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C-terminal interaction of translational release factors eRF1 and eRF3 of fission yeast: G-domain uncoupled binding and the role of conserved amino acids

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ABSTRACT

Translation termination in eukaryotes requires a stop codon-responsive (class-I) release factor, eRF1, and a guanine nucleotide-responsive (class-II) release factor, eRF3. *Schizosaccharomyces pombe* eRF3 has an N-terminal polypeptide similar in size to the prion-like domain of *Saccharomyces cerevisiae* eRF3 in addition to the EF-1α-like catalytic domain. By in vivo two-hybrid assay as well as by an in vitro pull-down analysis using purified proteins of *S. pombe* as well as of *S. cerevisiae*, eRF1 bound to the C-terminal one-third domain of eRF3, named eRF3C, but not to the N-terminal two-thirds, which was inconsistent with the previous report by Paushkin et al. (1997, *Mol Cell Biol 17*:2798–2805). The activity of *S. pombe* eRF3 in eRF1 binding was affected by Ala substitutions for the C-terminal residues conserved not only in eRF3s but also in elongation factors EF-Tu and EF-1α. These single mutational defects in the eRF1–eRF3 interaction became evident when either truncated protein eRF3C or C-terminally altered eRF1 proteins were used for the authentic protein, providing further support for the presence of a C-terminal interaction. Given that eRF3 is an EF-Tu/EF-1α homolog required for translation termination, the apparent dispensability of the N-terminal domain of eRF3 for binding to eRF1 is in contrast to importance, direct or indirect, in EF-Tu/EF-1α for binding to aminoacyl-tRNA, although both eRF3 and EF-Tu/EF-1α share some common amino acids for binding to eRF1 and aminoacyl-tRNA, respectively. These differences probably reflect the independence of eRF1 binding in relation to the G-domain function of eRF3 (i.e., probably uncoupled with GTP hydrolysis), whereas aminoacyl-tRNA binding depends on that of EF-Tu/EF-1α (i.e., coupled with GTP hydrolysis), which sheds some light on the mechanism of eRF3 function.

Keywords: EF-Tu homology; eRF1; eRF3; G domain; protein release factor; Sup35; Sup45; tRNA mimicry

INTRODUCTION

In eukaryotes, one translational release factor, eRF1, recognizes three stop codons (class-I), and another factor, eRF3, stimulates eRF1 activity and binds guanine nucleotides (class-II). The mechanism by which the eRF1 protein reads the stop codon and the G protein, eRF3, controls the mode of termination have been coding and translational problems for the three decades since the discovery of the genetic code (for a review, see Nakamura et al., 1996). Prokaryotes have two class-I release factors, RF1 and RF2, that recognize UAG/UAA and UGA/UAA, respectively. From a sequence comparison of release factors of different organisms, we have proposed a model in which the class-I release factors mimic the shape of tRNA for binding to the decoding site (A site) of the ribosome and mimic a tRNA anticodon for reading the stop codon ("RF-tRNA mimicry" hypothesis; Ito et al., 1996).

The mimicry of tRNA by protein has been identified by means of structural studies of bacterial elongation factors EF-G and EF-Tu complexed with guanine nucleotide(s) and aminoacyl-tRNA. The three-dimensional structure of *Thermus thermophilus* EF-G comprises five subdomains; the C-terminal part, domains III–V (Ævarsson et al., 1994; Czworkowski et al., 1994), appears to mimic the shapes of the acceptor stem, the anticodon helix, and the T stem of tRNA, respectively (Nissen et al., 1995). Class-I release factors share homology with domain IV of EF-G (Ito et al., 1996). Mutational

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studies have provided evidence that bacterial RF1 and RF2 encode a putative protein anticodon moiety (Ito et al., 1998b; for a review, see Nakamura & Ito, 1998). The model of RF-tRNA mimicry predicts that a class-II factor, eRF3, may be an EF-Tu-like vehicle protein to bring class-I proteins to the A site of the ribosome. Several lines of evidence support this view; eRF3 shows considerable C-terminal homology to EF-1α (for a review, see Stansfield & Tuite, 1994), and eRF3 and eRF1 bind in vivo and in vitro and exist as a heterodimer complex in yeast cell lysates (Stansfield et al., 1995; Zhouravleva et al., 1995; Ito et al., 1998a).

To extend the analysis of eukaryotic release factor function and interaction, we have cloned the eRF1 (identical to Sup45) and eRF3 (identical to Sup35) genes of the fission yeast *Schizosaccharomyces pombe* (Ito et al., 1996, 1998a). These genes are homologous to *Saccharomyces cerevisiae* counterparts and complement temperature-sensitive mutations in *sup45* and *sup35* of *S. cerevisiae*, respectively. *S. pombe* eRF3 is a protein with a molecular mass of 72.5 kDa (composed of 662 amino acids), and the deduced protein sequence of the C-terminal 430 amino acids is highly similar to that of *S. cerevisiae* eRF3 as well as to EF-1α. However, the 230 N-terminal amino acids do not share any sequence homology with *S. cerevisiae* eRF3. The C-terminal two-thirds are essential for viability and translation termination, while the N-terminal one-third is not conserved and is not essential for viability (Stansfield & Tuite, 1994). The N-terminal domain (250 amino acids) of *S. cerevisiae* eRF3 contains four tandem nonapeptide repeats (PQGGYQQYN), similar to mammalian prion repeats, and affects protein termination with prion-like properties (Stansfield & Tuite, 1994; Lindquist, 1997). However, *S. pombe* eRF3 does not contain any known prion-specific peptide repeats, but instead contains unique peptide repeats rich in Ala, Pro, Ser, and Thr (referred to as an APST repeat; Ito et al., 1998a) of yet unknown function.

Paushkin et al. (1997) have examined the eRF1-binding site(s) on eRF3 of *S. cerevisiae* by an in vitro pull-down analysis using immobilized eRF1 and yeast lysates containing differently truncated eRF3 fragments. By these analyses, two regions have been identified as participating in eRF1 binding: one, the middle one-third region between amino acid positions 254 and 480 (containing the G domain), and the other, the N-terminal one-third region between positions 1 and 240 (containing a prion-like domain), suggesting that the latter domain may act as a prion-dependent repressor of eRF1 in *S. cerevisiae* (Paushkin et al., 1997). In this study, we have independently characterized the *S. pombe* as well as *S. cerevisiae* eRF3 region(s) necessary for binding to eRF1 by an in vitro pull-down analysis using purified eRF1 and eRF3 fragments as well as by a yeast two-hybrid system. Our data disagree with those of Paushkin et al. (1997), showing that eRF1 binds to the C-terminal one-third domain (downstream of position 482) of *S. pombe* eRF3 as well as of *S. cerevisiae* and human eRF3s. These findings suggest that eRF1 binding to eRF3 may not be coupled with a GTP/GDP switching of the eRF3 protein conformation, which is in sharp contrast to the mode of aminoacyl-tRNA binding to the elongation factor EF-Tu/EF-1α.

RESULTS

Binding of eRF1 to the C-terminal domain of *S. pombe* eRF3

The N-terminally truncated EF-1α-like polypeptides of eRF3, referred to as eRF3* (Ito et al., 1998a), of *S. pombe, S. cerevisiae*, *Xenopus laevis*, and humans are known to bind to eRF1 both in vivo and in vitro (Stansfield et al., 1995; Zhouravleva et al., 1995; Ito et al., 1998a). To further map the eRF1-binding site(s), *S. pombe* eRF3 was truncated by *Kpn*I restriction enzyme at amino acid position 481, splitting into two fragments, eRF3ΔC and eRF3C (Fig. 1). The ability of these polypeptides to interact with *S. pombe* eRF1 was examined by the *GAL4*-based two-hybrid system (Fields & Song, 1989; Chien et al., 1991), as described previously (Ito et al., 1998a). eRF1 and eRF3 polypeptides were cloned in-frame downstream of the *GAL4* activation (ad) and binding (bd) domains, respectively, and the resulting plasmids were transformed in different pair-wise combinations into *S. cerevisiae* host strain HF7c (Feilotter et al., 1994). (Note that the reciprocal fusions between release factors and ad/bd vectors were also tested in all two-hybrid analyses shown here, which gave essentially the same result.) The HF7c yeast strain contained a reporter gene, *HIS3*, under the control of *GAL4*-responsive elements, and an in vivo protein–protein interaction enabled the reporter transformant to grow on histidine-free minimal medium. This two-hybrid assay indicated that the C-terminal segment eRF3C (amino acid positions 482–662) bound eRF1 (see Fig. 4A, sample 1) similarly to eRF3* (positions 212–662), whereas the N-terminal segment eRF3ΔC (positions 1–481) and eRF3*ΔC (positions 212–481) did not (data not shown; see Fig. 1).

