Mutations in the highly conserved GGQ motif of class 1 polypeptide release factors abolish ability of human eRF1 to trigger peptidyl-tRNA hydrolysis

LUDMILA YU. FROLOVA,^{1,3,4} RUSLAN YU. TSIVKOVSKII,² GALINA F. SIVOLOBOVA,²
NINA YU. OPARINA,¹ OLEG I. SERPINSKY,² VLADIMIR M. BLINOV,²
SERGEY I. TATKOV,² and LEV L. KISSELEV^{1,3}

¹Engelhardt Institute of Molecular Biology, 117984 Moscow, Russia

²Institute of Molecular Biology, NPO Vector, Koltzovo, Novosibirsk region, Russia

³U248 INSERM, Institut Curie, Paris 70005, France

⁴UPR41 CNRS, Université de Rennes 1, 350042 Rennes Cedex, France

ABSTRACT

Although the primary structures of class 1 polypeptide release factors (RF1 and RF2 in prokaryotes, eRF1 in eukaryotes) are known, the molecular basis by which they function in translational termination remains obscure. Because all class 1 RFs promote a stop-codon-dependent and ribosome-dependent hydrolysis of peptidyl-tRNAs, one may anticipate that this common function relies on a common structural motif(s). We have compared amino acid sequences of the available class 1 RFs and found a novel, common, unique, and strictly conserved GGQ motif that should be in a loop (coil) conformation as deduced by programs predicting protein secondary structure. Site-directed mutagenesis of the human eRF1 as a representative of class 1 RFs shows that substitution of both glycyl residues in this motif, G183 and G184, causes complete inactivation of the protein as a release factor toward all three stop codons, whereas two adjacent amino acid residues, G181 and R182, are functionally nonessential. Inactive human eRF1 mutants compete in release assays with wild-type eRF1 and strongly inhibit their release activity. Mutations of the glycyl residues in this motif do not affect another function, the ability of eRF1 together with the ribosome to induce GTPase activity of human eRF3, a class 2 RF. We assume that the novel highly conserved GGQ motif is implicated directly or indirectly in the activity of class 1 RFs in translation termination.

Keywords: eukaryotes; prokaryotes; site-directed mutagenesis; translation termination

INTRODUCTION

Termination of translation requires the positioning of peptidyl-tRNA at the ribosomal P site and of termination (stop) codon at the ribosomal decoding A site. Hydrolysis of peptidyl-tRNA is induced by class 1 polypeptide release factors (RF1 and RF2 in prokaryotes; eRF1 in eukaryotes, RF1 in Archaea) at the peptidyl transferase center (reviewed by Craigen et al., 1990; Tate & Brown, 1992; Kisselev & Frolova, 1995; Tate et al., 1996; Buckingham et al., 1997; Nakamura & Ito, 1998).

Primary structures of many prokaryotic and eukaryotic class 1 RFs are known, and class 1 RFs have also

been found in genome of Archaea (Bult et al., 1996; Klenk et al., 1997). They show resemblance to the highly conserved eukaryotic eRF1 sequences. The comparison of a number of prokaryotic class 1 RF sequences with yeast eRF1/Sup45 sequences led to the hypothesis that prokaryotic and eukaryotic class 1 RFs had some degree of similarity and possessed a common ancestor (Nakamura et al., 1995; Ito et al., 1996).

There are two major distinctions between prokaryotic and eukaryotic class 1 RFs: each of two bacterial class 1 RFs recognizes two out of the three stop codons (Scolnick et al., 1968) whereas eRF1 recognizes all three stop codons (Konecki et al., 1977; Frolova et al., 1994). The second distinction is related to their ability to form stable complexes with class 2 RFs, RF3 and eRF3, in the absence of ribosomes. Prokaryotic RF1 and RF2 lack this ability (Nakamura et al., 1996; Freistoffer et al., 1997; Grentzmann et al., 1998; Pel

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

et al., 1998) whereas eRF3 is able to form stable complexes with eRF1 in vivo and in vitro (Stansfield et al., 1995; Zhouravleva et al., 1995; Paushkin et al., 1997; Frolova et al., 1998; Ito et al., 1998; Merkulova et al., 1999).

