C-terminal domains of human translation termination factors eRF1 and eRF3 mediate their in vivo interaction

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Abstract At the termination step of protein synthesis, hydrolysis of the peptidyl-tRNA is jointly catalysed at the ribosome by the termination codon and the polypeptide release factor (eRF1 in eukaryotes). eRF1 forms in vivo and in vitro a stable complex with release factor eRF3, an eRF1-dependent and ribosome-dependent GTPase. The role of the eRF1•eRF3 complex in translation remains unclear. We have undertaken a systematic analysis of the interactions between the human eRF1 and eRF3 employing a yeast two-hybrid assay. We show that the N-terminal parts of eRF1 (positions 1–280) and of eRF3 (positions 1–477) are either not involved or non-essential for binding. Two regions in each factor are critical for mutual binding: positions 478–530 and 628–637 of eRF3 and positions 281–305 and 411–415 of eRF1. The GTP binding domain of eRF3 is not involved in complex formation with eRF1. The GILRY pentamer (positions 411–415) conserved in eukaryotes and archaebacteria is critical for eRF1's ability to stimulate eRF3 GTPase. The human eRF1 lacking 22 C-terminal amino acids remains active as a release factor and promotes an eRF3 GTPase activity whereas C-terminally truncated eRF3 is inactive as a GTPase.

Key words: Translation termination; Human polypeptide release factor; Complex formation; Interacting region; Yeast two-hybrid system

1. Introduction

Termination of protein synthesis proceeds on the ribosomes and requires the presence of a termination (stop) codon in the mRNA at the ribosomal A site and the polypeptide chain release factor (RF) of class 1 (reviewed in [1–6]). The termination reaction leads to hydrolysis of the peptidyl-tRNA, generating a free nascent polypeptide chain and a free (uncharged) tRNA followed by the release of these components from the ribosome.

In eukaryotes, translational termination is governed by a single RF which recognises all three termination codons [7]. After the structure of this factor was established it was termed eRF1 [8]. In contrast to earlier observations [9,10] where the termination reaction was accompanied by concomitant GTP hydrolysis, eRF1 appeared to be able to catalyse in vitro peptidyl-tRNA hydrolysis at a high termination codon concentration in the absence of GTP and other factors [8,11,12]. This apparent controversy was resolved when a second RF, termed eRF3, was identified and shown to be an eRF1-dependent and ribosome-dependent GTPase [11,12]. The initial experiments were done with eRF3 from *Xenopus laevis* and the same features were revealed later for the human eRF3 [13]. It was demonstrated that eRF1 and eRF3 bound to each other both in vitro and in vivo [12,14,15]. The complex is stable, even at high ionic strength, and complex formation is accompanied by significant conformational changes of eRF1 [13]. eRF1•eRF3 complex formation and eRF3 GTPase activity were tightly interrelated since eRF3 GTPase activity depended on the complex formation and eRF3 alone, even able to bind GTP, is virtually inactive in catalysing GTP hydrolysis on the ribosome [11].

The prokaryotic RF3 [16,17], also a GTP binding protein, differed significantly from eRF3: its GTPase activity is totally independent of RF1/RF2 [18] and no complex formation between RF1/2 and RF3 has been revealed so far [19,20]. The N-terminal fragment of *X. laevis* or human eRF3 proteins was non-essential for complex formation, and truncated eRF3 proteins retained their GTPase- and eRF1-stimulating activities [11–13]. It was suggested for *Saccharomyces cerevisiae* that eRF3/Sup35p possessed two large areas for eRF1/Sup45p binding, one of which was located within a part of the C-terminal domain, while the other is ascribed to the N-terminal and middle (M) domains of the molecule [15].

To gain further insight into the role of both factors in protein synthesis, we have analysed the molecular anatomy of interaction between the two human eRFs. To reach the goal, we employed a LexA-based yeast two-hybrid system as an assay for protein-protein interaction in vivo [21,22]. There are two major advantages of using this system. First, the protein-protein interaction proceeds in a living cell where the interacting macromolecules are surrounded by a huge excess of other compounds which may non-specifically or specifically interact with the proteins under investigation. Due to that, the interaction revealed in two-hybrid system tolerates all these interactions and therefore is of a highly specific nature. The second advantage stems from the high sensitivity of the system. If only some of the given protein molecules bind to each other, the system will respond to it.

The systematic application of two-hybrid strategy for both human termination factors performed in this work allowed us to characterise molecular anatomy of eRF1•eRF3 interaction

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Abbreviations: eRF, eukaryotic polypeptide chain release factor; EF, prokaryotic elongation factor; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; PP2Ac, protein phosphatase 2A (catalytic subunit); PCR, polymerase chain reaction; PMSF, phenylmethylsulphonyl fluoride; IPTG, isopropyl-1-thio-β-D-galactoside; NP40, Nonidet P40; PEI, polyethyleneimine; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside

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in vivo and to provide new data relevant to currently suggested models for eRF3 function(s).

