilar. The negatively charged C-terminal 17 amino acids of *S. pombe* eRF1 were found to be essential for eRF3 binding. Deletion of C-terminal 32 amino acids of *S. cerevisiae* eRF1 was lethal, whereas a shorter deletion of C-terminal 19 amino acids resulted in a termination defect in vivo. The C domain of human eRF1 essential for eRF3 binding encompasses two critical regions, including amino acid residues 281–305 and 411–415, but the C-terminal stretch of 22 amino acids (positions 416–437) is dispensable. Thus the entire C-terminal region of human eRF1 essential for eRF3 binding differs from that of yeast eRF1. In spite of these differences, it is obvious that the C domains are involved in mutual binding, whereas the N-terminal parts are not essential for this interaction (Ito et al., 1998a; Ebihara & Nakamura, 1999; Eurwilaichitr et al., 1999; Merkulova et al., 1999), which partially contradicts other observations (Paushkin et al., 1997; Hoshino et al., 1998).

The full-length human eRF1 and its C-terminally truncated eRF1$^{2-415}$ mutant are active in promoting peptidyl-tRNA cleavage in the absence of eRF3 and GTP (Frolova et al., 1994, 1996; Zhouravleva et al., 1995; Merkulova et al., 1999). *S. pombe* eRF1 without C-terminal 17 amino acids lacks the eRF3-binding capacity but remains active as RF in vivo, as it is able to complement the *Sup45ts* allele (Ito et al., 1998a). On the other hand, the eRF3 GTPase activity depends solely on the presence of the ribosome and eRF1 but not on the peptidyl-tRNA and/or mRNA (Frolova et al., 1996). Functional uncoupling in vitro of these two reactions, peptidyl-tRNA hydrolysis and GTP hydrolysis, implies that these activities are mutually independent and may be associated with different parts of the eRF1 protein. We assume that eRF1 might be composed of two domains, one of which is involved in triggering the termination reaction and the other in activation of eRF1-dependent and ribosome-dependent eRF3 GTPase.

The aim of the experiments described herein was to uncouple structurally the two major functional activities of eRF1, peptidyl-tRNA hydrolysis and eRF3 GTPase activation, and, if this was successful, to map the border between the eRF1 domains responsible for these activities. To reach this goal, a set of eRF1 deletion mutants was generated and analyzed. We arrived at the conclusion that the human eRF1 protein is composed of two nonoverlapping domains: a catalytically active "core" encompassing the N-terminal and middle parts (NM domain) of the molecule, and the C-terminal domain responsible for activation of eRF3 GTPase.

RESULTS

Release activity of eRF1 deletion mutants

To map the eRF1 region critical for RF activity, the N-terminally and C-terminally truncated eRF1 proteins were expressed in *Escherichia coli* and purified (Fig. 1A,B). The activity of these eRF1 mutants was tested in an in vitro RF assay in the presence of mini-messenger RNA (mini-mRNA) containing initiation and termination codons (Grentzmann & Kelly, 1997) or in the presence of the initiation codon and one of the three termination codons (Tate & Caskey, 1990).

We had already shown that deletion of the last 22 amino acid residues from the C terminus of human eRF1 did not impair its RF activity (Merkulova et al., 1999). Analysis of a set of drastically C-truncated eRF1 mutants (Fig. 2A) revealed that deletion of 167 C-terminal amino acids (eRF1$^{2-270}$) did not affect the

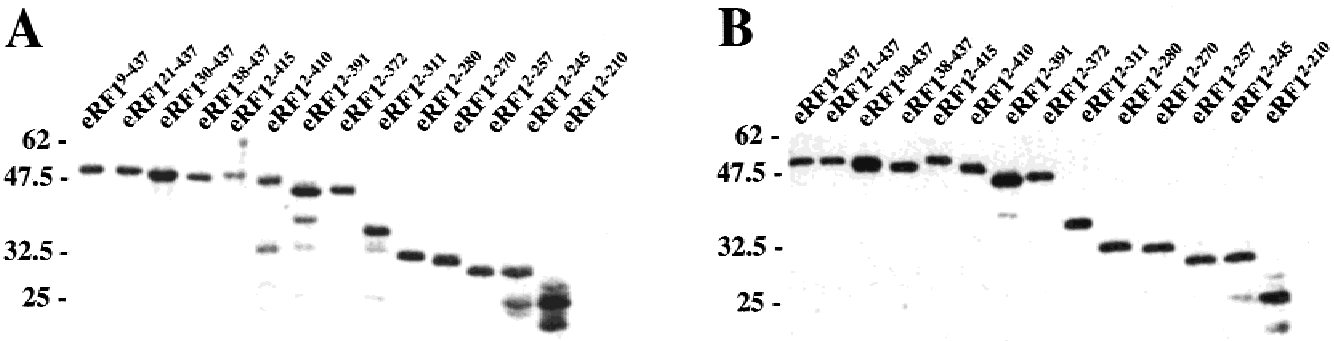

FIGURE 1. Electrophoretic mobility of bacterially expressed and purified human eRF1 deletion mutants. **A**: 10% PAGE-SDS followed by staining with Coomassie blue, 0.5 μg protein per track. **B**: Western blot analysis with anti-eRF1 antibodies, 0.05 μg protein per track. The positions of molecular mass markers are indicated on the left. Western blot analysis was performed as described (Frolova et al., 1998).

