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REPORT

Highly conserved NIKS tetrapeptide is functionally essential in eukaryotic translation termination factor eRF1

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ABSTRACT

Class-1 polypeptide chain release factors (RFs) play a key role in translation termination. Eukaryotic (eRF1) and archaeal class-1 RFs possess a highly conserved Asn-Ile-Lys-Ser (NIKS) tetrapeptide located at the N-terminal domain of human eRF1. In the three-dimensional structure, NIKS forms a loop between helices. The universal occurrence and exposed nature of this motif provoke the appearance of hypotheses postulating an essential role of this tetrapeptide in stop codon recognition and ribosome binding. To approach this problem experimentally, site-directed mutagenesis of the NIKS (positions 61–64) in human eRF1 and adjacent amino acids has been applied followed by determination of release activity and ribosome-binding capacity of mutants. Substitutions of Asn61 and Ile62 residues of the NIKS cause a decrease in the ability of eRF1 mutants to promote termination reaction in vitro, but to a different extent depending on the stop codon specificity, position, and nature of the substituting residues. This observation points to a possibility that Asn-Ile dipeptide modulates the specific recognition of the stop codons by eRF1. Some replacements at positions 60, 63, and 64 cause a negligible (if any) effect in contrast to what has been deduced from some current hypotheses predicting the structure of the termination codon recognition site in eRF1. Reduction in ribosome binding revealed for Ile62, Ser64, Arg65, and Arg68 mutants argues in favor of the essential role played by the right part of the NIKS loop in interaction with the ribosome, most probably with ribosomal RNA.

Keywords: human eRF1; NIKS motif; site-directed mutagenesis; stop codon recognition; translation termination

INTRODUCTION

Termination of translation requires the positioning of peptidyl-tRNA at the ribosomal P site and of the termination (stop, nonsense) codon at the decoding A site. Hydrolysis of peptidyl-tRNA is promoted by class-1 polypeptide chain release factors (RFs) at the peptidyl transferase center (reviewed by Kisselev & Buckingham, 2000; Poole & Tate, 2000). It has been demonstrated experimentally both for prokaryotes (Ito et al., 2000) and eukaryotes (Kervestin et al., 2001) that specificity of decoding of stop codons is associated with RFs rather than with the ribosome. Primary structures of many prokaryotic (RF1 and RF2), eukaryotic (eRF1), archaeal (aRF1), and mitochondrial (mtRF) class-1 RFs are known. RF1, RF2, and mtRF are structurally closely related and form one group, whereas eRF1 and aRF1 are also related and form the other group, which differs considerably from the first one (Frolova et al., 1994; Dontsova et al., 2000; Kisselev et al., 2000; Song et al., 2000). Despite these pronounced dissimilarities, all class-1 RFs share a common tripeptide GGQ that is functionally important because mutations of the amino acid residues in the GGQ and nearby cause complete or partial loss of the RF activity in vitro (Frolova et al., 1999; Seit-Nebi et al., 2000, 2001). Mutations of the GGQ in yeast eRF1 are lethal in vivo (Song et al., 2000). In crystal structure of human eRF1, the GGQ tripeptide is located at the tip of the middle (M) domain (Song et al., 2000). This location together with its universality and functional importance supports the hypothesis (Frolova et al., 1999) that GGQ is involved in promoting peptidyl-tRNA hydrolysis at the peptidyl transferase center located at the 50S/60S ribosomal subunit.

The second functionally essential site of class-1 RFs recognizing stop codons should be remote from the

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"catalytic" site and positioned near the 30S/40S and 50S/60S interface in the ribosomal particle. Mutagenesis of yeast eRF1 followed by in vivo genetic assay points to the N domain (Song et al., 2000) as a region of eRF1 where the termination codon recognition site (TCRS) is located (Bertram et al., 2000). The N-terminal domain (domain 1) of human eRF1 contains a conserved NIKS motif (positions 61–64 for human eRF1), common for Eukarya and Archaea (Kisselev et al., 2000; Song et al., 2000; Fig. 1A).

The third functional site(s), a ribosome binding site(s) (RBS), should be located at the N and M domains, as human eRF1 lacking the C domain binds to the ribosome and is functionally active in vitro (Frolova et al., 2000). This suggestion is consistent with the fact that these domains are topologically distinct (Song et al., 2000) and have to be fixed within the ribosome separately to ensure fidelity of two main joint functions of class-1 RFs, recognition of stop codons, and promotion of peptidyl-tRNA ester bond hydrolysis. Mutations in the M domain reduce the ribosome binding ability of human eRF1 (Seit-Nebi et al., 2001).