2. Materials and methods

2.1. Plasmid constructions

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 pGAD3s2x/eRF1 and pGADGH/eRF3 constructs, expressing corresponding eRF as a C-terminal part of the fusion proteins with the yeast GAL4 transactivation domain (GAL4AD) have been previously described [13]. N- and C-terminal deletion mutants for both eRF3 and eRF1 were generated by PCR amplification with *Pwo* polymerase (Boehringer, Mannheim) using the primers listed in Tables 1 and 2. In addition, deletion mutants were made by using the unique restriction sites within the GAL4AD expressing constructs (Tables 1 and 2). Briefly, the eRF3$^{379-637}$, eRF3$^{2-43}$, eRF3$^{2-295}$, eRF3$^{2-478}$ mutants were constructed by removing the *Spe*I-*Spe*I, *Apa*I-*Apa*I, *Xba*I-*Eco*RI or *Dra*III-*Eco*RI fragments, respectively, from pGADGH/eRF3 followed by filling in, if necessary, the incompatible ends of shortened parent constructs with Klenow DNA polymerase before ligation. The eRF3$^{2-606}$ mutant was obtained by subcloning the *Bam*HI-*Dra*I fragment of pUC19/*GST1-Hs* [23] between the *Bam*HI and *Sma*I sites of pGADGH. To create the eRF3$^{478-637}$ mutant, the eRF3 cDNA was first subcloned into pGAD3s2x as follows: a 115-bp fragment between the *Dra*I restriction site and the stop codon was generated by PCR using the following primers: forward, GSTDRA1, *Dra*I site underlined, 5′-GCCTTGAGACCTTTAAAGACTTCCC-3′; reverse, GSTSAL1, *Sal*I site underlined, 5′-AAGTCGACGTCTTTCTCTG-GAACCA-3′. The amplified fragment was ligated with the *Eco*RI-*Dra*I fragment of pUC19/*GST1-Hs* between the *Eco*RI and *Xho*I sites of pGAD3s2x. The *Eco*RI-*Dra*III fragment of the resultant pGAD3s2x/eRF3 was thereafter removed and the ends were filled in with Klenow DNA polymerase before ligation. The eRF1$^{228-437}$ and eRF1$^{1-230}$ mutants were obtained by subcloning the *Bam*HI-*Sal*I or *Eco*RI-*Bam*HI fragments of the PCR-amplified eRF1 cDNA [13] into the *Bam*HI-*Sal*I or *Eco*RI-*Bam*HI sites of pGAD3s2x. The eRF1$^{1-415}$ mutant was generated by subcloning of a *Eco*RI-*Asp*718 fragment of the amplified eRF1 cDNA into the *Eco*RI-*Asp*718 sites of pUC19 followed by subcloning of the *Eco*RI-*Xba*I fragment of the resultant construct into the *Eco*RI-*Xba*I sites of pGAD3s2x.

All PCR-amplified eRF3 deletion mutants, except the eRF3$^{139-606}$ mutant, were subcloned between *Bam*HI and *Sal*I sites into pGADGH in-frame with GAL4AD. To generate the pGADGH/eRF3$^{139-606}$ construct, *Bam*HI-*Eco*RI cloning sites were used. The eRF1$^{271-437}$, eRF1$^{306-437}$, eRF1$^{343-437}$ and eRF1$^{414-437}$ mutants were subcloned between *Bam*HI and *Xho*I sites, and the eRF1$^{2-410}$, eRF1$^{2-391}$ and eRF1$^{2-372}$ mutants were subcloned between *Eco*RI and *Xba*I sites into pGAD3s2x in frame with GAL4AD. Structure of PCR products and preservation of open reading frames for all constructs were confirmed by DNA sequencing (for details see [24]).

A set of 5′-unidirectional deletions for eRF1 was also generated using the Double-Stranded Nested Deletion Kit (Pharmacia Biotech) as recommended by the manufacturer. Briefly, pGAD3s2x/eRF1 construct was incubated with exonuclease III (ExoIII) at 25°C followed by linearisation using *Spe*I and *Eco*RI to create nuclease-resistant and nuclease-sensitive ends, respectively. *Spe*I was a first cut filled in with thionucleotides before *Eco*RI digestion. ExoIII-digested samples were removed at 3.5-min time intervals, then treated with S1 nuclease and analysed by agarose gel electrophoresis to determine the size of each deletion. The samples of interest were recircularised using T4 DNA ligase, pulled and used as a library of subclones with progressively deleted eRF1 cDNA for yeast transformation.

2.2. Yeast two-hybrid analysis

Two-hybrid deletion analysis of eRF1 and eRF3 interaction was performed using a mating procedure (for details see [25]). *S. cerevisiae* strain L40 (*MAT*a), containing two reporter genes *LacZ* and *HIS3* downstream of the DNA binding sequence for LexA, was transformed with the eRF1-LexBD or eRF3-LexBD fusion expression construct, and strain AMR 70 (*MAT*α) was transformed with the construct expressing GAL4AD fused to one of the deletion mutants of eRF. Transformants were selected for *trp1* or *leu2* complementation, respectively, mated on complete medium, and double-transformed diploids were selected on both tryptophan- and leucine-deficient medium. Expression of the *LacZ* reporter gene indicating the interaction was followed by monitoring β-galactosidase activity with X-Gal as a substrate [26]. Expression of the *HIS3* reporter gene was assessed by monitoring growth on leucine-, tryptophan- and histidine-deficient medium.