To confirm the interaction of truncated eRF3 polypeptides with eRF1 in vitro, the eRF3 segments were fused to their N-termini with glutathione *S*-transferase (GST), as described previously (Ito et al., 1998a), and immobilized onto glutathione-agarose beads for the pull-down analysis. *S. pombe* eRF1 was tagged at its N-terminus with a hexa-histidine sequence and purified by affinity to nickel-agarose beads (see Materials and Methods). The resin with bound GST-eRF3 polypeptides were incubated with purified His_6-eRF1 and then washed to remove nonspecific proteins. Bound proteins were eluted and analyzed by Western blotting to stain the eRF1-bearing histidine tag with an Ni-NTA-

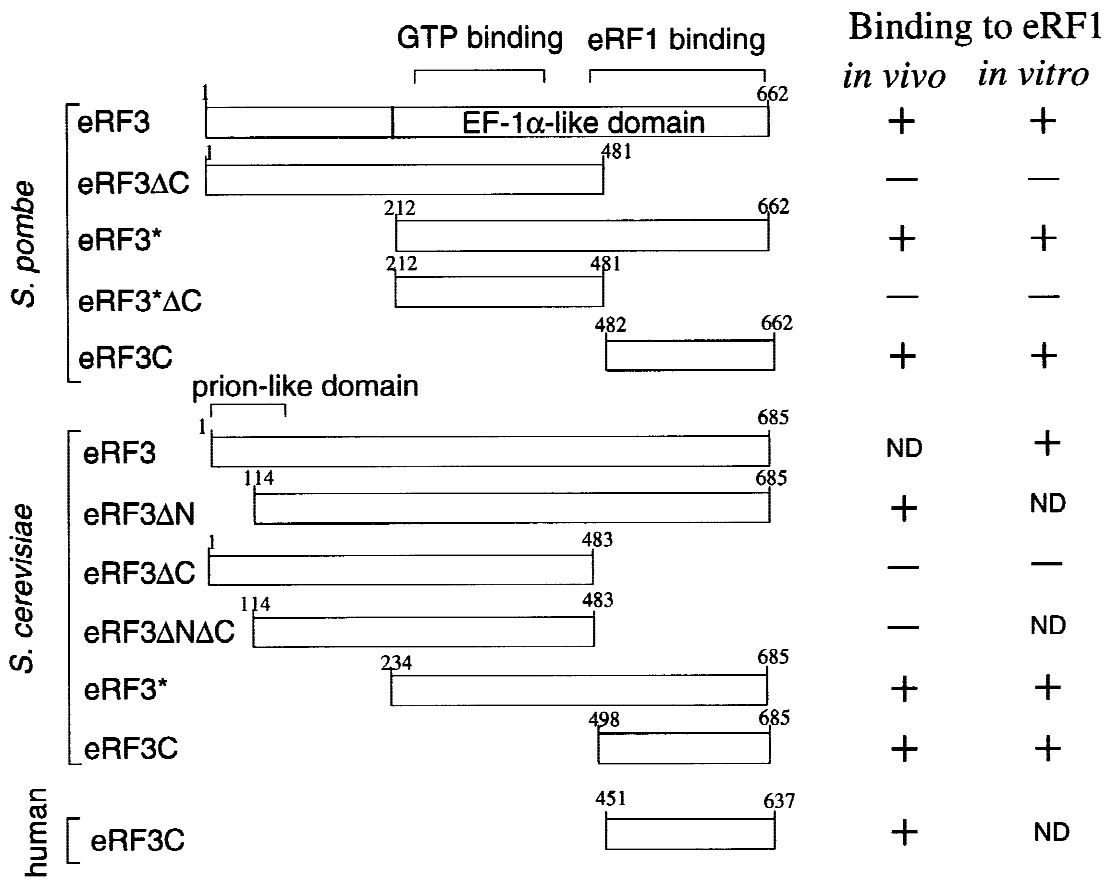

FIGURE 1. Truncated eRF3 proteins of *S. pombe*, *S. cerevisiae*, and humans and their binding to homogeneous eRF1. Truncated eRF3 polypeptides were generated by restriction enzyme digestion or by PCR amplification. Boxes indicate eRF3 segments cloned into *GAL4*-bd vector pGBT9 or fused to GST for overproduction and purification. The activity of various eRF3 segments for in vitro binding to eRF1 was examined by a pull-down analysis, as shown in Figure 2, and the data are summarized. +: binding; −: no binding. The number refers to the amino acid position from the translation start site. For in vivo binding analysis, the two-hybrid reporter strain HF7c was transformed with these pGBT plasmids in pair-wise combination with the *GAL4*-ad vector pGAD424 containing eRF1. The His phenotype of these transformants was monitored by growth on histidine-free minimal medium. +: growth (binding); −: no growth (no binding); ND: no data.

horseradish peroxidase conjugate. When the eRF1 and eRF3 derivatives were mixed, immobilized eRF3, eRF3*, and eRF3C efficiently precipitated eRF1, as shown in the SDS-polyacrylamide gel analysis (PAGE) (Fig. 2B, lanes 6, 8, and 10), whereas immobilized eRF3ΔC and eRF3*ΔC did not (Fig. 2B, lanes 7 and 9). These results indicated that the eRF1-binding site on eRF3 resides in positions 482–662 and that the G domain is not involved in the binding.

It is known that *S. pombe* eRF3 and eRF3* are able to restore growth of the temperature-sensitive eRF3 strain, *gst1-1* (Kikuchi et al., 1988) by intergeneric complementation (Ito et al., 1998a). Neither of the truncated polypeptides, eRF3C or eRF3ΔC, however, restored the viability of the *gst1-1* strain (data not shown).

Binding of eRF1 to the C-terminal domain of *S. cerevisiae* and human eRF3s

The above findings that eRF1 interacts with the C-terminal part, downstream of position 481, instead of with the N-terminal part of eRF3 conflict with the findings of Paushkin et al. (1997). This conflict could possibly be explained by the difference in the eRF3 species used by those investigators (*S. cerevisiae*) and by us (*S. pombe*). This explanation appears unlikely, however, based on the following experiments. *S. cerevisiae* eRF1 and a set of eRF3 truncations of *S. cerevisiae*, eRF3ΔC (positions 1–483), eRF3ΔN (positions 114–685), eRF3* (positions 234–685), eRF3ΔNΔC (positions 114–483), and eRF3C (positions 498–685) as well as eRF3, were fused downstream of the *GAL4*-ad and *GAL4*-bd sites, respectively. Note that *S. cerevisiae* eRF3C is structurally equivalent to *S. pombe* eRF3C, and eRF3ΔNΔC is equivalent to Sup35MCΔSp, which has been shown by Paushkin et al. (1997) to bind to eRF1 by an in vitro pull-down analysis. The two-hybrid analysis using these plasmids and the reporter yeast strain HF7c essentially confirmed the results with the *S. pombe* release factors, showing that the C-terminal part, downstream of position 498, of *S. cerevisiae* eRF3 binds to eRF1, but the N-terminal part