In spite of variability of amino acid sequences of class 1 RFs belonging to the three living kingdoms, a search for common (universal) motif(s) that may be essential for maintenance of RF structure and/or function seems justified. Class 1 RFs have a common feature, the ability to promote peptidyl-tRNA cleavage on the ribosome. This identical function should probably be reflected in common structural motif(s) in these molecules.

We have undertaken an extensive sequence comparison of class 1 RFs available so far, and after appropriate alignment, found a highly conserved unique amino acid sequence GGQ that is present in all known class 1 RFs. Site-directed mutagenesis has demonstrated that two glycyl residues in this universal amino acid stretch are essential for release activity of the human eRF1.

RESULTS

Comparison of class 1 release factor amino acid sequences

Alignment of bacterial, archaeobacterial, and eukaryotic class 1 RFs revealed a GGQ sequence that is absolutely conserved in all three living kingdoms (Fig. 1). On the C side of this sequence, the Ser residue is conserved in eukaryotes and Archaea, but much less conserved in prokaryotes. On the N side, GGQ sequence is flanked by an Arg conserved in eukaryotes, but not in archaeal and prokaryotic class 1 RFs (mostly replaced by Ala and Pro, and rarely by Lys and His).

Further toward the N end, a Gly residue is situated that is strictly conserved in eukaryotes and prokaryotes, and replaced by Lys and Arg in Archaea. A comparison of the amino acids in positions 181 and 182 (numbers correspond to human eRF1, the upper line on Fig. 1) shows that in Archaea and eukaryotes they are very similar (Gly and Arg in eukaryotes, Lys/Arg and Ala in Archaea), that is, a combination of small aliphatic (Gly or Ala) and positively charged (Arg or Lys) amino acid residues, but in inverted order. This local sequence feature distinguishes eukaryotes and Archaea from prokaryotes in which only the conserved Gly is present.

In addition, several other highly conserved amino acids are visible: P177 and K179 in eRF1, and Arg in prokaryotes, which is shifted by only one residue toward K179 in eukaryotes and seems to be equivalent to it (positively charged amino acids). Finally, a highly conserved VD sequence has been identified (positions 174 and 175) present in prokaryotes and eukaryotes, but not in Archaea.

Taken together, the structural analysis of this region points to its high conservation throughout evolution in class 1 RFs and presupposes that this region is essential for maintenance of RF structure and/or function.

The secondary structure of the eRF1 fragment containing GGQ motif was predicted using various programs (see Materials and Methods). It appears that this region of the protein adopts a coil (loop) structure devoid of α -helix and β -sheet (Fig. 2). This suggests that the GGQ motif is located on the surface of protein globule and is not involved in maintenance of the spatial structure. Rather, it might play an essential functional role in class 1 RFs.

Activities of the human eRF1 mutated at the universal GGQ motif

To test the hypothesis that the GGQ motif is functionally essential, site-directed mutagenesis was applied to the human eRF1 as representative of class 1 RFs. The mutations of four amino acid residues were introduced (Table 1). Two amino acids belong to the universal GGQ motif and two others are located in the partially conserved region upstream and adjacent to the GGQ motif (Fig. 1).

G181S and R182G mutations did not impair the release activity of eRF1 in the presence of any of the three stop codons. In contrast, G183R, G183A, and G184S mutations completely abolished the release activity of eRF1 in vitro and stimulation of the human eRF1 release activity by eRF3 in the presence of GTP and low stop codon concentration (Table 1). The functional inactivation of eRF1 caused by G183 and G184 mutations cannot be due to distortion of the local secondary structure because these substitutions do not affect the predicted secondary structure (Fig. 2).

The eRF1 G181S and R182G mutants that retained their activities in an in vitro RF assay were also active at low stop codon concentration in the presence of eRF3 and GTP (Table 1). The mutations that inactivated the eRF1 release activity did not affect another eRF1 function (Fig. 3), the ability of eRF1 and the ribosome to stimulate GTPase activity of eRF3 (Zhouravleva et al., 1995). This result implies that the common GGQ motif is not essential for eRF1–eRF3 interaction that is necessary for the eRF3 GTPase activity (Frolova et al., 1996, 1998) and is in a full agreement with earlier data demonstrating that the region encompassing positions 180–190 of the human eRF1 is not essential for binding to the human eRF3 (Merkulova et al., 1999).