Screen of the eRF1 cDNA 5′-deletion library for the shortest subspecies interacting with eRF3 was performed according to the standard two-hybrid technique [26]. The L40 yeast reporter strain was sequentially transformed with pVJL11/eRF3 and the cDNA library, and the double transformants expressing interacting proteins were selected on leucine-, tryptophan- and histidine-deficient medium. The filter replica method was applied using X-Gal to confirm the interaction. Plasmid DNA was prepared from colonies displaying a His$^+$/LacZ$^+$ phenotype and the insertion size within pGAD3s2X was determined by PCR with pGAD-specific primers. The pGAD3s2x plasmids containing the smallest insertions were recovered using HB101 as a recipient strain, selected on leucine-deficient M9 medium, and the inserts were sequenced.

2.3. Expression and purification of truncated eRF1 and eRF3 proteins

In order to express eRF1 and eRF3 truncated proteins, PCR-generated products were subcloned into pQE expression vectors (Qiagen) in frame with 6×His tag placed at the N-terminus of the resultant recombinant proteins. eRF3 and eRF1 deletion mutants were subcloned into pQE-31 and pQE-30, respectively, between the *Bam*HI and *Sal*I sites except for the eRF1$^{2-410}$ mutant where the *Asp*718

Table 1
PCR primers and restriction sites used to generate eRF3 deletion mutants for yeast two-hybrid analysis

Mutant	Forward primer or 5′-restriction site	Reverse primer or 3′-restriction site
eRF3$^{379-637}$	*Spe*I	*Bam*HI
eRF3$^{471-637}$	5′-AAGGATCCTGTGATGATGCCAAACAAG-3′	5′-TTGTCGACTTAGTCTTTCTCTGGAACC-3′
eRF3$^{478-637}$	*Dra*III	*Bam*HI
eRF3$^{531-637}$	5′-AAGGATCCATTTGATGCCCAGATAGTG-3′	5′-TTGTCGACTTAGTCTTTCTCTGGAACC-3′
eRF3$^{601-637}$	5′-AAGGATCCCATCTGCCTTGAGACCTT-3′	5′-TTGTCGACTTAGTCTTTCTCTGGAACC-3′
eRF3$^{2-43}$	*Bam*HI	*Apa*I
eRF3$^{2-295}$	*Bam*HI	*Xba*I
eRF3$^{2-478}$	*Bam*HI	*Dra*III
eRF3$^{2-606}$	*Bam*HI	*Dra*I
eRF3$^{139-617}$	5′-AAGGATCCCATGGAACTTTCAGAACCTAT-3′	5′-TTGTCGACTTATAAGGTGAAACGACCCAT-3′
eRF3$^{139-627}$	5′-AAGGATCCCATGGAACTTTCAGAACCTAT-3′	5′-TTGTCGACCTATCCAATTGCAATGGTCTT-3′
eRF3$^{139-606}$	5′-AAGGATCCCATGGAACTTTCAGAACCTAT-3′	5′-ATATGAATTCTTAAAAGGTCTCAAGGCAGATG-3′
eRF3$^{471-617}$	5′-AAGGATCCTGTGATGATGCCAAACAAG-3′	5′-TTGTCGACTTATAAGGTGAAACGACCCAT-3′
eRF3$^{471-627}$	5′-AAGGATCCTGTGATGATGCCAAACAAG-3′	5′-TTGTCGACCTATCCAATTGCAATGGTCTT-3′

Superscript numbers in mutants indicate positions of retained amino acids.

and *Pst*I cloning sites of pQE-30 were used. To generate the eRF3$^{139-478}$ mutant, the *Dra*III-*Sal*I fragment from the pQE31/eRF3$^{139-627}$ construct was removed followed by filling in the ends with Klenow DNA polymerase and ligation. For expression of the truncated cDNA encoding eRF1 terminated at Tyr-415, a TB3-1 cDNA [27] encoding full-length eRF1 [8] was amplified by PCR with *Pwo* DNA polymerase and the following oligonucleotides as primers: forward primer *Kpn*I (underlined), 5′-GCGGTACCGATGACGATGACAAAATGGCGGACGACGACCCCAGT-3′ and reverse primer, 5′-CGACTACCTAGTAGTCATC-3′, which is complementary to the nucleotide positions from +1301 to +1319 in TB3-1/eRF1 cDNA [27]. The resulting amplified PCR product was gel-purified, digested with *Kpn*I (*Kpn*I sites are in the forward primer and in the +1248 nucleotide position in TB3-1/eRF1 cDNA) and inserted into the *Kpn*I site of expression vector pQE-30. The expressed truncated protein, designated eRF1$^{1-415}$*, contained at the C-terminus after Tyr-415 an additional eight amino acids (PGSTCSQA) encoded in pQE-30 polylinker. Cells of *Escherichia coli* strain M15 [pREP4] were transformed with recombinant pQE-30 or pQE-31 with cDNA inserts encoding truncated forms of eRF1 or eRF3. The purification of truncated forms of eRF1 and eRF3 proteins containing His tags was performed on a Ni-NTA-agarose (Superflow, Qiagen) column as described [13].