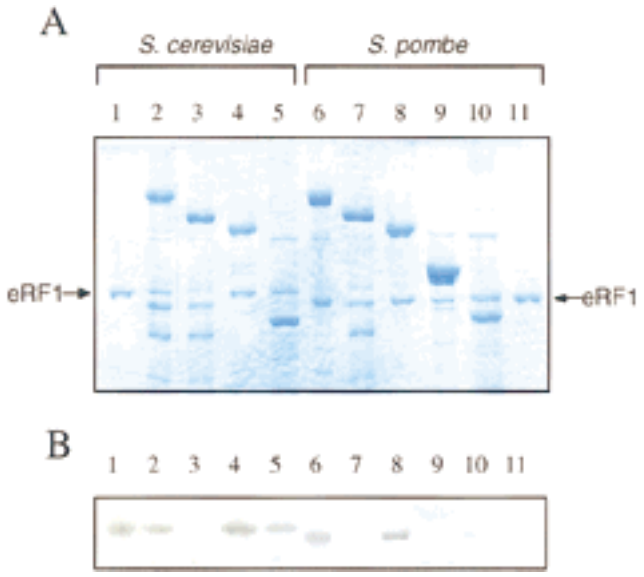

FIGURE 2. In vitro pull-down analysis of eRF1 with truncated eRF3s. The GST-fusions to eRF3 derivatives and histidine-tagged eRF1 were overexpressed and purified as described previously (Ito et al., 1998a). Immobilized-eRF3 polypeptide beads were mixed with His_6-eRF1 proteins (10 nmol) and incubated at 30 °C for 30 min. eRF1 proteins bound to the beads were collected by centrifugation, washed, and the eRF1-eRF3 polypeptide complexes were eluted from the beads by adding excess glutathione. These proteins were subjected to SDS-PAGE. **A**: Staining with Coomassie brilliant blue of complexes eluted from the beads. **B**: Western blot of eRF1 using an Ni-NTA-horseradish peroxidase conjugate essentially as described previously (Ito et al., 1998a). *S. cerevisiae* eRF1 (lanes 1–5) and *S. pombe* eRF1 (lanes 6–11) were mixed with homologous eRF3 derivatives. lane 1: *S. cerevisiae* eRF1 (control); lane 2: *S. cerevisiae* eRF3; lane 3: *S. cerevisiae* eRF3ΔC; lane 4: *S. cerevisiae* eRF3*; lane 5: *S. cerevisiae* eRF3C; lane 6: *S. pombe* eRF3; lane 7: *S. pombe* eRF3ΔC; lane 8: *S. pombe* eRF3*; lane 9: *S. pombe* eRF3*ΔC; lane 10: *S. pombe* eRF3C; lane 11: *S. pombe* eRF1 (control).

does not (Fig. 1). Expression of Gal4-bd fusions to eRF3 polypeptides in these transformants that were negative or positive in the two-hybrid assay was confirmed by Western blot analysis using anti-Gal4-bd antibody (data not shown).

The pull-down analyses of *S. cerevisiae* eRF1 (tagged with His_6) and eRF3 derivatives (fused to GST) confirmed these results; eRF3 with the intact C-terminal part bound to eRF1 (Fig. 2, lanes 2, 4, and 5) but not without the C-terminal part (Fig. 2, lane 3).

Finally, the human eRF3C segment (positions 451–637), equivalent to yeast eRF3C, was also able to interact with human eRF1 in the same two-hybrid analysis (Fig. 1). These results demonstrate that the observed conflict was not due to the species difference of the release factors being used but to some other experimental conditions or procedures (discussed shortly), and that the C-terminal one-third domain of eRF3 is sufficient to form a heterodimeric complex with eRF1.

C-terminal interaction between eRF1 and eRF3

We have already shown that the primary eRF3-binding site resides within the C-terminal 11 amino acids of eRF1; half of these amino acids are acidic residues (see Fig. 3). Four C-terminal deletions, ΔCα, ΔCβ, ΔCγ, and ΔC1 removed 2, 6, 11, and 17 amino acids, respectively, at the C-terminus (Ito et al., 1998a; see Fig. 3). By an in vivo two-hybrid test (Fig. 4A, samples 5–8) as well as by an in vitro pull-down analysis (Fig. 4B, lanes 2–4), eRF3C binding to eRF1 was not affected by the C-terminal two-amino-acid deletion but was completely eliminated in response to the deletion of more than six amino acids (see Fig. 5). Of the four N-terminal deletions tested, with ΔN6 through ΔN9 removing 227, 247, 276, and 349 amino acids (Ito et al., 1998a; see Fig. 5), only the largest deletion with ΔN9 diminished the activity of eRF1 to bind to eRF3C in the in vivo (Fig. 4A, samples 2–4) and in vitro (Fig. 4B, lanes 5 and 6; also data not shown) binding analyses (see Fig. 5). Therefore, it appears that two release factors, eRF1 and eRF3, interact with their C-terminal domains.

Decreased eRF1 binding by C-terminal amino acid substitutions of eRF3

The C-terminal sequence of *S. pombe* eRF3 was compared with those of human and *X. laevis* eRF3s as well as with the elongation factors EF-Tu and EF-1α. Although these proteins share high sequence homology in their N-terminal G-domain regions (data not shown), the conservation of several discrete, perhaps critical, amino acids is observed in the C-terminal regions that are capable of binding to eRF1 (Fig. 6). Among these, two residues, Tyr-577 and Asp-647, of *S. pombe* eRF3 are equivalent to Tyr-338 and Glu-390 of *Thermus aquaticus* EF-Tu, both of which are known to be involved in the binding to tRNA (Nissen et al., 1996; discussed shortly). We substituted Ala for five conservative and one nonconservative residues including these two sites and generated eRF3 and eRF3* derivatives carrying F560A, G576A, Y577A, S578A, F643A, and D647A substitutions. The binding of these eRF3 or eRF3* derivatives and eRF1 was examined by the same two-hybrid method as described above. The resulting reporter HF7c transformants grew on histidine-free minimal medium (Fig. 7A,B), showing that these conservative amino acid changes per se, or the Y577A-D647A double mutant, do not appreciably affect eRF3 binding to wild-type eRF1.

However, when these mutations were introduced into the eRF3C polypeptide, four of six derivatives, F560A, Y577A, F643A, and D647A, no longer bound to eRF1 in the two-hybrid system (Fig. 7C). This synergistic effect is seemingly consistent with the loss of binding of eRF3C (Fig. 4A, sample 6; Fig. 5), not eRF3* (Ito et al.,

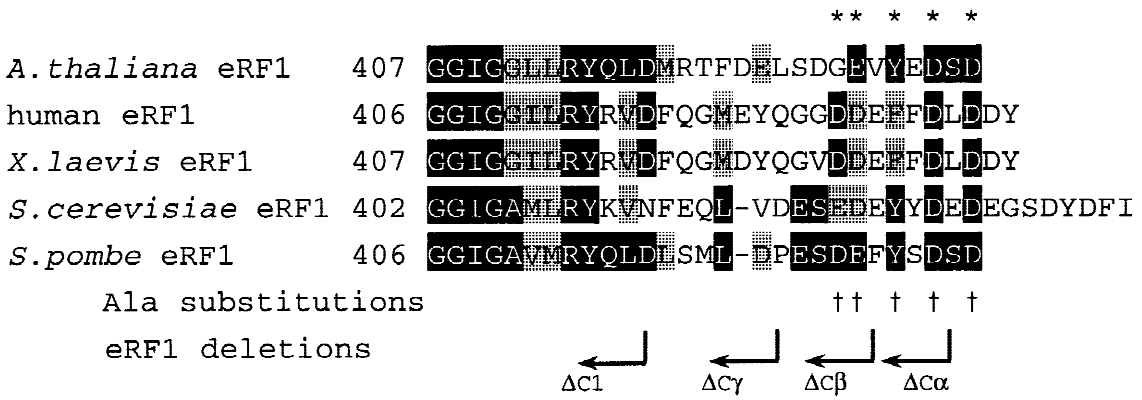

FIGURE 3. Comparison of the amino acid sequences of eukaryotic eRF1s. The similarity alignments of eRF1s were accomplished using the BESTFIT or PILEUP program from the GCG program package (Ito et al., 1998a). Identical residues compared with *S. pombe* eRF1 are boxed in black, and those similar to *S. pombe* eRF1 are boxed in gray. Asterisks mean complete or partial conservation of acidic amino acids at the indicated position. Daggers represent the position of amino acids mutated to alanine, and arrows represent the C-termini of *S. pombe* eRF1 deletions, ΔC1, ΔCγ, ΔCβ, and ΔCα.