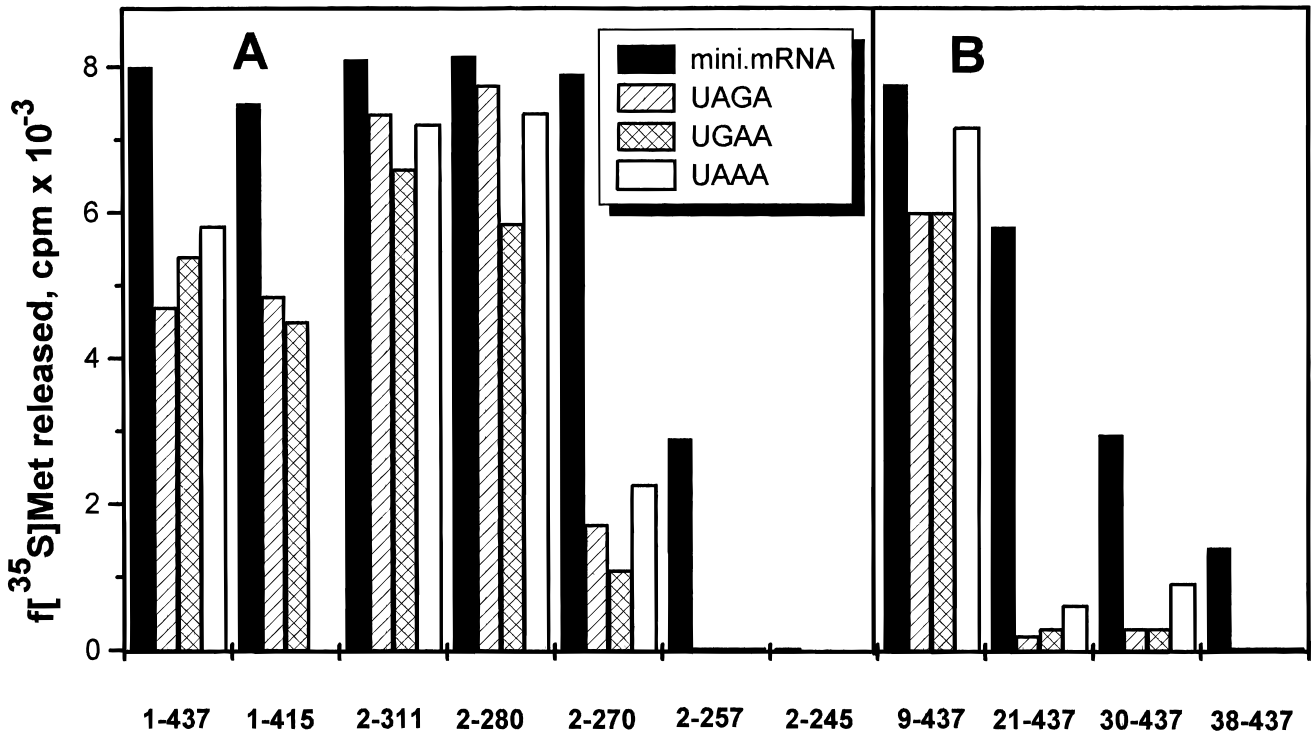

FIGURE 2. Release activity of the C-terminally (**A**) and N-terminally (**B**) truncated eRF1 mutants with various termination codons. The numbers under the abscissa indicate amino acid positions present in the wild-type eRF1 and its truncated mutants. Incubation mixtures (25 μL) contained 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 8 mM NH_4Cl, and 1.5 pmol of f[^{35}S]Met-tRNA$_f^{Met}$•UUCAUGUAAA•ribosome complex or 1.5 pmol of f[^{35}S]Met-tRNA$_f^{Met}$•AUG•ribosome complex plus 50 μM UAAA, UAGA, or UGAA. The concentration of the wild-type or mutant eRF1 was 0.15 μM. The samples were incubated for 20 min at 25 °C. The amount of f[^{35}S]Met released at zero time (1,000–1,500 cpm) for the given protein was subtracted from all values.

RF activity measured with mini-mRNA. We concluded that the C-terminal part of eRF1 was not essential for its ability to promote termination-codon-dependent and ribosome-dependent peptidyl-tRNA hydrolysis in vitro. The mini-mRNA was more efficient as a template than a combination of initiation and termination codons (Fig. 2A,B). The same difference was observed earlier with prokaryotic ribosomes and release factors (Grentzmann & Kelly, 1997).

The C-terminally and N-terminally truncated eRF1 mutants were inactive in the absence of stop-codon-containing tetraplets, or in the presence of a sense-codon-containing tetraplet UGGA, near cognate to UGA and UAG termination codons (data not shown). The wild-type human and *Xenopus laevis* eRF1 possess the same properties (Frolova et al., 1994). Therefore, the truncated active human eRF1 mutants are as specific toward the stop codons as the wild-type eRF1.

The stepwise shortening of eRF1 from the C terminus caused an increase in RF activity of the truncated mutants in the presence of UAG- or UAA-containing tetraplets (Fig. 2A), which did not exceed the level of the RF activity reached with mini-mRNA. Probably, the mini-mRNA binds better to the ribosome than tetraplets, and the removal of the C domain from eRF1 facilitates the binding of tetraplets with the ribosome, whereas for mini-mRNA closely resembling the natural mRNAs this effect is not manifested, as mini-mRNA binds efficiently even in the presence of the C domain.

When the calculated isoelectric points of the full-length eRF1 and its fragments were compared (Table 1), the charge distribution along the eRF1 polypeptide proved to be highly uneven. The positively charged amino acids prevailed within the NM domain (eRF1$^{1–270}$), whereas the negatively charged residues dominated in the C-ter-

TABLE 1. Isoelectric points and sizes of human eRF1 and its fragments.

eRF1	Positions of amino acid residues	Molecular mass (Da)	Isoelectric point (pI)[a]	Number of residues	
				Asp + Glu	Lys + Arg
Full-length	1–437	49,030.97	5.31	64	55
N domain[b]	1–189	20,715.96	9.85	20	27
NM domain	1–270	29,761.34	9.74	30	38
C domain	280–437	18,182.39	4.23	33	16

[a]The theoretical pI values were calculated using SWISS-PROT, search ExPASy, A.B.I.M.
[b]Defined arbitrarily.

minal part of the molecule (eRF1$^{280-437}$). Consequently, deletion of the C-terminal part converted eRF1 from an acidic to a basic protein and, in parallel, activated it. Presumably, the ribosomal A site, which accommodated eRF1, exhibited negative charges donated by rRNA and peptidyl-tRNA phosphate backbones surrounding this site. Owing to its positive charges, the eRF1$^{2-270}$ mutant appeared to bind to the ribosome more efficiently.

Comparison of the N termini of the eRF1 family showed that the first 12 amino acids varied significantly, whereas the amino acid stretch encompassing positions 13–39 in human eRF1 was conserved (Fig. 3A). In accord with that, the N-terminally truncated eRF1$^{9-437}$ was fully active in the RF assay with mini-mRNA or a mixture of initiation and termination codons (Fig. 2B). Further truncation from the N terminus (eRF1$^{21-437}$) caused a decrease of the RF activity in the presence of mini-mRNA, whereas eRF1$^{30-437}$ and eRF1$^{38-437}$ mutants were virtually inactive as RF in the presence of any of the three termination codons.

The gradual decrease in the RF activity observed for eRF1$^{2-270}$ deleted from the C terminus or for eRF1$^{9-437}$ deleted from the N terminus might be caused by reduction in their ribosome-binding ability. Consistent with this assumption are the data (Fig. 4) showing the inability of eRF1$^{2-245}$ and eRF1$^{38-437}$ mutants to compete with the full-length eRF1. In contrast, when one of the G residues in the GGQ motif of human eRF1 was mutated, although the RF activity was completely abolished, these inactive mutants were able to compete with the wild-type eRF1 for the ribosomal binding site and inhibited the RF activity (Frolova et al., 1999; Fig. 4).