The class-2 termination factors, eRF3/RF3, are known to be GTPases (Frolova et al., 1996; Freistroffer et al., 1997; Pel et al., 1998). The human eRF1 and eRF3 interact through their C termini (Merkulova et al., 1999) and this mutual binding is critical for manifestation of eRF3 GTPase activity within the ribosome (Frolova et al., 1996, 2000). This property can be used to detect the binding of eRF1 to eRF3 and to the ribosome. Because the N and M domains of the eRF1 molecule are required to activate eRF3 GTPase, although they are not involved in eRF3 binding, it means that the eRF1 binding to the ribosome is a prerequisite for its activating function toward eRF3 GTPase. Therefore, the capacity of mutant eRF1 to bind to the ribosome may be followed by measuring the ability of mutant eRF1 to activate eRF3 GTPase if mutations are located within N and/or M domains of eRF1 (Seit-Nebi et al., 2001).

Here, we approach the problem of structure and location of eRF1 functional sites, TCRS and RBS, by examining the properties of the human eRF1 NIKS tetrapeptide and adjacent amino acids by site-directed mutagenesis. This region seems to be a good candidate for being involved either in interaction with the ribosome and/or with stop codons due to their essential and specific features. The NIKS motif is composed of two invariant amino acids, Ile62 and Lys63 (numbering corresponds to human eRF1; Fig. 1A), and two conserved but not invariant residues, Asn61 and Ser64 (Kisselev et al., 2000; Song et al., 2000). The NIKS motif occupies a well-defined region at the extremity of the N domain (Song et al., 2000; Fig. 1B) and has been suggested to be involved both in the ribosome binding (Kisselev et al., 2000) and stop codon recognition (Knight & Landweber, 2000).

RESULTS AND DISCUSSION

We have substituted amino acids in the NIKS motif and around it, followed by measuring the ability of the mutant eRF1 to promote fMet-tRNA hydrolysis ("catalytic" or RF activity) and the ribosome binding capacity. In the latter case, eRF3 GTPase activity, which is entirely eRF1 dependent and ribosome dependent (Frolova et al., 1996), served as a measure of the eRF1 binding to the ribosome (Frolova et al., 2000; Seit-Nebi et al., 2001). It seems advantageous that this assay is performed in the absence of fMet-tRNA, mRNA, and stop codons (see Materials and Methods). Therefore, the GTPase activity measured in this work reflects the affinity of eRF1 toward the ribosome itself but not to mRNA and/or fMet-tRNA. From comparison of RF and GTPase activities, one may attempt to distinguish between amino acids involved in RBS or in TCRS or in both. To identify the TCRS, it is compulsory to follow the response of mutant eRF1 to all three stop codons. If the response varies toward different stop codons, it implies that the given amino acid residue of eRF1 could potentially be involved in stop codon recognition.

The nonconserved Ser60 neighboring the NIKS tetrapeptide from the N side seems to be insignificant for functional activity of human eRF1: Even in Ser60Val mutant, where a profound change of the chemical nature of amino acid is introduced, the mutant eRF1 remains functionally intact (Fig. 2A). In Ser60Glu and Ser60Thr mutants, both RF and binding activities were insignificantly affected (Fig. 2A). Therefore, as anticipated from the sequence alignment (Fig. 1A), position 60 is not directly implicated in eRF1 activity, but probably could weakly affect the surrounding amino acid sequences.

As is evident from Figure 2A, Asn61 is insignificant as a part of the RBS because none of the four amino acid substitutions caused any alteration of the eRF3 GTPase activity induced by mutant eRF1. However, the response toward three stop codons is different for all four mutants: The UGA response is much less affected by amino acid changes than UAA and UAG responses (Fig. 2A). Interestingly, the specificity toward stop codons is not lost because the sense codon UGG encoding tryptophan remains inactive (Asn61Ala, Asn61Gln, and Asn61Asp) in promoting eRF1 activity (not shown). Asn61 is essential for the UAA and UAG response, but not for the UGA recognition (Fig. 2A). This explains why *Euplotes* and other ciliate eRF1s that do not respond to UGA (Kervestin et al., 2001) tolerate amino acid variability at position 61 (Fig. 1A). The inability of *Euplotes* eRF1 to respond to UGA is related to other structural features of this eRF1 (yet unknown) and not to position 61. Potentially, Asn61 might affect recognition of the adenine in the second position of the stop codons in universal-code eRF1s, but this assumption requires further experimental verification.