It was shown (Grentzmann & Kelly, 1997) that the efficiency of the translation termination reaction in vitro with prokaryotic RFs and ribosomes increased in the presence of minimessengers containing initiation and termination codons instead of a mixture of AUG and one of the stop codons (Tate & Caskey, 1990). From the comparison of the data presented in Tables 1 and 2, it

sw P46055	<i>Homo sapiens</i>		VDLEKKKHGRGGQSALR 189
sw P35615	<i>Xenopus laevis</i>		VDLEKKKHGRGGQSALR 189
gb 2315514	<i>Caenorhabditis elegans</i>		VDLEKKKHGRGGQSAVR 197
sw P35614	<i>Arabidopsis thaliana</i>		VDLEKKKHGRGGQSALR 187
gb 3845214	<i>Plasmodium falciparum</i>		VDLEKKKHGRGGQSALR 185
gb d1010581	<i>Schizosaccharomyces pombe</i>		VDLEKKKHGRGGQSALR 186
sw P12385	<i>Saccharomyces cerevisiae</i>		VDLEKKKHGRGGQSALR 186
gb 2996008	<i>Podospora anserina</i>		VDLEKKKHGRGGQSALR 190
sw Q58239	<i>Methanococcus jannaschii</i>		SGVPGKFKAGGQSARR 189
gb 2621972	<i>Methanobact. thermoautotroph.</i>		SGVPGKHKAGGQSARR 185
gb d1031639	<i>Pyrococcus horikoshii</i>		SNVPGKTRAGGQSARR 186
gb 2649371	<i>Archaeoglobus fulgidus</i>		SMVPGKHRQGGQSVR 180
sw P45872	<i>Bacillus subtilis</i>		VDTFASSGPGGQSVNT 237
sw P28367	<i>Bacillus subtilis</i>	2	VDTYRASCAGGQHVNT 243
sw P30775	<i>Saccharomyces cerevisiae</i>	Mito1	VDIMRASCAGGQHVNT 290
sw P07012	<i>Escherichia coli</i>	2	IDVYRTSGAGGQHVNR 256
sw P07011	<i>Escherichia coli</i>	1	IDTFRSSGAGGQHVNT 239
sw P13654	<i>Salmonella typhimurium</i>	1	IDTFRSSGAGGQHVNT 239
sw P28353	<i>Salmonella typhimurium</i>	2	IDVYRASCAGGQHVNR 256
sw Q53915	<i>Streptomyces coelicolor</i>	1	VDVYRSSGPGGQGVNT 255
sw Q53915	<i>Streptomyces coelicolor</i>	2	VDVYRSSGPGGQGVNT 255
sw O05782	<i>Mycobacterium tuberculosis</i>	1	VDVYRSSGPGGQSVNT 264
sw O05782	<i>Mycobacterium tuberculosis</i>	2	VDVYRSSGPGGQSVNT 264
e332988	<i>Mycobacterium leprae</i>	1	VDVYRSSGPGGQSVNT 260
sw Z98271	<i>Mycobacterium leprae</i>	2	VDVYRSSGPGGQSVNT 260
gb BB08284	<i>Borrelia burgdorferi</i>	1	IDTYRASCAGGQHVNR 236
gb BB08284	<i>Borrelia burgdorferi</i>	2	IDVYRSSGAGGQHVNT 238
gb HPAE000529	<i>Helicobacter pylori</i>	1	IEVFRAGGHGCGQCVNT 237
gb HPAE000537	<i>Helicobacter pylori</i>	2	IDVYRSSGAGGQHVNR 255
sw D90915	<i>Synechocystis sp.</i>	1	ISTSHAGGKGGQNVNK 174
sw D90917	<i>Synechocystis sp.</i>	2	MSTARSGGAGGQNVNK 243
gb e1342580	<i>Rickettsia prowazekii</i>		IDTFRSSGAGGQHVNT 254
sw P43917	<i>Haemophilus influenzae</i>		IDTYRSSGAGGQHVNT 240
sw P96077	<i>Thermus thermophilus</i>	1	IDVIRASCAGGQGVNT 233
sw P71496	<i>Mycoplasma capricolum</i>	1	IDTYRASCAGGQHVNT 240
sw P47500	<i>Mycoplasma genitalium</i>	1	IDTYRASCAGGQHVNR 239
sw P75420	<i>Mycoplasma pneumoniae</i>	1	VDTYRASCAGGQHVNR 239