2.4. In vitro assays

The eRF1 activity was measured according to Tate and Caskey [28] with some modifications [8,13]. Rabbit reticulocyte ribosomal subunits were isolated as described [11,13]. The expression of full-length human eRF1 and eRF3 proteins in baculovirus system and their purification have been described [13]. eRF3 GTPase activity was followed by accumulation of [α-^{32}P]GDP after hydrolysis of [α-^{32}P]GTP using thin-layer chromatography on PEI-cellulose-coated plates (Macherey-Nagel) as described [11,13].

3. Results

3.1. Localisation of regions essential for binding of eRF3 and eRF1

In order to delineate the regions of the human eRF3 and eRF1 involved in complex formation in vivo, the binding of various truncated proteins was tested using the two-hybrid approach. AMR 70 yeast cells were transformed with plasmids expressing GAL4AD fused with eRF3 or eRF1 sequences, progressively deleted from either the N- or the C-terminus (Tables 1 and 2) and then mated with L40 yeast cells expressing full-length eRF1 or eRF3 fused to LexA (see Section 2). The resultant diploid yeast cells were tested for the expression of the two distinct reporter genes, *HIS3* and *LacZ*, to visualise eRF1 and eRF3 interaction. The results with these reporter genes were completely coincident: in no case did we observe any disagreement between the two assays for the given mutant. The difference between the binding and non-binding mutants (Fig. 1) for eRF1 and eRF3 in both assays was well defined: for non-binders, loss of yeast cell growth in the *HIS3* reporter assay and the absence of blue colour for the X-Gal assay. The binding efficiency of interacting mutants to the respective partners was comparable to the interaction between the full-length eRF1 and eRF3 as was evident from the growth rates (*HIS3* reporter) and intensity of blue colour (*LacZ* reporter).

To follow intrinsic transcriptional activation capacity or non-specific binding, mating with the cells possessing an empty vector was used. Transcriptional activation of reporter genes driven by interaction between full-length eRF1 and eRF3 [13] or by Ral and RLIP proteins [24] was used as controls.

The results of the analysis presented in Fig. 1A show that the N-terminal part of eRF3, including up to 477 amino acid residues, was non-essential for binding to eRF1. Further deletions from the N side abrogated the binding. None of the eRF3 C-terminal deletion mutants was able to bind eRF1. These results indicate that at least two regions in eRF3 spanning residues from 478 to 530 and from 628 to 637 seemed to be critical for eRF3•eRF1 interaction.

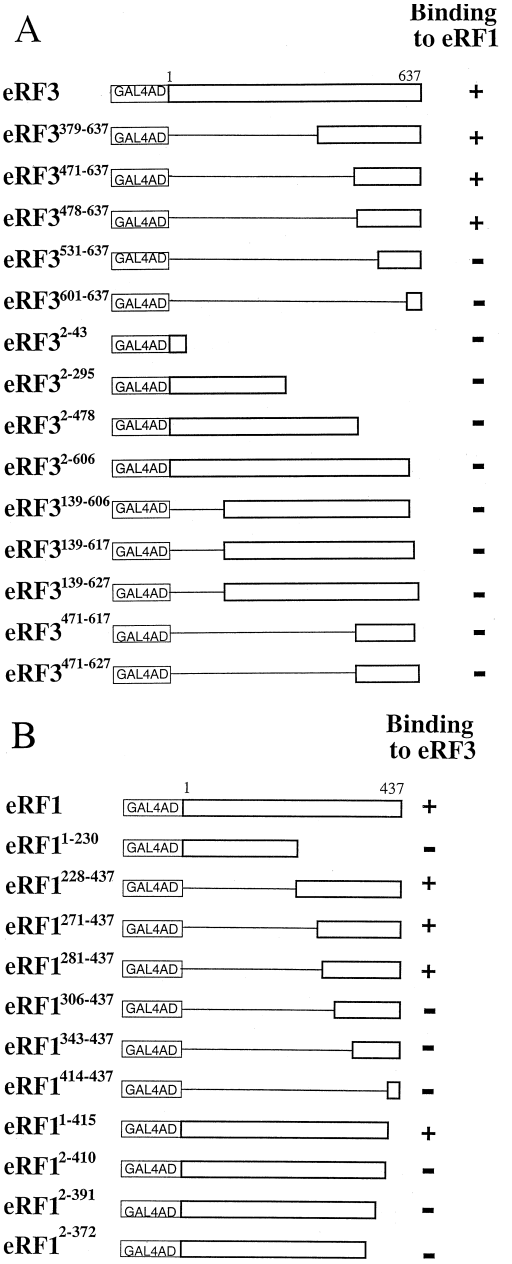

Fig. 1. Mapping of interacting regions in the human eRF1 and eRF3 using the yeast two-hybrid deletion analysis. Various DNA fragments encoding truncated eRF3 or eRF1 were cloned into the two-hybrid vectors pGADGH or pGAD3s2x, respectively, and the resultant plasmids were transformed into AMR 70 yeast cells mated with reporter yeast L40 cells transformed with pVJL12/eRF1 or pVJL11/eRF3. Diploids were replicated onto triple-selective leucine-, tryptophan- and histidine-deficient DO medium and/or on Whatman paper (No. 40) laid over DO medium lacking leucine and tryptophan to visualise β-galactosidase activity. Schematic representation of eRF3 (A) and eRF1 (B) deletion mutants used for the analysis. Boxes indicate the remaining amino acid sequences. The ability of various mutants to interact with the other factor, as follows from the expression of both reporter genes, is indicated on the right.