A

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eRF1 Homo sapiens [CAA57281]                          …GTASNIKSRVNRL… 69
eRF1 Xenopus laevis [A48061]                          …GTASNIKSRVNRL… 69
eRF1 Drosophila melanogaster [AAF51574]               …GTASNIKSRVNRL… 69
eRF1 Caenorhabditis elegans [T31907]                  …GTASNIKSRVNRL… 77
eRF1a Arabidopsis thaliana [BAB01727]                 …GTASNIKSRVNRQ… 67
eRF1b Arabidopsis thaliana [BAB11335]                 …GTASNIKSRVNRQ… 68
eRF1 Plasmodium falciparum [A71612]                   …GTASNIKSRVNRL… 65
eRF1 Podospora anserina [AAC08410]                    …GTASNIKSRVNRQ… 70
eRF1 Schizosaccharomyces pombe [T43243]               …GTASNIKSRVNRQ… 66
eRF1 Saccharomyces cerevisiae [NP_009701]             …GTASNIKSRVNRL… 66
eRF1 Dictyostelium discoideum [AAK07832]              …GTASNIKSRVNRL… 71
eRF1 Giardia intestinalis [AAF74402]                  …GTASNIKSRVNKN… 75
eRF1 Leishmania major [CAB77686]                      …GTASNIKSHTNKL… 71
eRF1a Euplotes aediculatus [AAK07829]                 …GTAESIKSRVNRQ… 65
eRF1a Euplotes octocarinatus [CAC14170]               …GTAESIKSKINRL… 65
eRF1b Euplotes aediculatus [AAK07830]                 …GTAVNIKSRVNRL… 65
eRF1b Euplotes octocarinatus [AAG25924]               …GTAVNIKSRVNRQ… 65
eRF1 Blepharisma americanum [AAK12089]                …GKSSNIKSRIVRQ… 67
eRF1 Oxytricha trifallax [AAK07828]                   …SAAQNIKSRITKQ… 73
eRF1 Stylonychia mytilis [AAK12091]                   …SAAQNIKSRITRQ… 73
eRF1 Stylonychia lemnae [AAK12092]                    …SAAQNIKSRITRQ… 73
eRF1 Paramecium tetraurelia [AAK66860]                …AEAASIKDKGNRI… 69
eRF1 Tetrahymena thermophila [AAK07831]               …SKATNIKDRVNRQ… 68
eRF1 Trypanosoma brucei [AAF86346]                    …GTASNIKSHTNKL… 70
aRF1 Thermoplasma acidophilum [CAC11674]              …STSSNIKSKSTRK… 67
aRF1 Archaeoglobus fulgidus [AAB90026]                …SQASNIKSKQTRT… 61
aRF1 Methanobact. thermoautotrophicum [C69217]        …SQSANIKSKQTKK… 64
aRF1 Halobacterium sp.NRC-1 [AAG20270]                …SEASNIKSKQTRT… 91
aRF1 Pyrococcus abussi [CAB49500]                     …STAQNIKSKTTRK… 66
aRF1 Pyrococcus horikoshii [BAA30696]                 …STAQNIKSKTTRK… 66
aRF1 Aeropyrum pernix [BAA80998]                      …SITDNIKLKRTRQ… 37
aRF1 Methanococcus jannaschii [AAB98828]              …SQASNIKSKSTRK… 68
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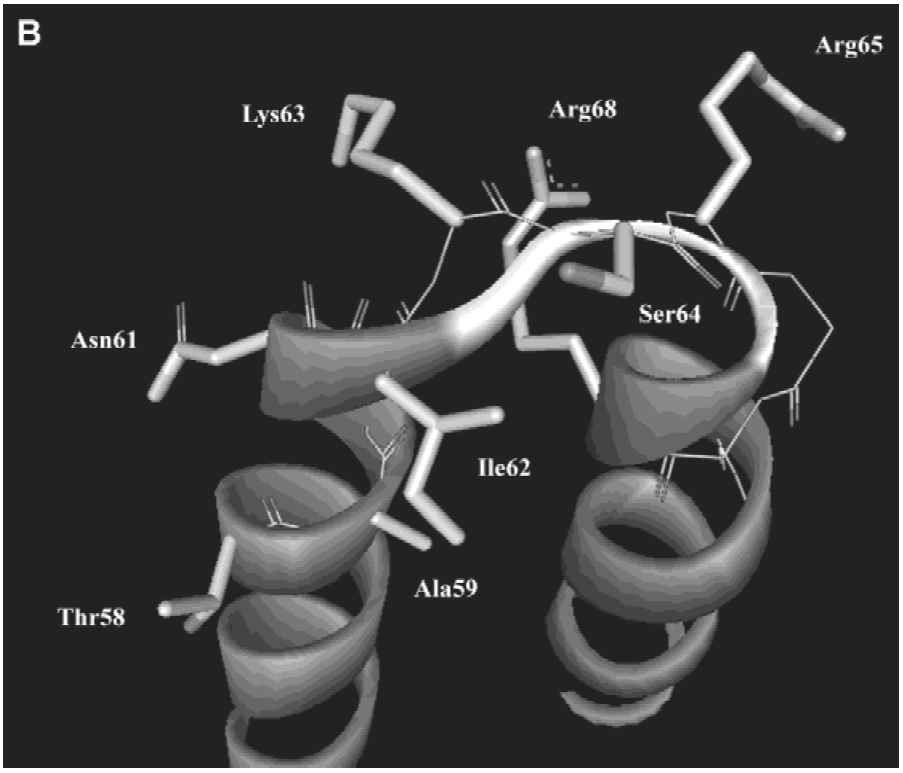