Interaction of eRF1 deletion mutants with eRF3 in vitro and in vivo

In addition to its major function, promotion of peptidyl-tRNA cleavage, eRF1 exhibited another, entirely distinct function, activation of the eRF3 GTPase activity. We analyzed the stimulating properties of eRF1 mutants toward eRF3 GTPase (Table 2). Truncation of human eRF1 from either end farther than position 9 or position 415 caused a dramatic reduction of the eRF1 ability to promote GTP hydrolysis catalyzed by eRF3 in the presence of the ribosome. These observations were fully consistent with the earlier data (Merkulova et al., 1999) that removal of the C domain from the human

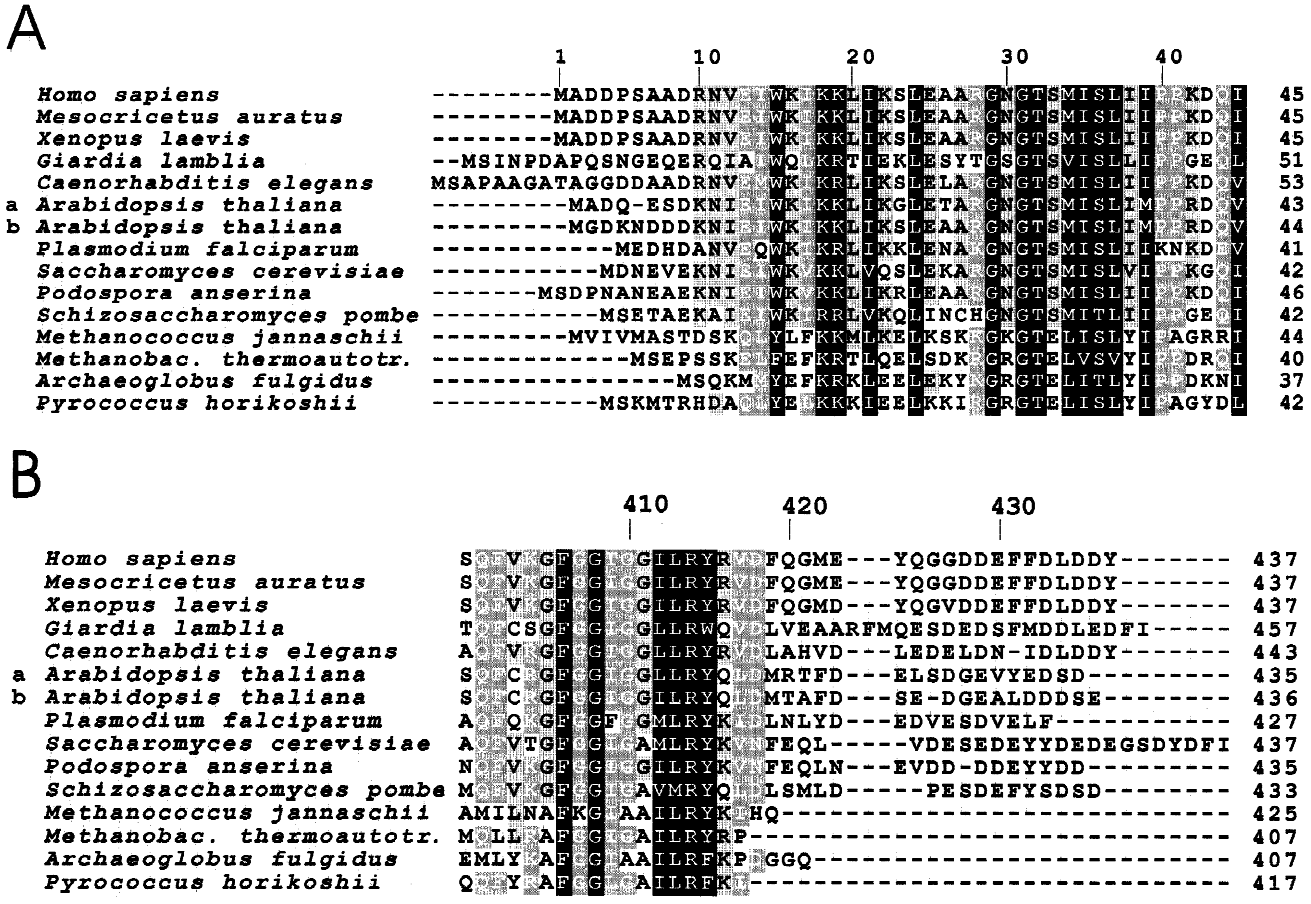

FIGURE 3. The N-terminal (**A**) and the C-terminal (**B**) sequences of class-1 release factors from eukaryotes and archaebacteria. The sequences were taken from SWISS-PROT and EMBL databases.

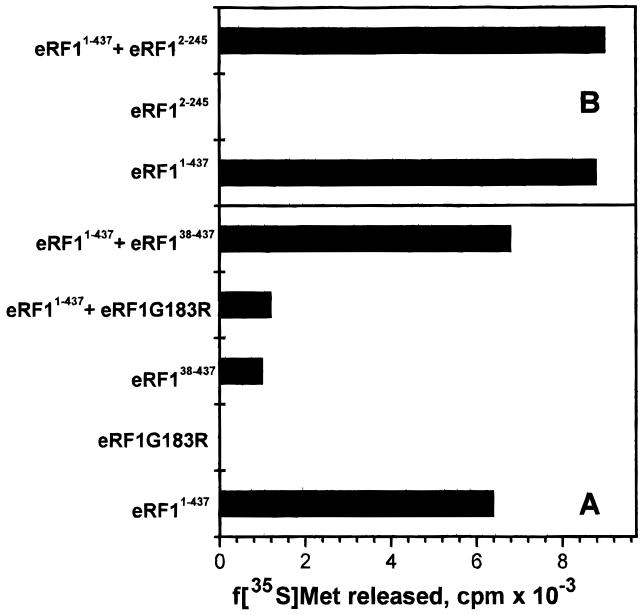

FIGURE 4. The influence of eRF1 deletion mutants on the in vitro RF activity of wild-type human eRF1. Incubation mixtures (25 μL) contained 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 8 mM NH_4Cl, and 1.5 pmol of f[^{35}S]Met-tRNA$_f^{Met}$•UUCAUGUAAA•ribosome complex. 2.5 pmol (**A**) or 3.2 pmol (**B**) of wild-type eRF1$^{1–437}$, 4.3 pmol of eRF1$^{38–437}$ or 6.0 pmol of eRF1G183R mutant, or 4 pmol of eRF1$^{2–245}$ were added alone or in different combinations as indicated. The samples were incubated for 20 min at 25 °C. The amount of f[^{35}S]Met released at zero time was subtracted from all values. The eRF1G183 mutant inactive in the RF assay and competing with wild-type human eRF1 was described earlier (Frolova et al., 1999) and used as a control.

TABLE 2. Stimulating activity of the wild-type and mutant eRF1 toward eRF3 GTPase.