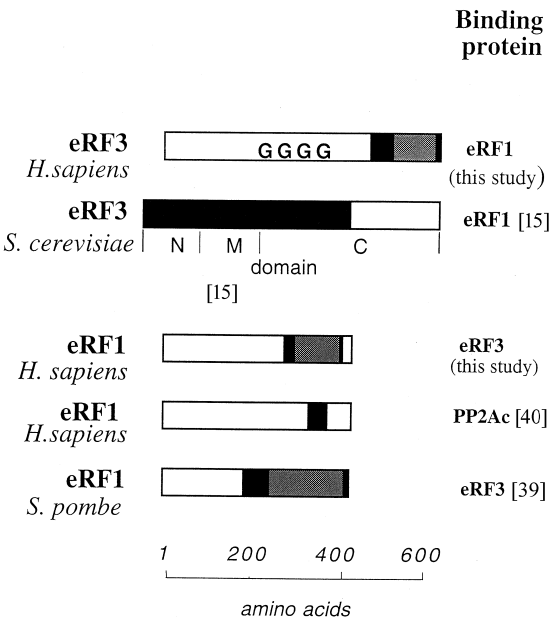

Fig. 2. Critical regions for the human eRF1 and eRF3 binding in comparison with other data. Critical binding regions, black boxes; regions non-essential or not involved in binding, white boxes; regions with unknown role in mutual binding, hatched boxes. G designates four putative GTP binding motifs. Subdivision of *S. cerevisiae* eRF3/Sup35p into three domains, N, M and C, is indicated.

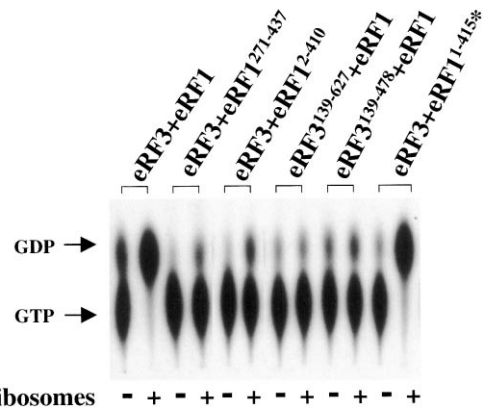

Fig. 3. Activity of the human eRF1 and eRF3 mutants in a GTPase assay. Incubation mixtures (12.5 μl) contained 5 μM [α-^{32}P]GTP (Amersham, specific activity 5000 cpm/pmol) and, where indicated, 0.02 μM rabbit ribosomes, full-length human eRF1, or eRF3, or their truncated forms (10 pmol each). The reaction was stopped by addition of 1 μl of a solution containing 20 mM EDTA and 5% SDS. 5-μl aliquots were spotted onto PEI-cellulose plates and resolved in 1 M acetic acid and 1 M LiCl during 1.5 h. The plates were dried and exposed to Hyperfilm-MP (Amersham). The human eRF1 and eRF3 were expressed in the baculovirus system and purified [13]. The truncated eRF1 and eRF3 proteins containing the N-terminal His tag were expressed in *E. coli* (see Section 2) and purified on Ni-NTA-agarose column [13]. The human eRF3 GTPase activity measured in the presence of eRF1 and ribosomes was used as a positive control. The human eRF3 lacking the N-terminal 139 amino acid residues is active as a GTPase in the presence of eRF1 and the ribosomes [13]. The residual GTPase activity detected in incubation mixture without ribosomes is due to trace amounts of *E. coli* GTPases remaining in the purified proteins.

To map the eRF1 binding regions involved in the interaction with eRF3, the N- and C-terminally truncated forms of eRF1 (Fig. 1B) were tested in the same experimental set-up as described above. In this case L40 cells were transformed with full-length eRF3 fused to LexA and mated with AMR 70 cells transformed with one of the eRF1 deletion mutants fused to GAL4AD. The results of mating experiments demonstrated that the N-terminal part of eRF1 is non-essential for binding, delineating the essential region for eRF3•eRF1 interaction. In order to establish the N-terminal boundary of this region more precisely, a set of 5′-terminally truncated eRF1 cDNA fragments was generated with ExoIII nuclease and used to construct an expression library which was screened for interaction with eRF3 (see Section 2). One hundred and ninety-two plasmids isolated from randomly selected His$^+$/LacZ$^+$ yeast colonies were analysed for insertion sizes. Five of them containing the shortest insertions were sequenced. The results of sequence analyses revealed that these eRF1 cDNAs encoded deleted products starting from amino acid positions 271, 272, 275 and 281. Among these mutants eRF1$^{281-437}$ represented the shortest form (Fig. 1B) still able to interact with eRF3. Thus, one eRF1 region critical for the interaction with eRF3 encompassed the amino acid sequence from position 281 to 305.

Among the eRF1 C-terminal deletions, the eRF1$^{1-415}$ and eRF1$^{2-410}$ mutants represented the shortest interacting and the longest non-interacting mutant proteins, respectively (Fig. 1B). Taken together, these results indicate that the second eRF1 region critical for eRF3 binding encompassed only five amino acids from 411 to 415. Consequently, two regions encompassing amino acids 281–305 and 411–415 seem to be indispensable for the interaction with eRF3.