FIGURE 1. A: Alignment of eRF1 and aRF1 amino acid sequences around the highly conserved NIKS tetrapeptide. Positions are numbered for human eRF1. Accession numbers are given after the names of the species. Numbers in brackets correspond to NCBI-Entrez-Proteins database accession numbers. Amino acids are shaded according to their identity percentage (white letters, black shading: 100%; white letters, dark grey shading: 80%; black letters, light grey shading: 70%). **B:** The ribbon diagram of the part of N-terminal domain of human eRF1 containing the NIKS motif derived from crystallographic data (Song et al., 2000) by WebLab Viewer-Lite program version 4.0 (Molecular Simulations Inc.). Side-chain residues of Thr58, Ala59, Asn61, Ile62, Lys63, Ser64, Arg65, and Arg68 displayed in a stick mode and backbone in a line mode. Front view.

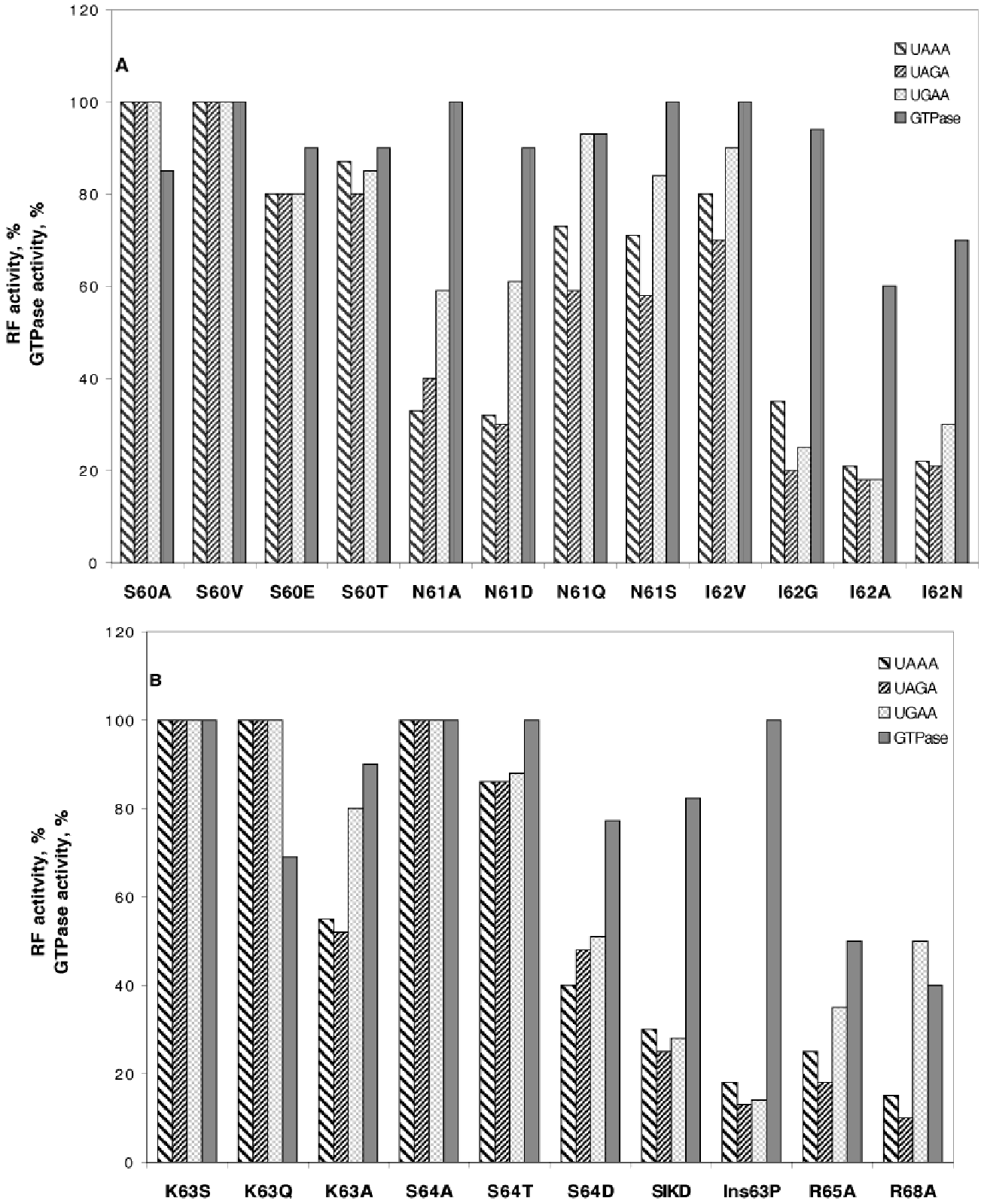

FIGURE 2. The in vitro functional activity of the human eRF1 mutants. The RF (hydrolytic) activity of human eRF1 toward the three stop codons and stimulating activity toward eRF3 GTPase were measured as described in Materials and Methods. The activities of the wild-type eRF1 were equal to 100%. Average from at least three independent experiments is presented. The standard deviation of the measurements was 11%.

Position 62, occupied by invariant isoleucine (Fig. 1A), is functionally important, as deep inactivation with all three stop codons has been observed for the three mutations (Ile62Gly, Ile62Ala, and Ile62Asn; Fig. 2A). It is unlikely that this effect is caused by weak binding to the ribosome as the stimulating activity toward eRF3 GTPase is much less affected than the catalytic activity (Fig. 2A). Even in Ile62Val eRF1 mutant where the chemical nature of both amino acids is very similar, some reduction of the response toward all stop codons is noticed. One may assume that Ile62 can affect