	[γ-^{32}P]P$_i$ released, cpm $\times$ 10^{-3}
eRF1$^{1–437}$, wild-type	25.5
C-terminally truncated mutants	
eRF1$^{2–415}$	28.7
eRF1$^{2–410}$	8.7
eRF1$^{2–391}$	2.5
eRF1$^{2–373}$	0.7
eRF1$^{2–340}$	0
N-terminally truncated mutants	
eRF1$^{9–437}$	22.5
eRF1$^{21–437}$	15.5
eRF1$^{30–437}$	9.0
eRF1$^{38–437}$	5.5

Incubation mixture (12.5 μL) contained 2 μM [γ-^{32}P]GTP (specific activity 10^4 cpm/pmol), 20 mM Tris-HCl, pH 7.5, 30 mM NH_4Cl, 15 mM $MgCl_2$, 0.1 μ ribosomes, 0.4 μ eRF1 and eRF3 each. The reaction was run at 37 °C for 20 min, and stopped by adding 0.75 mL of 5% charcoal suspension in 50 mM Na_2HPO_4 on ice. The mixture was vortexed and centrifuged at 10,000 rpm for 10 min at 4 °C, and the [γ-^{2}P]P$_i$ released into 0.5 mL of supernatant was quantitated by liquid scintillation counting. The release of [γ-^{32}P]P$_i$ in the absence of the ribosomes was subtracted from all values and constituted 5–8% of that in the presence of all components of the incubation mixture.

eRF1 completely abolished the ability of eRF1 to bind eRF3 in vivo. However, the N-terminal eRF1 fragment 2–280 was not essential for binding (Merkulova et al., 1999), and therefore the inability of the N-truncated eRF1 mutants to activate eRF3 GTPase could not be attributed to a loss of eRF3-binding ability. One of the possible explanations for this inactivity was that the N-terminal deletions attenuated the ribosome-binding ability of the eRF1 mutants, a prerequisite for both RF activity and stimulating activity toward eRF3 GTPase. It was noteworthy that gradual loss of the stimulating activity toward eRF3 GTPase and of the RF activity for the same eRF1 mutants took place in parallel (Fig. 2B; Table 2).

Comparison of the data presented here (Fig. 2; Table 2) with the data obtained earlier on the eRF1•eRF3 interaction in vivo (Merkulova et al., 1999) indicated that NM domain of eRF1 was in charge of its trigger activity toward peptidyl-tRNA hydrolysis, whereas the C domain is responsible for eRF3 binding.

This two-domain model was further examined by quantitative measurements of human eRF1•eRF3 binding in yeast two-hybrid system (Fig. 5). The efficiency of the interaction of eRF1•eRF3 was followed by measuring the activity of the reporter gene product, a β-galactosidase. The highest activity in this system (about 450 Miller units) was observed when eRF1 and eRF3 were fused with the LexA DNA-binding domain (BD) and the Gal4 activation domain (AD), respectively (Fig. 5). If the partners were exchanged, about a ninefold reduction in the efficiency of the reporter gene was observed. This result emphasized the necessity of appropriate controls when the deletion mutants were fused with LexA BD or Gal4 AD. The eRF1$^{306–437}$ mutant did not bind eRF3, whereas the eRF1$^{271–437}$ mutant is fully active, demonstrating the role of eRF1 fragment 271–305 in this interaction. The ability of some eRF1 mutants, weakly active or inactive in the two-hybrid system, to activate the wild-type eRF3 in an in vitro GTPase assay (Fig. 5; Table 2) was probably due to the presence of the ribosomes, which might have facilitated the mutual binding of the eRFs, in contrast to the yeast two-hybrid assay, where the factors interact in the absence of the ribosomes.

Because the C-terminal 17 amino acids of *S. pombe* eRF1 were found to be essential for eRF3 binding (Ito et al., 1998a) we reexamined the properties of the eRF1$^{2–415}$ mutant (Fig. 5). Surprisingly, its binding activity toward eRF3 was even higher than that of full-length eRF1, thus ruling out the involvement of the last 22 amino acid residues of human eRF1 in eRF3 binding by contrast to *S. pombe* eRF1. Furthermore, archaebacterial class-1 RFs lacked this stretch of amino acids or this region was shortened. Our conclusion that the extreme C terminus of human eRF1 was not essential for eRF3 binding was consistent with the non-

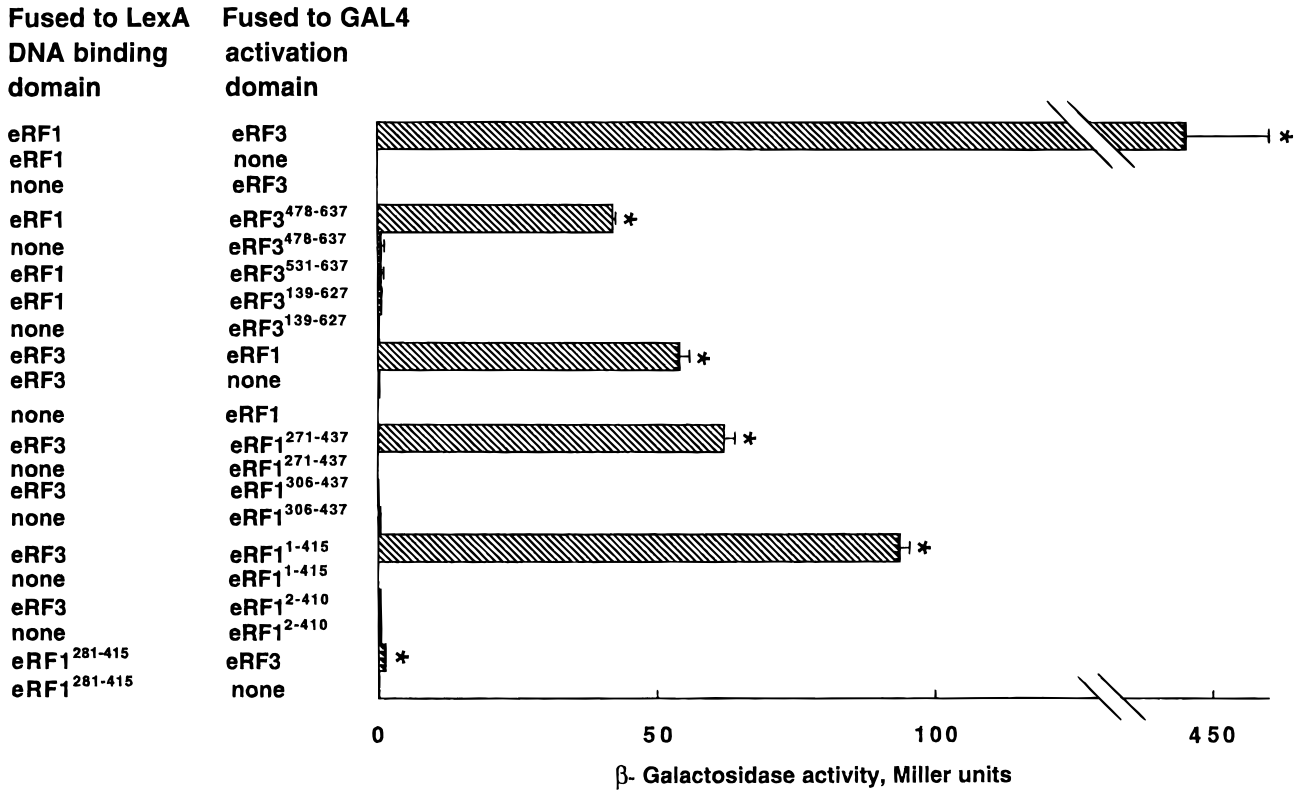

FIGURE 5. Quantitative analysis of the interaction between eRF1 and eRF3 and their deletion mutants in the yeast two-hybrid system. Interaction was scored as the activation of *lacZ* reporter gene in double-transformed diploids by β-galactosidase activity measurements in liquid assay with ONPG as a substrate (see Materials and Methods). Numbers represent the mean values from triplicates $\pm$ standard deviation. *$P < 0.001$ compared with the highest value of β-galactosidase activity in the diploids transformed with the corresponding construct and the empty opposite two-hybrid vector.

conserved primary structure of this region in eukaryotic class-1 factors (Fig. 3B). When five highly conserved amino acids of eRF1 were deleted (eRF1$^{2-410}$ mutant), its binding capacity toward eRF3 was abolished (Fig. 5).