3.2. Functional activity of eRF1 and eRF3 mutants

Although the C-terminus of eRF3 (positions 628–637) was critical for eRF1 binding, the C-terminus of eRF1 (positions 416–437) appeared to be non-essential (Figs. 1 and 2). To

Table 2
PCR primers and restriction sites used to generate eRF1 deletion mutants for yeast two-hybrid analysis

Mutant	Forward primer or 5′-restriction site	Reverse primer or 3′-restriction site
eRF1$^{228-437}$	*Bam*HI	*Sal*I
eRF1$^{271-437}$	5′-TTGGATCCTCTACTGAAGTCCTCTCC-3′	5′-TTGTCGACCTAGTAGTCATCAAGGTCA-3′
eRF1$^{306-437}$	5′-AAGGATCCGAAGATACACTAAAGGCTTT-3′	5′-TTGTCGACCTAGTAGTCATCAAGGTCA-3′
eRF1$^{343-437}$	5′-AAGGATCCATTCTCTATCTAACTCCAGA-3′	5′-TTGTCGACCTAGTAGTCATCAAGGTCA-3′
eRF1$^{414-437}$	5′-AAGGATCCCGGTACCGAGTAGATTTC-3′	5′-TTGTCGACCTAGTAGTCATCAAGGTCA-3′
eFR1$^{1-415}$	*Eco*RI	*Asp*718
eRF1$^{2-410}$	5′-ATATGAATTCTCGGTACCGCGGACGACCCCAGTGC-3′	5′-ATATTCTAGACTGCAGCTATCCAATTCCACCAAATCCTTT-3′
eRF1$^{2-391}$	5′-ATATGAATTCTCGGTACCGCGGACGACCCCAGTGC-3′	5′-ATATTCTAGACTGCAGCTAAATTTCCAACGTAGCTCCAAA-3′
eRF1$^{2-372}$	5′-ATATGAATTCTCGGTACCGCGGACGACCCCAGTGC-3′	5′-ATATTCTAGACTGCAGCTACATGCTCTCGATAAGCTCAT-3′
eRF1$^{1-230}$	*Eco*RI	*Bam*HI

Superscript numbers in mutants indicate positions of retained amino acids.

A

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                                  478                            510
                                   |                               |
Homo sapiens                  ...NVEVLGILSD-DVETDTVAPGENLKIRLKGIEEE
Xenopus laevis                ...IVEVLSLLSD-EVETELVAPGENLKLRLKGIEEE
Pichia pinus                  ...QVEVTTIYNETEAEADSAFCGEQVRLRLRGIEEE
Saccharomyces cerevisiae      ...AVEIQNIYNETENEVDMAMCGEQVKLRIKGVEEE
Candida albicans              ...PIEVLTIFNETEQECDTAFSGEQVRLKIKGIEEE
Nicotiana tabacum             ...AVKVLAIFCD-EDKVRHVGPGENVRVRLSGVEED
Caenorhabditis elegans        ...PVQVLQIWAD-DVETERVVAGDNIKFKLKGIEEN
Drosophila melanogaster       ...QVAVDQLFSD-DFEVTSVGPGENVKIKLKGIEEE

                              511                530   628       637
                               |                  |     |         |
Homo sapiens                  EILPGFILCDPNNLCHSGRT.....KVLKLVPEKD--
Xenopus laevis                EILPGFILCDPNNLCHSGRT.....KVLKLVPEKD--
Pichia pinus                  DLSAGYVLSSINHPVKTVTR.....KVTKLL------
Saccharomyces cerevisiae      DISPGFVLTSPKNPIKSVTK.....KIVKIAE-----
Candida albicans              DLQPGYVLTSPKNPVKTVTR.....KITKLL------
Nicotiana tabacum             DILSGFVLCSVAKPIHAVTE.....KITALPTVADNA
Caenorhabditis elegans        ELQGGFIICSPDSLAKTGRV.....KVLKVVE-----
Drosophila melanogaster       DVSPGFVLCDAANPIKTGKI.....KVLKVVE-----
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B

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                                     281                     305
                                      |                        |
Homo sapiens                      ...IQEKKLIGRYFDEIS-QDTGKYCFGV...
Mus musculus                      ...IQEKKLIGRYFDEIS-QDTGKYCFGV...
Mesocricetus auratus              ...IQEKKLIGRYFDEIS-QDTGKYCFGV...
Xenopus laevis                    ...IQEKKLIGRYFDEIS-QDTGKYCFGV...
Caenorhabditis elegans            ...IQEKKLIGGYFDEIS-QDTGKYVFGV...
Arabidopsis thaliana              ...IQEKKLIGKYFEEIS-QDTGKYVFGV...
Saccharomyces cerevisiae          ...VQEKKLLEAYFDEIS-QDTGKFCYGI...
Schizosaccharomyces pombe         ...VQEKKLIQRFFDEIS-LDSGKYCFGV...
Methanococcus jannaschii          ...MKEREAVQRFLKELIKEDGGLACYGE...
Archaeoglobus fulgidus            ...IREKNLMQRFLKEVA-RD-GLAAYGE...