recognition of uridine common to all three stop codons. Ile62 is located in between the helices in the NIKS subdomain (Fig. 1B) in a hydrophobic environment. Therefore, replacements of Ile62 may induce distortion in mutual orientation of the helices and lead to long-distance effects on the whole N domain.

As discussed above, it remains possible that Asn61-Ile62 dipeptide may affect or modulate recognition of the first and second nucleotides of the stop codons. However, it seems unlikely that the NIKS motif as a whole is the TCRS, as has been proposed (Knight & Landweber, 2000). This suggestion is also incompatible with the eRF1 sequences from organisms with variant genetic codes as pointed out earlier (Inagaki & Doolittle, 2001; Kervestin et al., 2001).

Although a priori invariant Lys63 (Fig. 1A) may be structurally or functionally essential, only one out of three mutants, Lys63Ala, possesses a twofold reduced activity with UAA and UAG whereas UGA response and the eRF3 GTPase activating ability is much less disturbed (Fig. 2B). Because species with variant genetic codes (*Paramecium*, *Tetrahymena*, and *Euplotes*) contain Lys63 (Fig. 1A) like the organisms with canonical genetic code, it seems unlikely that this amino acid takes part in the recognition of the second and third letters of the stop codons. Presumably, in the Lys63Ala mutant, conversion of a polar and charged amino acid residue to a nonpolar one may induce a distortion of the local spatial structure (Fig. 1B).

When proline residue was inserted between Ile62 and Lys63, the ribosome binding of mutant eRF1 was not affected, though the RF activity was profoundly diminished (Fig. 2B). The NIKS tetrapeptide in the three-dimensional structure of human eRF1 forms a loop (Fig. 1B). Insertion of proline residue may significantly distort spatial structure of the whole loop and in particular reduce its flexibility.