FIGURE 1. Comparison of the most conserved region in class 1 release factors. Asterisks indicate the amino acid residues in human eRF1 subjected to site-directed mutagenesis. Accession numbers and databases are indicated in the left column. For prokaryotes, RF1 and RF2 are marked by respective numbers. Mito1 stands for yeast mitochondrial prokaryotic-type factor. Identical amino acids are boxed in black. The numbers after the sequences indicate the position of the last amino acid in the indicated fragment of the appropriate release factor. Release factors are grouped into eukaryotes, archaeobacteria, and eubacteria from top to bottom. Additional prokaryotic and eukaryotic sequences identical or very similar to those shown in this figure were not included.

is clear that this holds true for mammalian ribosomes and the factor as well.

Addition of the inactive eRF1 mutants G183R, G183A, or G184S in fourfold molar excess over the wild-type eRF1 strongly inhibited eRF1 activity, whereas the addition of active eRF1 mutants G181S or R182G caused an increase of the RF activity (Table 2). Consequently, at nonsaturation levels of active eRF1, inactive eRF1 mutants were able to compete with the active factor for binding to the ribosome.

DISCUSSION

Here we present the first direct evidence that class 1 RFs possess a strictly conserved structural motif, GGQ, that is critical for the major function of these factors, triggering the stop-codon-dependent catalysis of peptidyl-tRNA hydrolysis at the ribosomal peptidyl transferase center. In earlier alignments of some prokaryotic RF1 and RF2 sequences with yeast eRF1/Sup45 sequences (Nakamura et al., 1995; Ito et al.,

eRF1	174	189
Wild-type	V D L P K K H G R G G Q S A L R h L L L l l l L L L l H H H H	
G181S	V D L P K K H S R G G Q S A L R h L L L l l l L L L l H H H H	
R182G	V D L P K K H G G G Q S A L R h L L L l l l L L L l H H H H	
G183R	V D L P K K H G R R G Q S A L R h L L L l l l L L L l H H H H	
G183A	V D L P K K H G R A G Q S A L R h l L L l l l L L L l H H H H	
G184S	V D L P K K H G R G S Q S A L R h L L L l l l L L L l H H H H	

FIGURE 2. Predicted secondary structure of the human eRF1 in the region of the GGQ motif. H: helix structure with high probability; L: loop structure with high probability; h: helix structure with low probability; l: loop structure with low probability. For programs used to calculate the secondary structure, see Materials and Methods.

1996) the GGQ motif common for prokaryotes and eukaryotes had not been identified.

According to the predicted secondary structure for class 1 RFs, the region encompassing positions 176–

TABLE 1. In vitro RF activity of the wild-type and mutant forms of the human eRF1 and the effect of the human eRF3 on eRF1 activity at low stop codon concentration.

		f[³⁵ S]Met released (cpm)					
		Stop codon added (μM)					
Release factors		UAAA		UAGA		UGAA	
eRF1	eRF3	50	2.5	50	2.5	50	2.5
Wild-type	–	2,600	0	2,500	0	2,700	0
G181S	–	2,120	0	2,520	0	2,800	0
R182G	–	2,100	0	2,300	0	2,200	0
G183R	–	0	nd ^a	0	nd	0	nd
G183A	–	0	nd	0	nd	0	nd
G184S	–	0	nd	0	nd	0	nd
Wild-type	+	nd	2,000	nd	1,850	nd	1,760
G181S	+	nd	1,920	nd	1,650	nd	1,600
R182G	+	nd	1,900	nd	1,500	nd	1,750
G183R	+	nd	0	nd	0	nd	0
G183A	+	nd	0	nd	0	nd	0
G184S	+	nd	0	nd	0	nd	0

The RF activities of the human eRF1 alone (0.16 μM) and in the presence of the human eRF3 (0.3 μM) were measured in the same incubation mixture (Zhouravleva et al., 1995) except that the concentration of the stop codons was 50 μM or 2.5 μM, respectively, and GTP (0.1 mM) was added where eRF3 was present. The value of f[³⁵S]Met released in the absence of stop codon for eRF1 or in the absence of GTP for eRF1 plus eRF3 was subtracted from all values.