1998a), to eRF1-ΔCβ that is partially affected in the eRF3 binding site. This effect is presumably due to the additional defect in the eRF1-binding ability upon removal of the middle domain, positions 212–481, of eRF3, which may indirectly influence the C-terminal activity.

Synergistic defects in eRF1-eRF3 interaction by their C-terminal alterations

The effect of Ala substitutions on the conservative amino acids of eRF3 became evident when the C-terminal eRF3-binding site of eRF1 was altered. In the C-terminal acidic amino acid stretch of eRF1, five conservative residues of eRF1 at positions 433 (Asp), 431 (Asp), 429 (Tyr), 427 (Glu), and 426 (Asp) were altered to Ala (see Fig. 3). The two-hybrid analysis indicated that no single substitutions or C-terminal triple substitutions (D433A D431A Y429A) of eRF1 reduced its binding to altered eRF3* polypeptides (data not shown). The quintuple mutant of eRF1 (referred to as eRF1-5A), however, failed to interact with the eRF3* that sustained the Y577A allele (Fig. 7D).

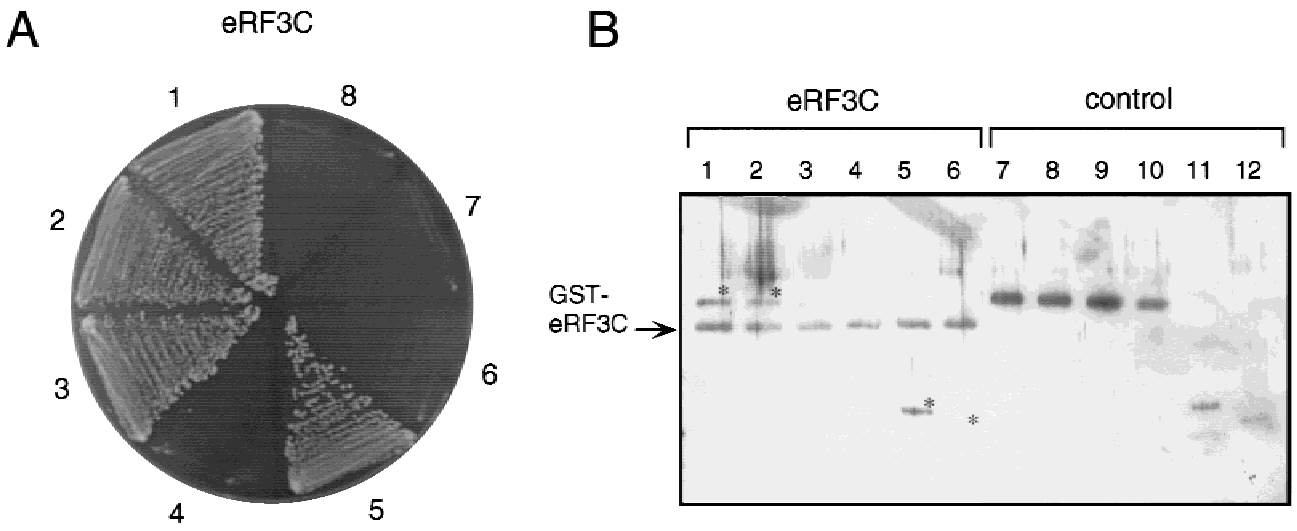

FIGURE 4. Truncated eRF1 proteins and their binding to the C-terminal domain of *S. pombe* eRF3. **A**: In vivo two-hybrid assay. *S pombe* eRF3C segment (amino acid positions 482–662) and truncated eRF1 segments were cloned into plasmids pGBT9 and pGAD424, respectively, and transformed into HF7c in different pair-wise combinations. Growth of these transformants on histidine-free minimal medium was monitored. pGAD424 samples: 1: wild-type eRF1; 2: eRF1-ΔN7; 3: eRF1-ΔN8; 4: eRF1-ΔN9; 5: eRF1-ΔCα; 6: eRF1-ΔCβ; 7: eRF1-ΔCγ; 8: eRF1-ΔC1. **B**: In vitro pull-down analysis. The GST-eRF3C fusion protein and histidine-tagged eRF1 polypeptides were purified, and their binding was examined as described in Figure 2. His_6-eRF1 polypeptides used for the binding assay (lanes 7–12) and those bound to the immobilized-eRF3C beads (lanes 1–6) were detected by standard Western blot techniques using an Ni-NTA-horseradish peroxidase conjugate essentially as described previously (Ito et al., 1998a). Lanes 1 and 7: wild-type eRF1; lanes 2 and 8: eRF1-ΔCα; lanes 3 and 9: eRF1-ΔCβ; lanes 4 and 10: eRF1-ΔCγ; lanes 5 and 11: eRF1-ΔN6; lanes 6 and 12: eRF1-ΔN7. Asterisks indicate eRF1 derivatives coprecipitated with eRF3C.

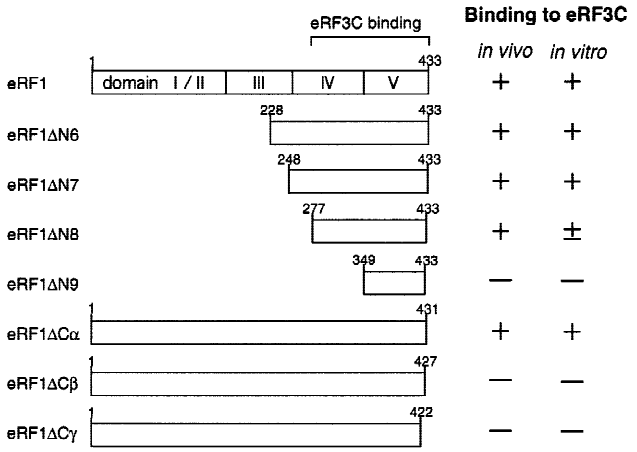

FIGURE 5. Truncated eRF1 proteins and their binding to the C-terminal domain of *S. pombe* eRF3. The activity of the eRF3C segment (amino acid positions 482–662) for binding to different truncated eRF1 polypeptides was examined by an in vivo two-hybrid system as well as by an in vitro pull-down analysis, as shown in Figure 4, and the data are summarized. +: binding, ±: weak binding; −: no binding. The number refers to the amino acid position from the translation start site. Sequence motifs homologous to domains III (acceptor stem mimicry), IV (anticodon helix mimicry), and V (T stem mimicry) of elongation factor EF-G are assigned (Ito et al., 1996, 1998a).

The necessity of having multiple C-terminal substitutions to diminish eRF1 binding to eRF3*-Y577A suggests that not a single specific residue but a peptide moiety consisting of multiple residues in the C-terminus of eRF1 is responsible for binding to eRF3. A two-hybrid analysis using eRF1-ΔCβ instead of eRF1-5A for the *GAL4*-ad construct essentially confirmed this result (Fig. 7E). Note that eRF3* binds to eRF1-ΔCβ even if eRF3C no longer binds (as described above). These observations indicate that Tyr-577 of eRF3 is involved in the interaction with eRF1 to form a heterodimer complex. The observed synergistic effects of altered eRF1 and eRF3 may be explained by assuming a partial inactivation of each release factor. It remains to be investigated whether these two loci interact directly.