Among many mutations within the N domain that affected the in vivo antisuppressor activity of yeast eRF1/Sup45, mutations of the NIKS tetrapeptide have not been revealed (Bertram et al., 2000). In principle, the procedure directed toward in vivo selection of mutants with altered stop codon specificity could select Asn61 mutants with different response toward various stop codons (Fig. 2A), but not other mutants where the response was insensitive toward the sequence of the stop codons. Inability of the in vivo genetic testing to detect the contribution of Asn61 to specific stop codon response might be due to several reasons. For example, low sensitivity of the in vivo testing, where cell growth rate rather than a defined biochemical activity is used to characterize the mutants. Moreover, indirect involvement of Asn61 in stop codon discrimination as follows from incomplete inactivation of Asn61 mutants (Fig. 2A) may appear insufficient to cause a switch in specific recognition of the stop codons probably required for in vivo detection. Finally, Asn61 mutants may become prone to proteolysis in yeast cells during slow growth. It is known that conformational changes induced by some point mutations cause proteolytic degradation of mutated proteins in vivo. If so, the Asn61 mutants may be degraded and for this reason not selected by genetic screening.

Given that all or some of the above mentioned factors may contribute to the in vivo testing, it does not seem surprising that one of the mutants among many could escape from the selection procedure. Therefore we do not consider our in vitro data with human eRF1 and the in vivo data with yeast eRF1 (Bertram et al., 2000) as contradictory.

The NIKS mutations that affect the eRF1 binding to the ribosome are Ile62Ala, Ile62Asn, Ser64Asp, and a double mutation Asn61Ser + Ser64Asp (Fig. 2A,B). Because Asn61Ser binds efficiently to the ribosome (Fig. 2A) the effect of double mutation is due to Ser64Asp substitution. A reduction in binding ability is probably responsible at least partly for the reduction of catalytic activity of these mutants.

It has been shown by a genetic approach that replacement of Arg65 for cysteine residue in yeast Sup45p later attributed to eRF1 (Frolova et al., 1994) confers an omnipotent suppressor phenotype to the mutant Sup45p/eRF1 (Mironova et al., 1986). At that time, these data were not explained, but now they are considered in view of potential location of the TCRS near this position (Song et al., 2000). Our data (Fig. 2B) provide evidence that, in fact, this position is essential both for ribosome binding and stop codon recognition (Arg65 in yeast eRF1 corresponds to Arg68 in human eRF1; see Fig. 1A). However, the genetic approach does not allow us to discriminate between omnipotent suppression caused by reduced eRF1 binding to the ribosome and the reduction of catalytic activity caused by distortion of the TCRS or peptidyl-tRNA interaction site.

Numerous biochemical, structural, and genetic data point to a functional role for rRNA in tRNA selection (see Green & Noller, 1997). The decoding domain of 16S rRNA formed by helices 18, 24, 27, 34, and 44 affects the translational accuracy (Lodmell & Dahlberg, 1997; O'Connor et al., 1997; Pagel et al., 1997). *Escherichia coli* 16S rRNA mutations cause defects in translation termination (Arkov et al., 1998). Crystallographic data (Carter et al., 2000; Ogle et al., 2001; Yusupov

et al., 2001) suggest that the first and the second base pairs in the minor groove of the codon–anticodon helix interact with positions 1492–1493 of 16S rRNA. G530 is also positioned in the minor groove of the A site codon–anticodon helix, near the second and the third base pairs. The C1054 projects toward the apex of the anticodon of tRNA located at the A site. Mutations at this position suppress UGA nonsense mutations (Murgola et al., 1988).

In eukaryotes, A1823 and A1824 in human 18S rRNA equivalent to *E. coli* G1491 and A1492, respectively, cross-react with the first position of the codon located at the A site (Demeshkina et al., 2000). Mutations in the 18S rRNA affect fidelity of the stop codon decoding (Velichutina et al., 2001, and references therein).

Collectively, all these data point to the involvement of small rRNA sequences in codon–anticodon and stop codon–eRF1 interactions. Coexistence of the elements of TCRS and RBS within the NIKS subdomain is entirely consistent with the close proximity of mRNA and small rRNA nucleotides at the A site. Amino acids at positions 64, 65, and 68 of human eRF1 that affect the ribosome binding properties of eRF1 in the absence of mRNA and tRNA (Fig. 2B) could interact with amino acid residues at positions 1492, 1493, and 530 of 18S rRNA (numeration as in *E. coli* 16S rRNA). Other nucleotides of 18S rRNA could be also implicated in this interaction. It means, that RBS may in fact be in close vicinity toward the TCRS or even overlap and these sites may be concomitantly affected by a single mutation.

We do not believe that stop codon recognition in eukaryotes is as simple as direct interaction between RF1/2 tripeptides and the second and third nucleotides of the stop codons (Ito et al., 2000). We share the opinion (Inagaki & Doolittle, 2001) that in eukaryotes, the decoding of stop codons within the ribosome is a complex process not yet understood.