^and: not determined.

TABLE 2. Inhibition of release activity of the human wild-type eRF1 by functionally inactive eRF1 mutants in an in vitro RF assay.

eRF1	f[³⁵ S]Met released	
	cpm	%
Wild-type	5,700	100
G181S	6,800	>100
R182G	6,500	>100
G183R	0	0
G183A	0	0
G184S	0	0
Wild-type + G181S	8,780	>100
Wild-type + R182G	9,270	>100
Wild-type + G183R	740	12.9
Wild-type + G183A	1,050	18.4
Wild-type + G184S	2,800	49.1

Incubation mixtures (25 μL) contained 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 8 mM NH₄Cl, 1.5 pmol of f[³⁵S]Met-tRNA^{Met}•UUGAUGUAAA•ribosome complex. Where indicated, human eRF1 was added in the following concentrations: wild-type: 0.1 μM; mutants alone: 0.1 μM; in mixtures of wild-type and mutants: G181S and R182G, 0.1 μM, and G183R, G183A, and G184S, 0.4 μM. The samples were incubated for 20 min at 25 °C. The amount of f[³⁵S]Met released at zero time (1,000–1,500 cpm) was subtracted from all values. In the mixture of two active factors, the values exceed the release caused by each individual factor because non-saturating concentrations for each active protein were used. The amount of f[³⁵S]Met released in the presence of active release factors was higher with minimessenger than with AUG and tetraplets (see Table 1) because of the higher efficiency of minimessenger in RF assay.

185 in human eRF1 is in a coil/loop conformation (Fig. 2). This prediction is strongly supported by the observation that *Escherichia coli* RF2 and RF1 are highly sensitive to limited proteolysis, and RF2 protein is split between Y244 and R245 by chymotrypsin (Mof-

FIGURE 3. Activity of human eRF1 mutants in stimulation of eRF1-dependent and ribosome-dependent GTPase activity of the human eRF3. Human eRF3 GTPase activity measured in the presence of wild-type human eRF1 and the ribosomes was used as a control. The GTPase activity detected in incubation mixtures without ribosomes is due to trace amounts of *E. coli* GTPase(s) remaining in the purified eRF1 preparations. This value varies between different eRF1 preparations.

fat & Tate, 1994). As shown in Figure 1, this position in *E. coli* RF2 is close to the common GGQ motif (five and six amino acids upstream from the GGQ motif) and is located within the predicted coil/loop (Fig. 2). Moreover, this cleavage produces a nicked RF2 in which the two parts of the protein remain associated, showing that Y244 and R245 are at the surface of a globule and not in the linker between the protein domains (Moffat & Tate, 1994). These data strengthen our interpretation that the GGQ motif is at the surface of a globule and that mutations inside this region should not affect the overall protein conformation.

Presumably, eRF1 possesses at least four binding regions (or sites): (1) mRNA recognition site, (2) ribosome binding site; (3) eRF3 binding site, and, probably, (4) peptidyl-tRNA binding site to promote hydrolytic reaction at the ribosomal peptidyl transferase center. Although functionally similar, prokaryotic and eukaryotic RFs differ in their stop codon specificity: eRF1 recognizes UAA, UGA, and UAG whereas RF1 responds to UAG and UAA and RF2 to UGA and UAA. For this reason it seems unlikely that the universal GGQ motif directly contributes to stop codon recognition as part of site (1). Indeed, it was shown (Ito et al., 1998) that a single E167K mutation in *E. coli* RF2 converted the E167K mutant into eRF1-like factor in terms of its ability to recognize all three stop codons (Konecki et al., 1977; Frolova et al., 1994), though the mutant protein possessed rather low release activity. The E167 in RF2 is far from the GGQ motif (Fig. 1), indicating that the latter seems not to be involved directly in stop codon recognition. The total inability (Table 1) of the G183R, G183A, and G184S eRF1 mutants to respond to any of the three stop codons (rather than partial inactivation or change in specificity) implies that the GGQ motif is probably essential for other eRF1 function(s) rather than for specific stop codon recognition. This interpretation is consistent with the fact that in RF assay, the inactive eRF1 mutants, G183R, G183A and G184S, strongly compete with wild-type eRF1 or with active eRF1 mutants, G181S and R182G (Table 2), indicating the conservation of the spatial structure, ribosome binding, and probably stop-codon recognition ability in the inactive eRF1 mutants.