                                     411  415                   437
                                      |    |                     |
Homo sapiens                      ...GILRYRVDFQGMEYQGGDDEFFDLD---DY---
Mus musculus                      ...GILRYRVDFQGMEYQGGDDEFFDLD---DY---
Mesocricetus auratus              ...GILRYRVDFQGMEYQGGDDEFFDLD---DY---
Xenopus laevis                    ...GILRYRVDFQGMDYQGVDDEFFDLD---DY---
Caenorhabditis elegans            ...GLLRYRVDLAHVDLED-ELDNIDLD---DY---
Arabidopsis thaliana              ...GLLRYQLDMRTFDELS-DGEVYEDS---D----
Saccharomyces cerevisiae          ...AMLRYKVNFEQLVDES-EDEYYDEDEGSDYDFI
Schizosaccharomyces pombe         ...AVMRYQLDLSMLDPES--DEFYSDS---D----
Methanococcus jannaschii          ...AILRYKIHQ------------------------
Archaeoglobus fulgidus            ...AILRFKPD-------GGQ---------------
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Fig. 4. Amino acid sequences of eRF3 (A) and eRF1 (B) involved in mutual binding. Amino acid identities (black boldface), similarities (grey boldface) and dissimilarities (italic) are indicated. Positions corresponding to the boundaries of the interacting regions in human factors are in boldface. GenBank accession numbers for eRF3 are: for *Homo sapiens*, X17644; for *Xenopus laevis*, L37045; for *Pichia pinus*, P23637; for *Saccharomyces cerevisiae*, P05453 and P05420; for *Candida albicans*, AF020554; for *Nicotiana tabacum*, L38828; for *Caenorhabditis elegans*, Z92835; for *Drosophila melanogaster*, U88868. GenBank accession numbers for eRF1 are: for *Homo sapiens*, P46055; for *Mus musculus*, D87691; for *Mesocricetus auratus*, X81626; for *Xenopus laevis*, P35615; for *Caenorhabditis elegans*, AF016452; for *Arabidopsis thaliana*, X69375; for *Saccharomyces cerevisiae*, X04082; for *Schizosaccharomyces pombe*, D63883; for *Methanococcus jannaschii*, Q58239; for *Archaeoglobus fulgidus*, AE001020 and AE000782. ClustalW Multiple Sequence Alignment Program (version 1.7, June 1997) was applied.

extend this observation using another approach, we measured the functional activity of eRF1 mutants in two assay systems. First, we showed (Fig. 3) that the C-terminally truncated $eRF1^{1-415}$* mutant promoted eRF3 GTPase activity in the presence of the ribosomes. This observation implied that deletion of the last 22 amino acid residues of eRF1 did not interfere with eRF1•eRF3 complex formation in vitro since eRF3 GTPase exhibited activity only if bound to eRF1 [11]. Second, the $eRF1^{1-415*}$ mutant was also active as a release factor (not shown) promoting hydrolysis of formylmethionyl-$tRNA_f^{Met}$ in the presence of the ribosomes and any of the three stop codons. This implied that the C-terminus of eRF1 is not only dispensable for eRF3 binding but is also non-essential for its own catalytic activity.

The GTPase-stimulating activity of the $eRF1^{271-437}$ mutant was low (Fig. 3) although it contained both critical eRF3 binding regions. The lack of a considerable portion of the eRF1 sequence (positions 1–270) where the ribosome binding site was thought to be located might abrogate the stable eRF1 binding to the ribosome and prevent ternary eRF1•eRF3•ribosome complex formation, a prerequisite for eRF3 GTPase activity.

When the critical region at the C-terminus of eRF1 was deleted (positions 411–415) the $eRF1^{2-410}$ mutant not only

lost its eRF3 binding capacity in vivo (Fig. 1) but its stimulating activity towards eRF3 GTPase was profoundly diminished (Fig. 3). From these observations we concluded that the eRF1 region encompassing amino acids 411–415 is essential for eRF3 binding and for stimulating eRF3 GTPase activity.

To prove further the functional role of the C-terminal region of eRF3, the eRF3$^{139-627}$ mutant was tested for GTPase activity and found to be inactive (Fig. 3). Deletion of the 138 N-terminal amino acids in this mutant did not impair its functional activity [11–13]. Therefore, its inactivation was caused by the lack of the C-terminal decamer. The second eRF3 mutant, eRF3$^{139-478}$, lacking both critical regions for eRF1 binding, was inactive as well (Fig. 3). In both of these eRF3 mutants the GTP binding domain was retained (Fig. 2). Consequently, the abolition of eRF1 binding correlated with loss of eRF1-dependent GTPase activity. GTPase activity of the N-terminally truncated eRF3 proteins was not tested because of deletion of their GTP binding motifs.

4. Discussion

4.1. Structural-functional anatomy of eRF3

Two regions are critical in the eRF3 sequence for eRF1 binding: the C-terminal decamer (positions 628–637) and the longer stretch (positions 478–530) inside the C-terminal domain. As is evident from the alignment (Fig. 4), positions 634–637 are non-conserved in the eRF3 family whereas the upstream positions 628–633 are conservative. Therefore, we deduce that for eRF1 binding the C-terminal tetramer might be dispensable while the adjacent upstream hexamer is critically important. Positions 478–530 are composed of highly conserved (75%) and non-conserved (25%) amino acids. Since this stretch is relatively long we suppose that conserved residues (Fig. 4) are essential for eRF1 binding while non-conserved amino acids are dispensable.