MATERIALS AND METHODS

Cloning and mutagenesis of human *eRF1*

The full-length cDNA encoding eRF1 with C-terminal His-tag fusion was cloned into pET23b(+) vector (Novagen) under control of phage T7 RNA polymerase promoter. For this, the coding region of the cDNA from TB3-1 clone (14) was amplified using the forward primer (RFNde) 5′-GAGATATA CATATGGCGGACGACCC-3′ (*Nde*I site underlined) and the reverse primer, 5′-GTGGTGCTCGAGGTAGTCATCAAG GTC-3′ (*Xho*I site underlined). Then, the PCR product was subsequently treated with the restriction endonucleases *Nde*I and *Xho*I and inserted into pET23b(+) vector treated with the same endonucleases. The resulting construct was verified by DNA sequencing and used to perform mutagenesis.

The mutagenesis procedure was simplified by introducing into human *eRF1* cDNA a unique *Bst98*I site affecting neither amino acid sequence nor the reading frame of human eRF1 using GeneEditor in vitro site-directed mutagenesis kit (Promega). For this purpose the RFBst primer 5′-CCATT CTTAAGCGGGCAAAACGCAAGG-3′ (*Bst98*I site underlined) was used. The resulting construct pERF4B containing the unique *Bst98*I site within the gene encoding human eRF1 at positions 576–581 (T576C substitution) from the start ATG codon was used for mutagenesis of human eRF1.

The mutagenesis procedure was performed according to PCR-based "megaprimer" method (Sarkar & Sommer, 1990). The PCR primers used for generation eRF1 mutants are listed in Table 1. The direct primer, one of those mentioned in Table 1 (except Asn61Ser reverse primer), and the reverse primer (RFBst) 5′-CCATTCTTAAGCGGGCAAAACGCA AGG-3′ (*Bst98*I site underlined) were used at the first step of PCR. The resulting 400-bp PCR product was purified in low-melting NuSieve GTG agarose (FMC Bioproducts) and used as the reverse megaprimer together with the direct primer (RFNde), 5′-GAGATATACATATGGCGGACGACCC-3′ (*Nde*I site underlined), at the second step of PCR. The resulting 590-bp PCR product was purified in low-melting agarose, hydrolyzed with *Nde*I and *Bst98*I, and ligated with pERF4B plasmid, treated with the same endonucleases. The ligated mixture was transformed into *E. coli*, strain JM 109. The cloned DNAs were sequenced and appropriate clones were used for expression of the mutant eRF1. DNA amplifications were carried out in 50-μL reaction mixtures containing 20 ng of pERF4B DNA, 0.4 μM each primer, 0.24 mM each deoxynucleoside triphosphate, 1× of commercial *Pfu* DNA polymerase reaction buffer, and 2.5 U of *PfuTurbo* DNA polymerase (Stratagene). Amplifications were run for 1 cycle at 95 °C for 3 min, 25 cycles at 95 °C for 30 s, 43 °C for 30 s, 72 °C for 45 s in a thermocycler.

Single Asn61Ser and double Asn61Ser + Ser64Asp mutants were obtained by the same scheme, but at the first step

TABLE 1. PCR primers used to generate the constructs for bacterial expression of human eRF1 mutant proteins.