Thus the regions in human eRF1 important for eRF3 binding were located within conserved amino acid sequences at both termini (Fig. 3). For the C terminus of yeast eRF1 involved in interaction with eRF3 (Ito et al., 1998a; Ebihara & Nakamura, 1999; Eurwilaichitr et al., 1999) this was not the case, although the overall structural similarity between eRF1 from these species was high (Frolova et al., 1994). These apparent differences between higher and lower eukaryotes deserve further elucidation.

The eRF1$^{281-415}$ mutant truncated from both ends possessed very low but significant binding capacity, although the eRF1$^{271-437}$ and eRF1$^{2-415}$ mutants were as active as the full-length eRF1 in eRF3 binding (Fig. 5). This result implied that the eRF1 region between positions 272 and 280 could also be essential for eRF3 binding. Alternatively, simultaneous truncation from both ends might cause destabilization of the eRF1$^{281-415}$ structure, leading to reduction of its binding capacity.

The binding of eRF3$^{478-637}$ mutant to eRF1 was about 50 times higher than that of eRF3$^{531-637}$ (the binding of the eRF3$^{531-637}$ mutant with the full-length eRF1 was the same as for the pair of the full-length eRF1 and the empty vector) confirming that eRF3 fragment 478–530 was critical for eRF3 binding to eRF1 (Merkulova et al., 1999). The N-terminally truncated mutant eRF1$^{271-437}$ retained its binding capacity, indicating the noninvolvement of the NM domain of eRF1 in eRF3 binding, shown qualitatively earlier (Ito et al., 1998a; Merkulova et al., 1999). Deletion of the C-terminal 10 amino acids (eRF3$^{139-627}$) caused profound loss of binding capacity.

In summary, in vitro data on the RF activity of the truncated eRF1 mutants and in vivo data on eRF1•eRF3 interaction are consistent with the two-domain model. The border between the two domains is located most probably around positions 270–280 of human eRF1.

DISCUSSION

The high conservation of the eRF1 family (Frolova et al., 1994) is probably associated with the multiplicity of functional sites in this protein. Because eRF1 at the ribosomal A site triggers the hydrolytic reaction at the peptidyl transferase center of the ribosome, eRF1 should contain a ribosome-binding site (RBS). If class-1 RFs interact directly with the termination codon at the ribo-

somal A site (Brown & Tate, 1994; Ito et al., 1996, 1998b; Tate et al., 1996; Nakamura & Ito, 1998), a termination codon-binding site (TCBS) might also exist. The eRF1-triggered cleavage of the bond between peptidyl and tRNA moieties is a stop-codon-dependent and ribosome-dependent reaction. Consequently, one may anticipate the existence of a peptidyl-tRNA binding site (PRBS). Finally, as considered above, eRF1 binds eRF3, and an eRF3-binding site essential for activation of eRF3 GTPase should be also present in eRF1. The multifunctional nature of eRF1 manifested in the four binding sites for ribosome, termination signal, eRF3, and probably peptidyl-tRNA, might be associated with its multidomain organization. The primary goal in elucidating the functional anatomy of eRF1 is to map these sites along the polypeptide chain.

The eRF1$^{2–270}$ mutant drastically truncated from the C terminus for more than one-third of total protein mass remains fully active in the RF assay (Fig. 2A). Hence three binding sites (RBS, TCBS, and PRBS) critical for the RF activity are nested in the NM domain of the eRF1 polypeptide. As shown earlier (Merkulova et al., 1999) and quantitatively confirmed here (Fig. 5), the C domain of human eRF1 is involved in eRF3 binding. This conclusion is consistent with the observation that eRF1 mutants truncated from the C side farther than 22 amino acids, which are not essential, lose considerably their stimulating activity toward eRF3 GTPase (Table 2). Consequently, we separated physically two eRF1 activities: the release activity that resides in the NM domain (positions 9–270) and the eRF3-binding ability tightly associated with the GTPase activating function, which resides in the C-terminal third of the polypeptide chain.

The NM domain of human eRF1 active on its own in promoting peptidyl-tRNA hydrolysis may be designated as a “core” domain (Fig. 6). From the functional point of view, the C-terminal domain may be named an eRF3-binding domain and it extends from position 275 $\pm$ 5 to position 415. Presumably, there is a short sequence that links the two domains, probably located between positions 270 and 280.

The result that the core domain is fully competent as RF (Fig. 2A) is consistent with our earlier data showing noninvolvement of the core domain in eRF3 binding (Merkulova et al., 1999) and dispensability of eRF3 and GTP for eRF1 activity (Frolova et al., 1994, 1996). All these observations taken together strongly argue against the hypothesis that the translation termination reaction (peptidyl-tRNA hydrolysis) is mediated by formation of a heterodimer between eRF1 and eRF3 (Stansfield et al., 1995a, 1995b).

It has been suggested (Ito et al., 1996; Nakamura et al., 1996; partly revised in Ito et al., 1998a; Nakamura & Ito, 1998) that class-2 RFs (RF3 and eRF3) may be a functional equivalent of EFTu or eEF1α (elongation factors of prokaryotes and eukaryotes, respectively). As shown in this work, the core domain of human eRF1 is active as a release factor (Fig. 2A) being completely devoid of the eRF3-binding activity (Fig. 5). In contrast, for aminoacyl-tRNA binding to the ribosome, the presence of eEF1α is essential. Therefore, we suppose that eRF3 is not functionally similar to eEF1α.

The peculiar feature of the core domain is its amino acid composition highly enriched in positively charged amino acid residues, in contrast to the C domain, which is acidic (Table 1). From this profound difference in calculated isoelectric points between the two domains, one may anticipate some interesting consequences. First, the two domains may electrostatically interact with each other, and this interaction may affect the properties of both domains. In fact, removal of the C domain enhances the RF activity with stop codons (Fig. 2A) and removal of the core domain abolishes the activating capacity of the C domain toward GTPase, though the binding ability is preserved (Table 2; Fig. 5). Second, the A site of the ribosome (probably negatively charged, in part because of the presence of rRNA sequences) and the C domain of eRF1 (with low pI) could compete with each other for the positively charged core domain (high pI). This interplay may add a new dimension in the regulation of the termination step of protein synthesis. For example, binding of the C domain of eRF1 to eRF3 may facilitate interaction of the core domain with the ribosome and thereby may stimulate the eRF1 activity at low stop codon concentration (Zhouravleva et al., 1995). Enhancement of the RF activity with stop-codon-containing tetraplets in the absence of the eRF3-binding domain (Fig. 2A) is in line with this suggestion.