At the C-terminal domain of eRF3 two regions essential for binding and the fragment between them are remote from GTP binding motifs (Fig. 2). Hence, when eRF1 binds to eRF3, the GTPase activation is probably mediated via a conformational change of eRF3 induced by eRF1 binding. The non-overlapping of GTP and eRF1 binding regions (Fig. 2) is in agreement with earlier data [12] showing that GTP binding to eRF3 is not affected by eRF1.

Very recently the mouse genes GSPT1 and GSPT2, structurally related to human eRF3, have been cloned and sequenced [29] although the catalytic activity of their protein products has not been demonstrated. The GSPT1 and GSPT2 proteins were able to form a complex with human eRF1 in the two-hybrid assay [29] as was shown earlier for human eRF1 and eRF3 [13]. Deletion of the C-terminal domain (205 amino acids) of the GSPT2 protein (presumably mouse eRF3) caused impairment of binding capacity towards eRF1 in full agreement with our more detailed data (Fig. 1). It is very surprising that the highly variable and functionally non-essential N-terminal domain of eRF3/GSPT2 affected binding with the highly conserved human eRF1.

4.2. Structural-functional anatomy of eRF1

A considerable portion of the eRF1 sequence could be deleted from the N-terminus without affecting eRF3 binding (Fig. 1). Furthermore, 22 C-terminal amino acids may also be deleted without loss of the binding capacity and functional activity (Figs. 1 and 3). On the other hand, deletion of fragment 281–305 or 411–415 impairs the binding of the truncated eRF1 to eRF3 (Fig. 2). These two critical regions are highly conserved in the eRF1 family (Fig. 4). The 411–415 amino acid sequence is rather hydrophobic and may interact with hydrophobic regions in eRF3. In contrast to the 411–415 region, the 281–305 region is hydrophilic (Fig. 4) and has a potential for electrostatic interactions with eRF3.

In addition to eRF3, eRF1 binds to a catalytic subunit of protein phosphatase 2A both in vitro and in vivo [30]. By deletion analysis, the PP2Ac binding domain was found to be located between amino acid residues 338 and 381 of the human eRF1. This region is located exactly between the two regions critical for eRF3 binding (Fig. 2). Therefore, the C domain of eRF1 seems to be open for protein-protein interactions while the N domain looks to be more adapted for nucleic acid-protein interactions.

Recently, the eRF1 binding sites for eRF3 were analysed for eRF1/Sup45p from *Schizosaccharomyces pombe* [31]. It was concluded that the stretch of acidic amino acids at the C-terminus of eRF1/Sup45p is a primary binding site for eRF3. As shown here, this region is essential neither for the human eRF3 binding nor for the human eRF1 activity. Our conclusion is supported by the fact that the last 11 amino acids of the eRF1 protein family are weakly conserved (Fig. 4). The other difference concerns the internal essential binding region: in the human eRF1 it encompasses positions 281–305 while in fission yeast this region was found to be much broader (positions 187–247) and displaced towards the N-terminus (Fig. 2). Surprisingly, both internal and C-terminal regions for eRF3 binding do not overlap in the human and *S. pombe* eRF1. On the other hand, our conclusion that the 22 C-terminal amino acids of the human eRF1 are non-essential for eRF1 function in vitro is consistent with the data on in vivo complementation of C-terminally truncated fission yeast eRF1/Sup45p with the thermosensitive Sup45p mutant from *S. cerevisiae* [31].

As is well known (see [32,33]) G proteins interact with at least two types of proteins, GAP, a GTPase activating protein, and GEF, a guanine nucleotide exchange factor. It was suggested earlier [11,12] that eRF1 and the ribosome may be regarded as a composite GAP by analogy with other G proteins. In prokaryotes, RF3 GTPase activity depends solely on the ribosome, not on RF1/2 [18], and EF-Ts is a GEF for EF-Tu. For this reason one may assume that eRF3 is also activated mostly or solely by the ribosome, and if so, eRF1 may predominantly serve as a GEF for eRF3.

The two-hybrid system does not make it possible to detect the complex inside the ribosomal particle. However, since eRF3 GTPase is not only ribosome-dependent, but also eRF1-dependent, there is little doubt that this complex is also present on the ribosome at a certain stage of the translation cycle. It is noteworthy that GTP hydrolysis is required for eRF3 function, not simply GTP binding [12].

The deletion analysis employed in this work was based on truncation of both ends of the polypeptide chains of the factors and no internal deletions were introduced. Therefore, we cannot yet decide whether the amino acid sequences between the critical regions of each factor (positions 531–627 for eRF3 and positions 306–410 for eRF1) are essential or non-essential for mutual binding (Fig. 2). The second issue which remained unresolved in these studies concerns continuity of the critical

regions of both factors. In principle, these regions may be independent and topologically remote. Alternatively, the critical regions of each factor may neighbour each other in space forming a single continuous composite binding site. To resolve this alternative one has to know the 3D structure of each factor and of the complex. Regretfully, this information is so far not available.

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