Involvement of conservative amino acids of eRF3 for eRF1 binding in vitro

To establish the above argument, we carried out the in vitro binding assay using immobilized GST-eRF3* with or without Y577A and/or D647A alterations, and His_6-eRF1 with or without the 5A or ΔCβ mutation. The GST-eRF3* derivatives (immobilized to beads) and His_6-eRF1 derivatives were mixed, and the protein fractions precipitated with beads were eluted with glutathione and analyzed by SDS-PAGE (Fig. 8A). The intensities of His_6-eRF1 and GST-eRF3* bands were quantified by Western blotting with a histidine tag-directed Ni-NTA-horseradish peroxidase conjugate and anti-GST antibody, respectively, using an enhanced chemiluminescence system and densitometry scanning. The efficiency of eRF1-eRF3* binding was estimated by the relative value of His_6-eRF1 to GST-eRF3*, which represents the internal control throughout the experiment (Fig. 8B). The data indicate that the activity of eRF1 to bind eRF3* was reduced to 40 and 20% of the wild-type levels by eRF1-5A and eRF1-ΔCβ alterations, respectively (Fig. 8B, lanes 1–3), and that the residual 20% binding of eRF1-ΔCβ to eRF3* was further decreased by the Y577A single or Y577A-D647A double mutant of eRF3* (Fig. 8B, lanes 6 and 12). It is noteworthy that the defect in binding to wild-type eRF1 by Y577A-D647A doubly altered eRF3* was not shown by the in vivo two-hybrid assay (Fig. 7B, sample 8) but was shown by the in vitro pull-down assays (Fig. 8B, lane 10), reflecting the sensitivity of the respective assay systems. These synergistic defects in the eRF1-eRF3 interaction again confirm that the conservative amino acids, Tyr-577 and probably Asp-647, of eRF3 as well as the C-terminus of eRF1 are directly involved in the formation of the heterodimer.

DISCUSSION

The C-terminal domain of eRF3 for binding to eRF1

This study has indicated that the C-terminal one-third region, eRF3C, of *S. pombe* eRF3 is necessary and sufficient for binding to eRF1, and that several C-terminal amino acids conserved not only in eRF3s but also in elongation factors EF-Tu/EF-1α are involved in the interaction with eRF1. The eRF3 C-terminal interaction was also confirmed by using the equivalent eRF3C segments of *S. cerevisiae* (by means of in vivo and in vitro binding) and humans (by means of in vivo binding). These results are in sharp conflict with those reported by Paushkin et al. (1997), who have argued that the N-terminal one-third and the middle one-third regions of *S. cerevisiae* eRF3 are responsible for binding to eRF1. In this study, however, we failed to reproduce their results. The reason for these discrepancies is not immediately obvious, but we assume that some of the experimental conditions employed in the previous study might have interfered with the bimolecular interaction of eRF1 and eRF3, or unknown action(s) might have overlapped. One could argue that because these authors primarily employed the pull-down analysis using immobilized eRF1 and yeast lysates containing truncated eRF3 fragments, a heterotrimeric complex could have been formed by involving an adapter protein such as *S. cerevisiae* Upf1 that is known to interact with both eRF1 and eRF3 (Czaplinski et al., 1998). Alternatively, the N-terminal prion domain of *S. cerevisiae* eRF3 might have indirectly influenced the eRF1 binding. It is noteworthy, however, that Paushkin et al. have not directly examined, and hence have not excluded, the potential

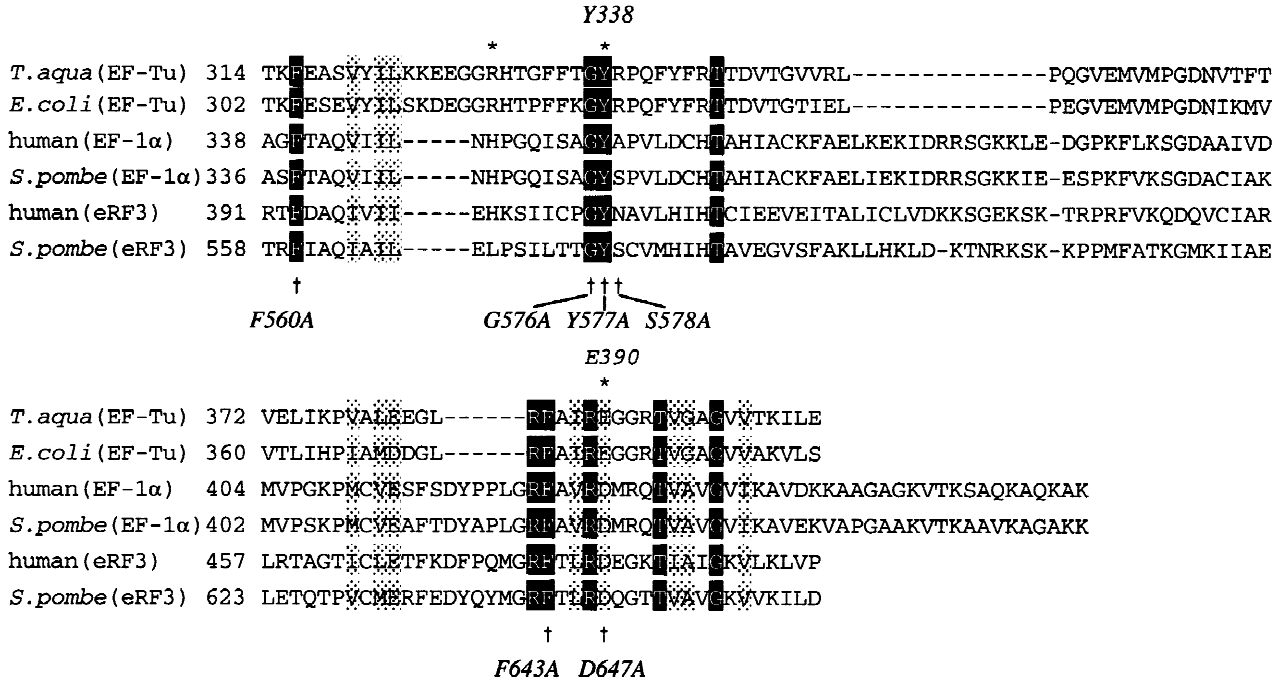

FIGURE 6. Comparison of the amino acid sequences of eRF3s and elongation factors EF-Tu and EF-1α. The similarity alignments of eRF1s were accomplished using the PILEUP program from the GCG program package (Devereux et al., 1984). Identical and similar amino acids are boxed in black and gray, respectively. Asterisks indicate amino acids of *T. aquaticus* EF-Tu that are involved in tRNA binding in the three-dimensional structure (Nissen et al., 1996). Daggers represent amino acids of *S. pombe* eRF3 that were mutated to alanine. The number refers to the amino acid position counted from the N-terminal Met.

interaction of the C-terminal one-third domain of eRF3 per se with eRF1. The present study has also confirmed the importance of the C-terminal acidic amino acid stretch of *S. pombe* eRF1 for binding to eRF3 (Ito et al., 1998a). Following near completion of the revised manuscript after submission, we became aware of a recent study that warrants mention. Merkulova et al. (1999) have reported the similar C-terminal domain activity of human eRF3 for binding to eRF1.