It was shown that the eRF3 binding site (3) is located in the C domain of human eRF1 as deletion of the N-terminal domain of the human eRF1 (positions 1–277) including the GGQ motif (positions 183–185) did not impair the eRF3 binding (Merkulova et al., 1999). Consequently, the GGQ motif is not involved in eRF3 binding. Consistent with this is the observation that the eRF1 dependence of eRF3 GTPase activity controlled by complex formation between two factors and the ribosome (Frolova et al. 1996) is not impaired (Fig. 3).

Because eRF3 GTPase activity is entirely dependent on eRF1 and the ribosome, it is possible to explore this activity as a measure of eRF1 binding to the ribosome.

Thus, the maintenance of the GTPase-stimulating activity in all of the eRF1 mutants inactive in the RF assay indicates that the GGQ motif is not essential for eRF1 binding to the ribosome (site (2)).

For prokaryotes, several indirect arguments have been presented in favor of the notion that peptidyl-tRNA hydrolysis proceeds at the ribosomal peptidyl transferase center and that certain regions of small (16S) and large (23S) rRNAs are involved in translation termination (for reviews and references, see Murgola, 1996; Tate et al., 1996; Arkov et al., 1998a, 1998b). In line with these views, the role of class 1 RFs could be to trigger the activity of the peptidyl transferase center toward peptidyl-tRNA hydrolysis, that is, to activate the ribosomal catalytic site. If so, the termination process may be considered as a transfer of a termination signal from any of the three stop codons to peptidyl-tRNA mediated by rRNAs and class 1 RFs. In this model, the two adjacent Gly residues of the GGQ motif might provide high local flexibility to the polypeptide chain important for signal transduction. Any substitutions that introduce side chains at these positions could reduce the conformational mobility of eRF1 needed for functioning of the factor as a molecular trigger.

At the termination step of protein synthesis, the CCA end of tRNA covalently bound to the carbonyl group of the last amino acid residue is an element common to all living organisms, whereas the peptidyl and tRNA moieties of the peptidyl-tRNA are highly variable. We assume that this common element is recognized by another common element, the universal GGQ motif. The main argument in favor of this hypothesis is that these elements are the only structural features known so far to be universal for the translation termination machinery in all living organisms. This concept stems from the idea that when the mechanism of termination evolved, it was conserved. Our assumption is consistent with the observation that nicked RF2 from *E. coli* that is able to interact with stop codons is inactive in promoting peptidyl-tRNA hydrolysis (Moffat & Tate, 1994). This cleavage site between Y244 and R245 is close to the essential G183 and G184 of the human eRF1 and is located in the same loop (Figs. 1 and 2). It should be emphasized that the postulated interaction is mediated via (e)RF1/2 interaction with appropriate stop codon, as the termination reaction is entirely stop codon dependent.

In contrast to all other natural amino acids, glycine possesses no side chain and is the smallest amino acid residue in polypeptides. For this reason, it may leave room for a water molecule when eRF1 interacts with the peptidyl-tRNA in the peptidyl transferase center of the ribosome. The GGQ motif may participate in the formation of a channel-type structure providing access to the water molecule to perform the hydrolysis reaction in the peptidyl transferase center. When Gly residues are substituted by more bulky or charged res-

idues as in the G183R and G184S mutants, the channel structure might be perturbed leaving no space for a water molecule.

Mutations of partially conserved G181 or nonconserved R182 in human eRF1 do not abolish eRF1 activity. This implies that the functionally essential G183 is flanked on its N side by nonessential residues. On the other hand, the strictly conserved Q185 is a reasonable candidate that could play a functional role. Experiments are in progress on site-directed mutagenesis of Q185 in the human eRF1.

MATERIALS AND METHODS

Materials

L-[³⁵S]methionine (specific activity 10⁴ cpm/pmol) and [α -³²P]GTP were from Amersham, pQE-30 plasmid and NiNTA-agarose from Qiagen, Talon-agarose was from Clontech, and PEI-cellulose plates from Macherey-Nagel. AUG, tetraplets containing stop codons and the minimessenger RNA, UUCAUGUAAA, were synthesized by A. Veniaminova and M. Ryabkova (Novosibirsk State University). Restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, and the Klenow fragment of DNA polymerase were from Sib-Enzym (Novosibirsk).