The GTPase activity depends not only on eRF3 and the ribosome (Frolova et al., 1996), but also on the

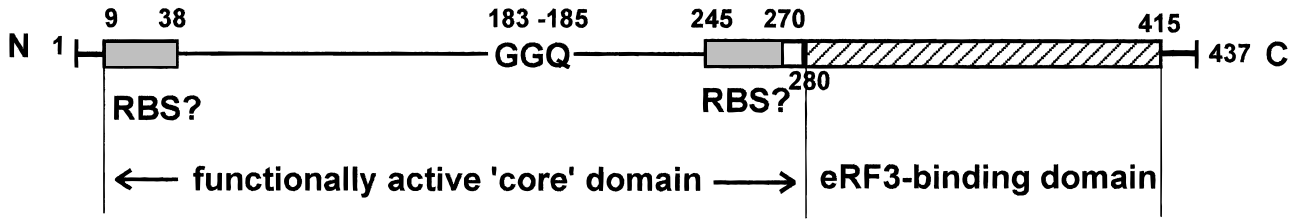

FIGURE 6. Two-domain organization of human eRF1. RBS: ribosome binding site; white box: linker between domains; solid black lines: nonessential regions at the ends of the polypeptide.

presence of both domains of eRF1. In the absence of the core domain or eRF3-binding domain the eRF3 GTPase activity is abolished (Table 2). The eRF3-binding domain of eRF1 interacts with the C-terminal domain of eRF3 (Merkulova et al., 1999; Ebihara & Nakamura, 1999), whereas the association of the eRF1•eRF3 complex with the ribosome is governed by the core domain, which contains RBS(s).

The two-domain model had already been suggested for prokaryotic class-1 RFs (Moffat & Tate, 1994), based on the observation that limited proteolysis of the *E. coli* RF2 generated a nicked protein that was able to interact with the ribosome and probably with stop codons but was inactive in promoting peptidyl-tRNA hydrolysis. Two domains of the prokaryotic RF1/2 functionally correspond to the core domain of the eukaryotic model suggested in this work. However, archaeal genomes do not contain genes encoding RF3 (see Nakamura & Ito, 1998), although the primary structures of archaeal RF1 are much closer to eRF1 than to prokaryotic RF1/2. Therefore, it was anticipated that this living kingdom should contain eRF3-like genes. Because no information is available regarding the biochemical properties of RF1 from Archaea, two possibilities may be considered: RF3 is encoded in archaeal genomes but has not yet been identified because of its divergence from both eukaryotic and prokaryotic class-2 RFs, or, alternatively, the C domain of archaeal RF1 binds another protein(s), not RF3.

Entirely different functions of the core and eRF3-binding domains, their topological separation along the eRF1 polypeptide chain, and distinct physicochemical features point to the possibility that these domains originated from different genes and fused later in evolution.

The full competence of the eRF1 core domain in triggering peptidyl-tRNA hydrolysis (Fig. 2A) implies that RBS(s) is (are) located inside this domain. This conclusion is consistent with the observation that *S. cerevisiae* eRF1 lacking the C-terminal 32 amino acids still retained its ability to bind to the yeast ribosomes (Eurwilaichitr et al., 1999). Where is (are) RBS(s) located in the core domain? A gradual decrease in RF activity occurs when eRF1 is truncated either from the N terminus, positions 9–38 (Fig. 2B), or from the C terminus, positions 280–245 (Fig. 2A). In both cases, these eRF1 mutants, either inactive or with strongly diminished RF activity, weakly compete or do not compete at all with the full-length eRF1 in the RF assay (Fig. 4). The N-terminally truncated mutants eRF1$^{30-437}$ and eRF1$^{38-437}$ also have reduced ability to activate eRF3 GTPase (Table 2), though the eRF3-binding domain is preserved. We have shown (Merkulova et al., 1999) that the eRF3-binding domain together with the ribosome are unable to induce eRF3 GTPase. All these data taken together are consistent with the assumption that RBS sequences flank the core domain on both sides (Fig. 6). Consequently, we propose that at least two segments of the core domain are involved in ribosome binding, from conserved positions 13 to 38 and from positions 246 to 275 $\pm$ 5. It is noteworthy that the 9–38 fragment contains six positively charged residues (Fig. 3A) and therefore might possess RNA-binding ability.

In conclusion, human and most probably other eukaryotic class-1 RFs are composed of two functionally distinct domains that are topologically separated along the polypeptide chain of eRF1, are different in size and physicochemical properties, and presumably originated from different parts of the eukaryotic genome by gene fusion. This novel conclusion provides a basis for further progress in understanding translation termination in eukaryotes.

MATERIALS AND METHODS

Materials

L-[^{35}S]methionine (specific activity 10^4 cpm/pmol) and [γ-^{32}P]GTP (specific activity 10^4 cpm/pmol) were from Amersham, pQE-30 plasmid from Qiagen, Talon™ metal affinity resin from Clontech, Pwo polymerase from Boehringer Mannheim; AUG, tetraplets containing stop codons, and the mini-mRNA UUCAUGUAAA were synthesized by A. Veniaminova and M. Ryabkova (Novosibirsk State University).

Bacterial strains, vectors, and cloning

A pQE-30 plasmid was used to clone the full-length human eRF1 and its truncated forms. *E. coli* strain M15 [pREP4] was used for the production of recombinant eRF1. Preparation of competent *E. coli* cells, transfections, and isolation of plasmid DNAs were performed by standard protocols (Sambrook et al., 1989).

Yeast two-hybrid analysis

The pVJL12/eRF1 and pVJL11/eRF3 constructs expressing corresponding eRFs as C-terminal parts of fusion proteins with the bacterial LexA DNA-binding domain (LexA) and the pGAD3s2x/eRF1 and pGADGH/eRF3 constructs expressing corresponding eRFs as C-terminal parts of the fusion proteins with the yeast Gal4 transactivation domain (GAL4AD) have been described previously (Frolova et al., 1998). Preparation of the series of N-terminally and C-terminally truncated forms of both proteins within corresponding pGADs was described (Merkulova et al., 1999). The eRF1$^{281-415}$ mutant was generated by PCR using Pwo polymerase and the following primers: forward, 5′-ATATGGATCCATTCAAGAGA AGAAATTAATAG-3′; reverse, 5′-ATATGTCGACCTAGTACC GCAAGATACCTCC-3′. The PCR product was subcloned into *Bam*HI and *Sal*I sites of pVJL12, and its structure was confirmed by sequencing (for details see Jullien-Flores et al., 1995).

Two-hybrid analysis of the interaction between eRFs was monitored by expression of two reporter genes, *LacZ* and

HIS3 in diploids, obtained after mating the *S. cerevisiae* strain L40 (*MAT***a**), transformed with a pVJL expressing one of the full-length eRFs, and the *S. cerevisiae* strain AMR70 (*MATa*), transformed with a pGAD expressing one of the truncated versions of another eRF (Frolova et al., 1998). Expression of the *LacZ* reporter was also followed by measuring β-galactosidase activity with *o*-nitrophenyl-β-D-galactoside (ONPG) as a substrate. In this case, double-transformed diploids, selected on tryptophan- and leucine-deficient medium, were inoculated in the same liquid medium and grown overnight at 30 °C to a density of about 1.5 at A_{600}. Yeast cells from 1 mL of culture were harvested by centrifugation, resuspended in 0.5 mL of 0.1 M sodium phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, permeabilized by vortexing with 25 μL of $CHCl_3$, and incubated at 30 °C for 10 min. The reaction was initiated by addition of 100 μL of ONPG (4 mg/mL in water) and stopped after 5 or 30 min of incubation at 30 °C by addition of Na_2CO_3 to a final concentration of 0.2 M. Accumulation of *o*-nitrophenol was measured at A_{420}. β-Galactosidase activity was expressed in Miller units, calculated as follows: $A_{420} \times (1{,}000/\text{time, min}) \times (1/A_{600})$. All assays were done in triplicate.