eRF1 mutant	Primers
Thr58Lys	5′-GAGTTTGGAAAGGCATCTAACATTAAGTC-3′
Ser60Ala	5′-ACTGCAGCTAACATTAAGTCACGAGTAAACCG-3′;
Ser60Glu	5′-ACTGCAGAGAACATTAAGTCACGAGTAAACCG-3′;
Ser60Thr	5′-ACTGCAACAAACATTAAGTCACGAGTAAACCG-3′
Ser60Val	5′-ACTGCAGTTAACATTAAGTCACGAGTAAACCG-3′
Asn61Ala	5′-CTGCATCTGCCATTAAGTCACGAGTAAACCG-3′
Asn61Asp	5′-CTGCATCTGACATTAAGTCACGAGTAAACCG-3′
Asn61Ser	5′-CTTAATTGAAGATGCAGTTCCAAACTCATCCG-3′
Asn61Gln	5′-CTGCATCTCAGATTAAGTCACGAGTAAACCG-3′
Ile62Ala	5′-GCATCTAACGCTAAGTCACGAGTAAACCG-3′
Ile62Gly	5′-CTGCATCTAACGGGAAGTCACGAGTAAACCG-3′
Ile62Asn	5′-CTGCATCTAACAACAAGTCACGAGTAAACCG-3′
Ile62Val	5′-CTGCATCTAACGTGAAGTCACGAGTAAACCG-3′
Lys63Arg	5′-CTGCATCTAACATTCGCTCACGAGTAAACCG-3′
Lys63Ala	5′-CTGCATCTAACATTGCCTCACGAGTAAACCG-3′
Lys63Gln	5′-CTGCATCTAACATTCAGTCACGAGTAAACCG-3′
Ins63Pro	5′-GGAACTGCATCTAACATTCCTAAGTCACGAGTAA ACCGC-3′
Ser64Thr	5′-CTGCATCTAACATTAAGACTCGAGTAAACCG-3′
Ser64Ala	5′-CTGCATCTAACATTAAGGCCCGAGTAAACCG-3′
Ser64Asp	5′-CTGCATCTAACATTAAGGACCGAGTAAACCG-3′
Arg65Ala	5′-GTTTACTGCTGACTTAATGTTAGATGC-3′
Arg68Ala	5′-GTAAACGCCCTTTCAGTCCTGGG-3′

of mutagenesis the RFNde direct and the N61S reverse primers were used. At the second step of PCR, the resulting 200-bp PCR product (the direct megaprimer), RFBst (the reverse primer), and pERF4B for a single mutant and pERF4B-Asn64Asp for a double mutant (DNA template) were used.

Arg68Ala and Arg65Ala + Arg68Ala mutants were obtained as a Asn61Ser + Ser64Asp double mutant. For Arg65Ala + Arg68Ala double mutant, pERF4B-Arg68Ala was used as a DNA template for PCR reaction at the second step of PCR.

Expression and purification of human eRF1

Wild-type human eRF1 and its mutants containing His-tag at the C terminus was expressed in *E. coli,* strain BL21(DE3), and purified using Ni-NTA resin, Superflow (Qiagen), as described (Frolova et al., 1994, 2000).

Cloning and expression of human *eRF3* in *E. coli* and purification of eRF3

Cloning of the full-length human *eRF3* was performed as described earlier for the carboxy-terminal part of human eRF3 (eRF3Cp) (Frolova et al., 1998), using eRF3 cDNA inserted into pUC19 (Hoshino et al., 1989) as a template for PCR reaction. The forward primer was 5′-CCCGAATTCATATGGA TCCGGGCGG-3′ (*Nde*I underlined). Human eRF3 containing His-tag at the C terminus was synthesized in *E. coli,* strain BL21(DE3), and purified as previously described (Frolova et al., 1998).

Ribosomes

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

In vitro RF assay

The eRF1 activity was measured as described (Caskey et al., 1974; Frolova et al., 1994) at saturating levels (50 μM) of one out of the three stop-codon-containing tetraplets. The incubation mixture (25 μL) contained 20 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 8 mM NH_4Cl, 1.5 pmol of f[^{35}S]Met-tRNA$_f^{Met}$-AUG-ribosome complex and 4 pmol of eRF1. The reaction was run at 20 °C for 20 min. In these conditions, the amount of eRF1 was rate limiting. The background was measured without tetraplet and subtracted from all values. The amount of f[^{35}S]Met released without stop codon was 500–800 cpm. AUG and ribotetraplets were synthesized by A. Veniaminova and M. Ryabkova (Institute of Bioorganic Chemistry, Novosibirsk).

Assays for GTPase activity

GTPase activity was followed by accumulation of [^{32}P]P_i using a modified charcoal precipitation assay as described (Frolova et al., 1996). Incubation mixture (12.5 μL) contained 2 μM [γ-^{32}P]GTP (10,000 cpm/pmol), 20 mM Tris-HCl, pH 7.5, 30 mM NH_4Cl, 15 mM $MgCl_2$, 0.36 μM ribosomes, 0.24 μM human eRF1, and 0.24 μM human eRF3. In these conditions, the amount of factors was rate limiting. The reaction was run at 30 °C for 20 min, stopped by adding 0.5 mL of a 5% charcoal suspension in 50 mM NaH_2PO_4 on ice. The mixture was vortexed and centrifuged at 10,000 rpm for 10 min at 4 °C, and the [^{32}P]P_i released into 375 μL of supernatant was quantified by liquid scintillation counting. Protein-independent release of [^{32}P]P_i due to decomposition of the labeled GTP in the solution was measured simultaneously and this value was subtracted from all samples.

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