Uncoupling between eRF1 binding and G-domain function

The eRF3C domain that is sufficient for binding to eRF1 does not include the G-domain motifs. This is in sharp contrast with other translational G proteins, elongation factors EF-Tu and EF-1α, or initiation factors IF2 and eIF-2, whose aminoacyl-tRNA or N-formylmethionyl-tRNA binding is controlled by G-domain function: GTP stimulates the association and GDP dissociates the complex. There have been numerous reports that the N-terminal domain, including the G domain, of EF-Tu and EF-1α plays a crucial role in the binding of aminoacyl-tRNA directly or indirectly: the binding is diminished by mutations of Lys-4 (Laurberg et al., 1998), Arg-7 (Mansilla et al., 1997), Lys-9 (Laurberg et al., 1998), Arg-58 (Knudsen & Clark, 1995), Lys-89 (Wiborg et al., 1996), Asn-90 (Wiborg et al., 1996), Gly-94 (Knudsen et al., 1995), His-118 (Jonak et al., 1994), and Glu-259 (Pedersen et al., 1998) of *E. coli* EF-Tu; Thr-62 of *T. thermophilus* EF-Tu (Ahmadian et al., 1995); and Gly-280 of *Salmonella typhimurium* EF-Tu (Tubulekas & Hughes, 1993). Some of these substitutions, however, are known to affect the stability of the GTP form of EF-Tu/EF-1α relative to the GDP form, and thereby diminish the binding of aminoacyl-tRNA. Because of the functional requirement for continuous delivery of aminoacyl-tRNA during protein elongation, the G-domain activity influences, directly or indirectly, the binding of aminoacyl-tRNA.

On the other hand, guanine nucleotides do not seem to influence the eRF1-eRF3 interaction. They form a complex in vitro both in the presence (Zhouravleva et al., 1995) or absence (Stansfield et al., 1995; Frolova et al., 1998) of GTP. Therefore, the G-domain function of eRF3 may not be to change the binding of eRF1, but instead to change the binding of the ribosome or to catalyze final translocation of the ribosome. Once eRF3 is associated with eRF1 before or after binding to the ribosome, the two probably remain associated via their C-termini interaction until their release from the ribosome, showing a clear functional difference between eRF3 and EF-Tu/EF-1α.

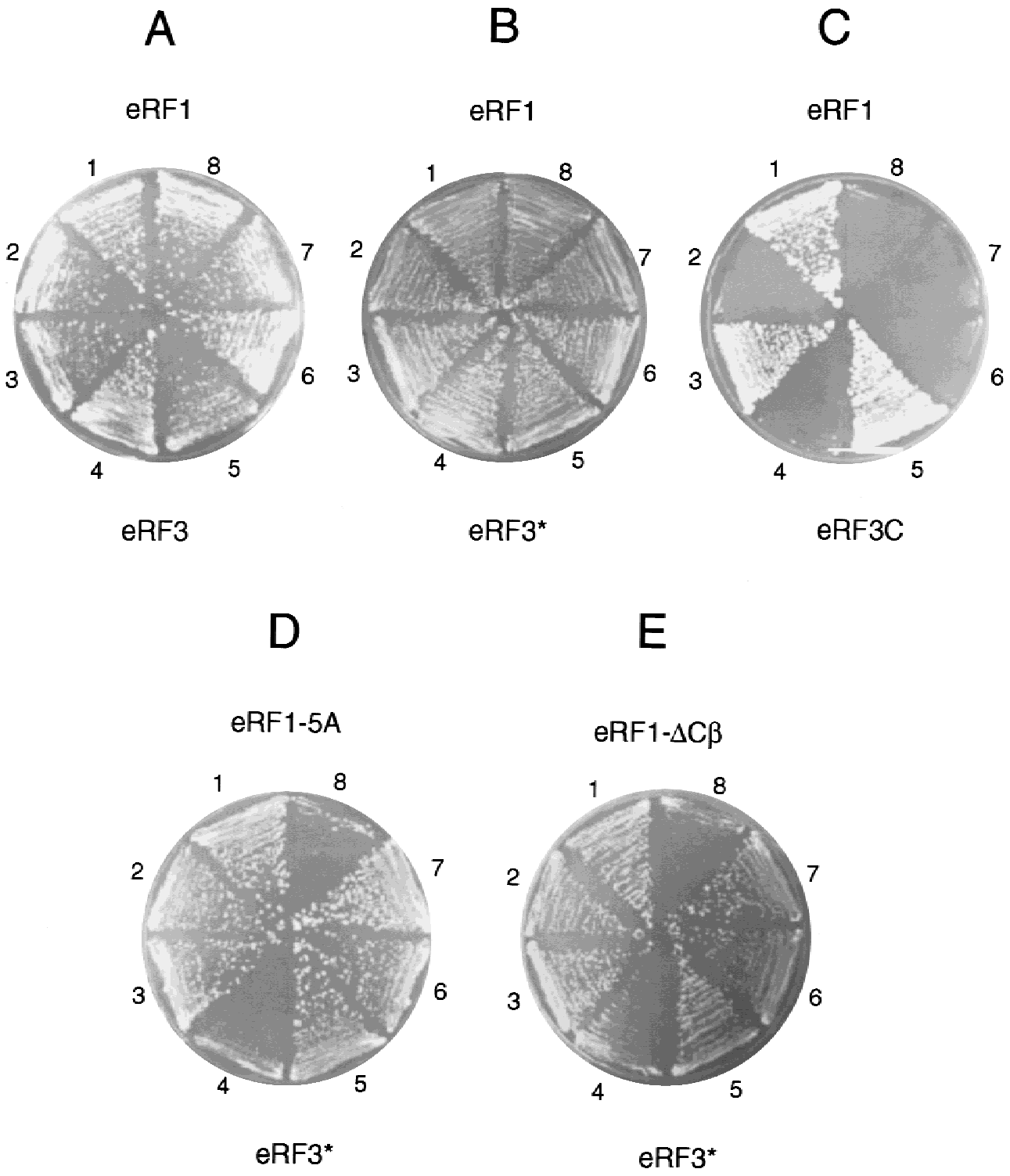

FIGURE 7. Contact site mutations and synergistic defects in the eRF1–eRF3 interaction. The activity of several eRF3* and eRF3C mutants in binding to wild-type or altered eRF1 was examined by a yeast two-hybrid assay. The eRF1 and eRF3 derivatives were cloned into plasmids pGBT9 and pGAD424, respectively, and the HF7c transformants were tested for growth on a histidine-free minimal medium plate. **A**: Interaction between eRF1 and eRF3 derivatives. **B**: Interaction between eRF1 and eRF3* derivatives. **C**: Interaction between eRF1 and eRF3C derivatives. **D**: Interaction between eRF1-5A and eRF3* derivatives. **E**: Interaction between eRF1-Δ$C\beta$ and eRF3* derivatives. pGAD424 samples: 1: wild-type eRF3*; 2: eRF3*-F560A; 3: eRF3*-G576A; 4: eRF3*-Y577A; 5: eRF3*-S578A; 6: eRF3*-F643A; 7: eRF3*-D647A; 8: eRF3*-Y577A-D647A.