Bacterial strains, vectors, and cloning

Phage M13mp19 was used to clone the full-length human eRF1, and pQE-30 plasmid to clone the truncated form of the human eRF1. The TB3-1 cDNA encoding the full-length human eRF1 (Grenett et al., 1992; Frolova et al., 1994) was inserted into M13mp19 cleaved with *EcoRI* followed by ligation with phage vector (M13/eRF1). *E. coli* strains TG1 or M15 (pREP4) were used for the production of recombinant eRF1. Preparation of competent *E. coli* cells, transfections, and isolation of single-stranded and double-stranded phage and plasmid DNAs were performed as described (Sambrook et al., 1989).

Mutagenesis

Mutagenesis was performed using M13-derived recombinant vectors (Zoller & Smith, 1982) harboring the human eRF1 sequence. The oligonucleotides used for site-directed amino acid substitutions were for G181S, 5'-AAAGAAACACAGTA GAGGAGGTCAGTC-3'; for R182G, 5'-AAGAAACACGGTG GTGGAGGTCAGTC-3'; for G183R, 5'-CACGGTAGAAGAG GTCAGTCAGTCAGCC-3'; for G183A, 5'-AACACGGTAGA GCGGGTCAGTCAGC-3'; and for G184S, 5'-CACGGTAGA GGAAGTCAGTCAGCC-3'. Selection of mutant clones was performed by hybridization of the cloned DNA with the appropriate oligonucleotide used for mutagenesis. Point mutations in recombinant DNA were confirmed by sequencing (Sanger et al., 1977). The primer used to verify the nucleotide sequence of the mutated region was 5'-ACTTTTGGCAC ACTCC-3'.

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

To generate expression of wild-type and mutant eRF1, M13/eRF1 construct was digested with *Bgl*III and *Sa*I, positions 40 and 1321 in the human eRF1 cDNA (Grenett et al., 1992), respectively. In parallel, pQE-30/eRF1Tyr415 plasmid (Merkulova et al., 1999) carrying cDNA encoding eRF1 terminated with Y415 was digested with the same two restriction enzymes. The pQE-30 vector containing the 40 N-terminal nucleotides of eRF1 after treatment with *Bgl*III and *Sa*I was ligated with the fragment from phage vector encoding the other part of the human eRF1 molecule (nt 40–1321) containing the wild-type or mutated fragments of eRF1. DNA clones were selected based on the high yield of wild-type or mutant proteins produced as measured by SDS-PAGE. Purification of the eRF1 proteins was achieved using NiNTA-agarose or Talon-agarose affinity chromatography as described earlier (Frolova et al., 1998). The wild-type gene was expressed in the same plasmid and the resulting protein was purified exactly as described for mutants and served as reference.

Assays for eRF1 activities in vitro

The eRF1 release activity was measured in an in vitro assay as stop-codon-dependent hydrolysis of f[³⁵S]Met-tRNA^{Met} associated with the AUG•80S ribosome complex according to Tate and Caskey (1990) with some modification (Frolova et al., 1994), or as hydrolysis of f[³⁵S]Met-tRNA^{Met} associated with 80S ribosome programmed with the minimessenger UUCAUGUAAA, containing initiation (AUG) and termination (UAA) codons as described (Grentzmann & Kelly, 1997). eRF3 stimulation activity toward eRF1 was measured as described earlier (Frolova et al., 1996). Isolation of rabbit reticulocyte ribosomal subunits and expression and purification of the human eRF3 were as described (Frolova et al., 1996, 1998). eRF3 GTPase activity was followed by accumulation of [α -³²P]GDP after hydrolysis of [α -³²P]GTP using thin-layer chromatography on PEI-cellulose-coated plates (Frolova et al., 1996).

Software

Alignment of class 1 RF proteins was performed by the ClustalX program with visual control. Prediction of eRF1 secondary structure was performed by M.L.R. combination of SOPMA, GOR4, and SIMPA at Network Protein Sequence Analysis at IBCP, France.

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