Expression and purification of wild-type and mutant human eRF1 proteins

Subcloning of PCR-generated products for $eRF1^{271-437}$, $eRF1^{306-437}$, $eRF1^{2-415}$, and $eRF1^{2-410}$ into a pQE30 expression vector in frame with a His-tag placed at the N terminus of the resultant recombinant proteins has been described (Merkulova et al., 1999). All other eRF1 deletion mutants as well as the PCR primers used for their generation are listed in Table 3. PCR products for $eRF1^{9-437}$, $eRF1^{21-437}$, $eRF1^{30-437}$, and $eRF1^{38-437}$ were subcloned into *Sac*I and *Sal*I sites of pQE30. For subcloning of PCR products for all C-terminally truncated eRF1 forms, *Asp*718 and *Pst*I sites of pQE30 were used. The structures of all constructs were verified by sequencing.

TABLE 3. Mutants and PCR primers used to generate the constructs for bacterial expression of truncated eRF1 proteins.

eRF1 mutant	Primers
$eRF1^{9-437}$	5′-ATATGAGCTCGACAGGAACGTGGAGAT-3′
$eRF1^{21-437}$	5′-ATATGAGCTCATTAAGAGCTTGGAGGC-3′
$eRF1^{30-437}$	5′-ATATGAGCTCAATGGCACCAGCATGAT-3′
$eRF1^{38-437}$	5′-ATATGAGCTCATCATTCCTCCCAAAGA-3′
$eRF1^{2-340}$	5′-ATATCTGCAGCTACTCTTCTGTGCCTTGGCAA-3′
$eRF1^{2-311}$	5′-ATATCTGCAGCTAAGCCTTTAGTGTATCTTCAAC-3′
$eRF1^{2-280}$	5′-ATATCTGCAGCTAGAATTTCACGTTGGAGAGGA-3′
$eRF1^{2-270}$	5′-ATATCTGCAGCTATAACTCAATAGCTTGGTTGAAT-3′
$eRF1^{2-257}$	5′-ATATCTGCAGCTAGGATATATCAACTAATTTTAAAAC-3′
$eRF1^{2-245}$	5′-ATATCTGCAGCTACCTCTGATCAAACATATCAGA-3′
$eRF1^{2-210}$	5′-ATATCTGCAGCTACACAGCAGTCTCTGCTACT-3′

5′-ATATGAATTCTCGGTACCGCGGACGACCCCAGTGC-3′ was used as a forward primer to generate C-terminal deletion mutants; 5′-TTGTCGACCTAGTAGTCATCAAGGTCA-3′ was used as a reverse primer to generate N-terminal deletion mutants.

Protein expression was induced by adding IPTG to a final concentration of 0.4 mM. The bacterial pellet was resuspended in 20 mL of lysis buffer containing 50 mM Tris-HCl, pH 7.5, 0.2 M KCl, 10 mM imidazole, 1% Nonidet P40, 10% glycerol, 2 mM β-mercaptoethanol, 1 mM PMSF, and 0.2 mg/mL of lysozyme, incubated 15 min at room temperature, sonicated, and centrifuged at 10,000 $\times$ g for 30 min. Then, 150 μL of Talon™ metal affinity resin equilibrated with buffer A (20 mM Tris-HCl, pH 8.0, 0.2 M KCl, 10% glycerol) containing 10 mM imidazole was added to the supernatants. After 30 min incubation at room temperature the Talon™ suspension was transferred into a column and the resin was washed with 10 vol of buffer A plus 10 mM imidazole. His-tagged protein was eluted with buffer A containing 0.1 M imidazole; 0.2-mL fractions were collected and analyzed by 10% PAGE-SDS. Protein-containing fractions were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10% glycerol, 0.1 mM EDTA, and 1 mM DTT. In some cases, protein eluted from the column was diluted 1:10 with the same buffer without glycerol and concentrated in an Ultrafree concentration unit (Millipore). Protein concentration was determined with the Bio-Rad protein assay reagent using BSA as a standard.

Assays for eRF1 activities in vitro

The eRF1 release activity was measured in an in vitro assay as stop-codon-dependent hydrolysis of f[^{35}S]Met-$tRNA_f^{Met}$ associated with the AUG•80S ribosome complex according to Tate and Caskey (1990) with some modifications (Frolova et al., 1994), or as hydrolysis of f[^{35}S]Met-$tRNA_f^{Met}$ associated with 80S ribosomes programmed with mini-mRNA UUCAUGUAAA containing the initiation (AUG) and termination (UAA) codons (Grentzmann & Kelly, 1997). Isolation of rabbit reticulocyte ribosomal subunits, expression, and purification of the human eRF3 were described (Frolova et al., 1996, 1998). The eRF3 GTPase activity was followed by accumulation of [γ-^{32}P]P_i after hydrolysis of [γ-^{32}P]GTP using a charcoal assay (Frolova et al., 1996).

ACKNOWLEDGMENTS

We are grateful to Armand Tavitian, Michel Philippe, Jean de Gunzburg, and Jacques Camonis for generous support, to Sylvain Blanquet and Yves Mechulam for providing *E. coli* $tRNA_f^{Met}$ and formylase, to Peter Simonenko for providing rabbit ribosomes and to Nina Oparina for help in preparation of the figures. Critical remarks of both referees and Alexander Galkin were very helpful in preparing the revised version of the manuscript. This work was supported by the Human Frontiers Science Program (grant RG0032/1997-M), by the INTAS-96-1706 grant, by Chaire Internationale Blaise Pascal (T.M. and L.K.), by the French Ministère de l'Education Nationale de Recherche et de la Technologie (contrat triennal, L.F.), by PICS du Centre National de la Recherche Scientifique (L.F. and L.K.), by the Russian Foundation for Basic Research (L.K.), and by the Program of Support for Russian Scientific Schools (L.K.).

Received July 9, 1999; returned for revision September 15, 1999; revised manuscript received October 14, 1999

REFERENCES

Brown CM, Tate WP. 1994. Direct recognition of mRNA stop signals by *Escherichia coli* polypeptide chain release factor two. *J Biol Chem 269*:33164–33170.

Buckingham RH, Grentzmann G, Kisselev L. 1997. Polypeptide chain release factors. *Mol Microbiol 24*:449–456.

Ebihara K, Nakamura Y. 1999. C-terminal interaction of translational release factors eRF1 and eRF3 of fission yeast: G-domain uncoupled binding and the role of conserved amino acids. *RNA 5*:739–750.