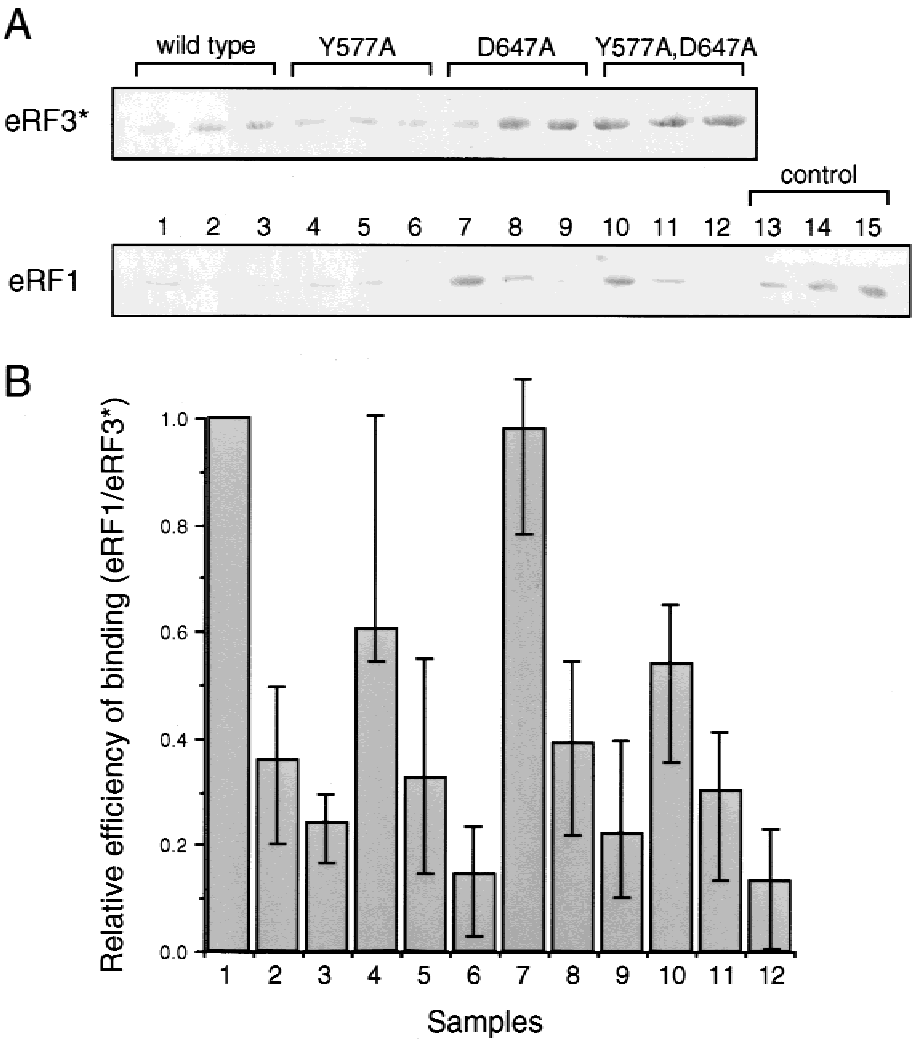

FIGURE 8. In vitro binding of eRF1 and eRF3* proteins carrying contact site mutations. Experimental procedures and conditions are described in Materials and Methods. **A**: Immobilized wild-type or mutant GST-eRF3* were mixed with wild-type or mutant His_6-eRF1 proteins, and the coprecipitated proteins were analyzed by Western blotting after SDS-PAGE. The immunoblots of bound His_6-eRF1 (lower) and GST-eRF3* (upper) proteins were detected by chemiluminescence. eRF3* proteins: lanes 1–3: wild-type; lanes 4–6: Y577A mutant; lanes 7–9: D647A mutant; lanes 10–12: Y577A-D647A double mutant. eRF1 proteins: lanes 1, 4, 7, 10, and 13: wild-type; lanes 2, 5, 8, 11, and 14: eRF1-5A; lanes 3, 6, 9, 12, and 15: eRF1-$\Delta C\beta$. Lanes 13–15 are in-put eRF1 controls. **B**: Binding efficiency of eRF1 proteins to immobilized eRF3* proteins in the presence of amino acid substitutions. The intensity of His_6-eRF1 and GST-eRF3* bands was quantified, and the binding efficiency was estimated as a ratio of His_6-eRF1 to GST-eRF3* (internal control) and is presented as a relative value to that of wild-type eRF1 and eRF3* binding (lane 1). Experiments were performed independently at least three times, and the mean values are expressed with raw experimental errors.

Prediction by RF-tRNA mimicry hypothesis

The "RF-tRNA mimicry" hypothesis predicts that, of the three tRNA-mimicry domains III–V, eRF1 regions equivalent to domains III and V of EF-G should mimic the acceptor stem and the T stem of tRNA (Ito et al., 1996). We have previously assigned two eRF3-contact sites on *S. pombe* eRF1, one to the internal region between amino acid positions 187–247, and the other to the C-terminal region as the primary and strongest binding site for eRF3 (Ito et al., 1998a). The importance of the latter for binding to eRF3 was confirmed in this work. Given that these two sites correspond to domains III and V, respectively, the C-terminus of eRF1 may mimic the T stem of tRNA. The C-terminal region of eRF1 conserves a number of amino acids, particularly acidic residues, in eukaryotic RFs, but not in prokaryotic RFs (see Fig. 3). The three-dimensional structure of the ternary complex of Phe-tRNA, EF-Tu, and GDPNP has revealed that the contacts are located in three regions: (1) binding of the CCA-Phe end to domain 2 of EF-Tu and its interface to domain 1; (2) binding of the 5′ end and a part of the acceptor stem at the intersection of the three domain interfaces and to the GTPase switch regions; and (3) binding of one side of the T-stem to the surface of domain 3 of EF-Tu (Nissen et al., 1996). These features of aminoacyl-tRNA have been interpreted to define a general aminoacyl-tRNA motif that EF-Tu:GTP recognizes on all ordinary elongator aminoacyl-tRNA molecules. The importance of the former two regions of EF-Tu have been demonstrated by mutational analyses by several investigators (as described above). Of these contacts, the T stem-EF-Tu interaction is thought to be important for recognition of all ordinary elongator aminoacyl-tRNA molecules, which was shown first by a three-dimensional study (Nissen et al., 1996). The detailed resolution of atoms involved in this T stem-EF-Tu interaction has revealed that the negatively charged phosphate backbone of tRNA is essential for binding to EF-Tu (Nissen et al., 1996; Nakamura & Ito, 1998), suggesting that the stretch of negatively charged amino acids at the C-terminus of eRF1 may mimic the negatively charged phosphate backbone of the T stem of tRNA to bind eRF3 (Ito et al., 1998a).

Based on the sequence resemblance between EF-Tu and eRF3, the C-terminal part of eRF3, which is equivalent to domain 3 of EF-Tu, might interact with the C-terminal peptide of eRF1, which mimics the T stem of tRNA. The contact site of *T. aquaticus* EF-Tu with the T stem of tRNA has been assigned to Glu-390 and its neighboring residues as well as to Tyr-338 and Arg-330 (Nissen et al., 1996; see Fig. 6). It is noteworthy that eRF3 from different species exclusively conserves glutamate or aspartate at the positions equivalent to Glu-390 of EF-Tu, that it well conserves other residues around this position, and that it conserves fewer but significantly homologous or identical residues corresponding to Tyr-338 and Arg-330. This reinforces the model that eRF3 and EF-Tu are homologs that bind eRF1 and tRNA, respectively, using the same conserved residues. The RF-tRNA mimicry model also predicts that the putative equivalent to domain III (acceptor stem mimicry) of eRF1 (positions 187–247) may make contact with the N-terminal peptide of eRF3 (Nissen et al., 1996). This interaction, however, is less likely, as has been shown in this study. The present findings provide further clarification of the similarities and the distinction between eRF3 and EF-Tu in terms of binding to eRF1 (a putative tRNA-mimicry protein) and tRNA, respectively, shedding light on the release factor-tRNA mimicry hypothesis from eRF3.

MATERIALS AND METHODS

Strains, media, and plasmids

The yeast and bacterial strains used are listed in Table 1. Yeast cultures were grown using standard conditions (Sherman, 1991) in YPD liquid medium (2% w/v Bacto-peptone, 1% w/v yeast extract, 2% w/v glucose). Yeast transformants were grown in synthetic minimal (SD) media (2% w/v glucose, 0.67% w/v yeast nitrogen base without amino acids

TABLE 1. Strains and vectors used in this study.[a]

Strain or vector	Genotype or relevant description	Source or reference
A. *S. cerevisiae* strains		
HF7c	Two-hybrid host, *MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 carf gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4 17-mers)$_3$-CYC1–lacZ*	Feilotter et al., 1994 (CLONTECH)
B. Plasmid vectors		
pYK807	Complete eRF3 (Sup35) gene of *S. cerevisiae* cloned in YCp50	Kikuchi et al., 1988
pGH5	Human eRF3 gene cloned in pUC19, Ap^R	Hoshino et al., 1989
pET15b	Multi-cloning-site expression vector with a hexa-histidine sequence under the control of T7 RNA polymerase promoter, Ap^R	Novagen, Inc.
pET-Sp-eRF1	*S. pombe* eRF1 (Sup45) gene cloned in pET15b, Ap^R	Ito et al., 1998a
pET-Sp-eRF1-ΔN	pET-Sp-eRF1 derivatives containing N-terminal deletions in eRF1	Ito et al., 1998a
pET-Sp-eRF1-ΔC	pET-Sp-eRF1 derivatives containing C-terminal deletions in eRF1	Ito et al., 1998a
pET-Sc-eRF1	*S. cerevisiae* eRF1 (Sup45) gene cloned in pET15b, Ap^R	This study
pGEX-5X-3	Multi-cloning-site expression vector of GST-protein fusion under the control of *lacI-tac* promoter, Km^R	Smith & Johnson, 1988
pGEX-Sp-eRF3	pGEX-5X-3 derivative expressing a fusion protein between GST and *S. pombe* RF3 (full length)	Ito et al., 1998a
pGEX-Sp-eRF3*	pGEX-5X-3 derivative expressing a fusion protein between GST and *S. pombe* RF3* (212–662)	Ito et al., 1998a
pGEX-Sp-eRF3C	pGEX-5X-3 derivative expressing a fusion protein between GST and *S. pombe* eRF3C (482–662)	This study
pGEX-Sp-eRF3ΔC	pGEX-5X-3 derivative expressing a fusion protein between GST and *S. pombe* eRF3ΔC (1–481)	This study
pGEX-Sp-eRF3*ΔC	pGEX-5X-3 derivative expressing a fusion protein between GST and *S. pombe* eRF3*ΔC (212–481)	This study
pGEX-Sc-eRF3	pGEX-5X-3 derivative expressing a fusion protein between GST and *S. cerevisiae* RF3 (full length)	This study
pGEX-Sc-eRF3*	pGEX-5X-3 derivative expressing a fusion protein between GST and *S. cerevisiae* RF3* (234–685)	This study
pGEX-Sc-eRF3C	pGEX-5X-3 derivative expressing a fusion protein between GST and *S. cerevisiae* eRF3C (498–685)	This study
pGEX-Sc-eRF3ΔNΔC	pGEX-5X-3 derivative expressing a fusion protein between GST and *S. cerevisiae* eRF3ΔNΔC (114–483)	This study
pGBT9	Two-hybrid DNA-binding domain vector, GAL4 bd (1–147), *TRP1*, Ap^R	Fields & Song, 1989 (CLONTECH)
pGAD424	Two-hybrid activation domain vector, GAL4 ad (768–881), *LEU2*, Ap^R	Fields & Song, 1989 (CLONTECH)

[a]Ap^R: ampicillin resistance; Km^R: kanamycin resistance; ts: temperature sensitive; GST: glutathione *S*-transferase.

[Difco], supplemented with the required amino acids and cofactors) or in synthetic complete (SC) media (Sherman, 1991). Bacteria were grown in LB broth (1% Bacto-tryptone, 0.5% yeast extract, and 0.5% NaCl; see Sambrook et al., 1989) supplemented with the relevant antibiotics for selection (50 μg/mL ampicillin or 50 μg/mL kanamycin).

Most of the *E. coli* and yeast plasmids used for the overproduction of *S. pombe* eRF1 and eRF3 derivatives have been described previously (Ito et al., 1998a) and are listed in Table 1. To truncate *S. pombe* eRF3 at its C-terminal one-third position, the eRF3 gene was cleaved at the *Kpn*I site, generating two split segments, eRF3ΔC (positions 1–481) and eRF3C (positions 482–662). The segments were cloned into plasmid pGEX-5X-3 or pGBT9 for in vitro pull-down analysis or in vivo two-hybrid assay. The C-terminal segment was fused in frame to the initiator AUG using primer 5′-CCGGATCCATATGATCTTAGAAGGAAAGATTGAG-3′. Similar to *S. pombe* eRF3, the *S. cerevisiae* eRF3 gene was truncated with restriction enzymes *Eco*RV, *Sal*I, and *Xba*I to generate polypeptides equivalent to those described by Paushkin et al. (1997): eRF3ΔN (positions 114–685), eRF3ΔC (positions 1–483), eRF3* (positions 234–685), eRF3ΔNΔC (positions 114–483), and eRF3C (positions 498–685). The truncated eRF3* and eRF3ΔC sequences were fused in frame to the initiator AUG using primers, 5′-GGGAATTCACCATG GCTGATGCCTTGATCAA-3′ and 5′-CCGGATCCAGATGAA GGATCTAGGTACCAT-3′, respectively. The intact N-terminal polypeptides, eRF3 and eRF3ΔC, employed a primer 5′-CCGGATCCATATGTCGGATTCAAACCAAGG-3′ for reclonning into other expression vectors. The human eRF3 gene was also truncated using primer 5′-CCGGATCCATATGGGC ACTGTGGTCCTGGG-3′ to generate human eRF3C equivalent (positions 451–637). These segments were cloned into pGBT9 for in vivo two-hybrid assay. Base substitutions in *S. pombe* eRF1 and eRF3 genes were introduced by a polymerase chain reaction (PCR) using sets of sense and antisense primers designed for the respective changes: eRF1-5A (5′-GGGGGTCGACTTAGGCGGAGGCGGAAGCAAATGCA GCAGATTCAGGATCAAGCAT-3′), eRF3-F560A (5′-TGCTA CTACTCGTGCCATTGCACAAATT-3′ and 5′-AATTTGTGC AATGGCACGAGTAGTAGCA-3′), eRF3-G576A (5′-ATTTTG ACAACTGCTTATTCTTGTGTA-3′ and 5′-TACACAAGAATA AGCAGTTGTCAAAAT-3′), eRF3-Y577A (5′-TTGACAACTG GTGCTTCTTGTGTAATG-3′ and 5′-CATTACACAAGAAGC ACCAGTTGTCAA-3′), eRF3-S578A (5′-GACAACTGGTTAT GCTTGTGTAATGCA-3′ and 5′-TGCATTACACAAGCATAAC CAGTTGTC-3′), eRF3-F643A (5′-GTATATGGGCCGTGCCA CTTTGCGTGAT-3′ and 5′-ATCACGCAAAGTGGCACGGC CCATATAC-3′), and eRF3-D647A (5′-TTCACTTTGCGTG CTCAGGGTACTACG-3′ and 5′-CGTAGTACCCTGAGCAC GCAAAGTGAA-3′). DNA fragments amplified by PCR in these experiments were sequenced to avoid mutations in any constructs.

In vitro protein binding

S. pombe and *S. cerevisiae* eRF1 derivatives were cloned into plasmid pET15b (Novagen, Inc., Madison, Wisconsin), and histidine-tagged eRF1 proteins were overproduced and purified using the T7 RNA polymerase-dependent expression system and Probondq™ Resin (Invitrogen), as described (Ito et al., 1998a). *S. pombe* as well as *S. cerevisiae* eRF3 derivatives were cloned into plasmid pGEX-5X-3 (Pharmacia), and GST-eRF3 fusions were recloned into plasmid pET30a (Novagen), overexpressed, and isolated using glutathione-agarose beads (Sigma), as described (Ito et al., 1998a). Pull-down analysis using immobilized GST-eRF3 proteins was performed by the same procedure as described previously (Ito et al., 1998a). His_6-eRF1 proteins were stained by Western blotting after SDS-PAGE using a Ni-NTA-horseradish peroxidase conjugate (Qiagen) that detects the histidine tag according to the manufacturer's instructions, GST-eRF3* proteins, using mouse anti-GST antibody and peroxidase conjugated sheep secondary antibody. After washing, the membranes were developed by means of enhanced chemiluminescence using ECL Western Blotting Detection System (Amersham) according to the manufacturer's instructions.

Other methods

Yeast transformation, in vivo two-hybrid assay, and DNA manipulations were carried out by the same procedures and conditions as described previously (Ito et al., 1998a).

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