Eurwilaichitr L, Graves FM, Stansfield I, Tuite M. 1999. The C-terminus of eRF1 defines a functionally important domain for translation termination in *Saccharomyces cerevisiae*. *Mol Microbiol 32*:485–496.

Freistroffer DV, Pavlov MYu, MacDougall J, Buckingham RH, Ehrenberg M. 1997. Release factor RF3 in *E. coli* accelerates the dissociation of release factors RF1 and RF2 from the ribosome in a GTP-dependent manner. *EMBO J 16*:4126–4133.

Frolova L, Le Goff X, Rasmussen HH, Cheperegin S, Drugeon G, Kress M, Arman I, Haenni A-L, Celis JE, Philippe M, Justesen J, Kisselev L. 1994. A highly conserved eukaryotic protein family possessing properties of polypeptide chain release factor. *Nature 372*:701–703.

Frolova L, Le Goff X, Zhouravleva G, Davydova E, Philippe M, Kisselev L. 1996. Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase. *RNA 2*:334–341.

Frolova LYu, Simonsen JL, Merkulova TI, Litvinov DY, Martensen PM, Rechinsky VO, Camonis J, Kisselev LL, Justesen J. 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 256*:36–44.

Frolova LYu, Tsivkovskii RYu, Sivolobova GF, Oparina NYu, Serpinsky OI, Blinov VM, Tatkov SI, Kisselev LL. 1999. Mutations in the highly conserved GGQ motif of class 1 polypeptide release factors abolish ability of human eRF1 to trigger peptidyl-tRNA hydrolysis. *RNA 5*:1014–1020.

Grentzmann G, Brechemier-Baey D, Heurgue V, Mora L, Buckingham RH. 1994. Localization and characterization of the gene encoding release factor RF3 in *Escherichia coli*. *Proc Natl Acad Sci USA* 91:5848–5852.

Grentzmann G, Kelly PJ. 1997. Ribosomal binding site of release factors RF1 and RF2. A new translational termination assay in vitro. *J Biol Chem 272*:12300–12304.

Grentzmann G, Kelly PJ, Laalami S, Shuda M, Firpo MA, Cenatiempo Y, Kaji A. 1998. Release factor RF-3 GTPase activity acts in disassembly of the ribosome termination complex. *RNA 4*:973–983.

Hoshino S-i, Imai M, Mizutani M, Kikuchi Y, Hanaoka F, Ui M, Katada T. 1998. Molecular cloning of a novel member of the eukaryotic polypeptide chain-releasing factors (eRF). Its identification as eRF3 interacting with eRF1. *J Biol Chem 273*:22254–22259.

Ito K, Ebihara K, Nakamura Y. 1998a. The stretch of C-terminal acidic amino acids of translational release factor eRF1 is a primary binding site for eRF3 of fission yeast. *RNA 4*:958–972.

Ito K, Ebihara K, Uno M, Nakamura Y. 1996. Conserved motifs of prokaryotic and eukaryotic polypeptide chain release factors: tRNA–protein mimicry hypothesis. *Proc Natl Acad Sci USA 93*: 5443–5448.

Ito K, Uno M, Nakamura Y. 1998b. Single amino acid substitution in prokaryotic polypeptide release factor 2 permits it to terminate translation at all three termination codons. *Proc Natl Acad Sci USA 95*:8165–8169.

Jullien-Flores V, Dorseuil O, Romero F, Letourneur F, Saragosti S, Berger R, Tavitian A, Gacon G, Camonis JH. 1995. Bridging Ral GTPase to Rho pathways. RLIP76, a Ral effector with CDC42/Rac GTPase-activating activity. *J Biol Chem 270*:22473–22477.

Konecki DS, Aune KS, Tate WP, Caskey CT. 1977. Characterization of reticulocyte release factor. *J Biol Chem 252*:4514–4520.

Merkulova TI, Frolova LY, Lazar M, Camonis J, Kisselev LL. 1999. C-terminal domains of human translation termination factors eRF1 and eRF3 mediate their in vivo interaction. *FEBS Lett 443*:41–47.

Mikuni O, Ito K, Moffat J, Matsumura K, McCaughan K, Nobukuni T, Tate W, Nakamura Y. 1994. Identification of the *prfC* gene, which encodes peptide-chain release factor 3 of *Escherichia coli*. *Proc Natl Acad Sci USA 91*:5798–5802.

Moffat JG, Tate WP. 1994. A single proteolytic cleavage in release factor 2 stabilizes ribosome binding and abolishes peptidyl-tRNA hydrolysis activity. *J Biol Chem 269*:18899–18903.

Nakamura Y, Ito K. 1998. How proteins read the stop codon and terminate translation. *Genes Cells 3*:265–278.

Nakamura Y, Ito K, Isaksson LA. 1996. Emerging understanding of translation termination. *Cell 87*:147–150.

Nakamura Y, Ito K, Matsumura K, Kawazu Y, Ebihara K. 1995. Regulation of translation termination: Conserved structural motifs in bacterial and eukaryotic polypeptide release factors. *Biochem Cell Biol 73*:1113–1122.

Paushkin SV, Kushnirov VV, Smirnov VN, Ter-Avanesyan MD. 1997. Interaction between yeast Sup45p (eRF1) and Sup35p (eRF3) polypeptide chain release factors: Implications for prion-dependent regulation. *Mol Cell Biol 17*:2798–2805.

Pel HJ, Moffat JG, Ito K, Nakamura Y, Tate WP. 1998. *Escherichia coli* release factor 3: Resolving the paradox of a typical G protein structure and atypical function with guanine nucleotides. *RNA* 4:47–54.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning. A laboratory manual.* 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Scolnick E, Tompkins R, Caskey T, Nirenberg M. 1968. Release factors differing in specificity for termination codons. *Proc Natl Acad Sci USA 61*:768–774.

Stansfield I, Jones KM, Kushnirov VV, Dagkesamanskaya AR, Poznyakovski AI, Paushkin SV, Nierras CR, Cox BS, Ter-Avanesyan MD, Tuite MF. 1995a. The products of the *SUP45* (eRF1) and *SUP35* genes interact to mediate translational termination in *Saccharomyces cerevisiae*. *EMBO J 14*:4365–4373.

Stansfield I, Jones KM, Tuite MF. 1995b. The end in sight: Terminating translation in eukaryotes. *Trends Biochem* Sci *20*:489–491.

Tate WP, Brown CM. 1992. Translational termination: 'Stop' for protein synthesis or 'pause' for regulation of gene expression. *Biochem 31*:2443–2450.

Tate WP, Caskey CT. 1990. Termination of protein synthesis. In: Speding G, ed. *Ribosomes and protein synthesis. A practical approach*. Oxford: IRL Press. pp 81–100.

Tate WP, Poole ES, Mannering SA. 1996. Hidden infidelities of the translational stop signal. *Prog Nucleic Acids Res Mol Biol 52*: 293–335.

Zhouravleva G, Frolova L, Le Goff X, Le Guellec R, Inge-Vechtomov S, Kisselev L, Philippe M. 1995. Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. *EMBO J 14*:4065–4072.