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Eukaryotic release factor 1 (eRF1) abolishes readthrough and competes with suppressor tRNAs at all three termination codons in messenger RNA

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ABSTRACT

It is known from experiments with bacteria and eukaryotic viruses that readthrough of termination codons located within the open reading frame (ORF) of mRNAs depends on the availability of suppressor tRNA(s) and the efficiency of termination in cells. Consequently, the yield of readthrough products can be used as a measure of the activity of polypeptide chain release factor(s) (RF), key components of the translation termination machinery. Readthrough of the UAG codon located at the end of the ORF encoding the coat protein of beet necrotic yellow vein furovirus is required for virus replication. Constructs harbouring this suppressible UAG codon and derivatives containing a UGA or UAA codon in place of the UAG codon have been used in translation experiments *in vitro* in the absence or presence of human suppressor tRNAs. Readthrough can be virtually abolished by addition of bacterially-expressed eukaryotic RF1 (eRF1). Thus, eRF1 is functional towards all three termination codons located in a natural mRNA and efficiently competes *in vitro* with endogenous and exogenous suppressor tRNA(s) at the ribosomal A site. These results are consistent with a crucial role of eRF1 in translation termination and forms the essence of an *in vitro* assay for RF activity based on the abolishment of readthrough by eRF1.

INTRODUCTION

Termination of protein synthesis occurs when the ribosome translation machinery encounters an in-frame termination codon, either UAG (amber), UGA (opal) or UAA (ochre), on the mRNA. Hydrolysis of the ester bond linking the polypeptide chain and the last tRNA is triggered by the peptidyltransferase center of the ribosome and requires specific release factors (RFs) and GTP (for recent reviews see 1–3).

In higher eukaryotes, the situation concerning peptide chain termination remained largely unexplored following the identification and partial purification from rabbit reticulocytes of one RF recognizing any of the three termination codons (4,5). Recently, the rabbit RF was purified to homogeneity and four of its proteolytic peptides sequenced (6). These peptides were identical or very similar to peptides deduced from the amino acid sequences derived from the human TB3-1, *Xenopus laevis* Cl1, *Saccharomyces cerevisiae* sup45 and *Arabidopsis thaliana* sup45-like cDNAs. Expression of the human and *X.laevis* genes in yeast and *Escherichia coli* respectively, yielded proteins endowed with RF activity. Since all these proteins possess similar amino acid sequences, it was proposed that they belong to a highly conserved protein family designated eRF1 (6). No GTP binding domain was detected in the eRF1 family (6), and indeed, GTP is not an obligatory component of eRF1 activity (7). A second eRF was identified and characterized from *X.laevis* (7) that behaves *in vitro* similarly to *E.coli* RF3 (8,9). This protein possesses GTP-binding domains as do the human GSPT1, *S.cerevisiae* sup35 and *Pichia pinus* sup35 proteins. It binds to eRF1 and stimulates eRF1 activity in the presence of GTP. This family of proteins is designated eRF3 (7) by analogy with the *E.coli* RF3. eRF3 belongs to small G proteins and is an eRF1- and ribosome-dependent GTPase (10).

The assay devised to test termination in prokaryotes (11) was adapted for eukaryotic systems (12). The preformed intermediate is composed of AUG and *E.coli* fMet-tRNA^{fMet} bound to rabbit reticulocyte ribosomes. Release of fMet is accomplished by incubating the intermediate with eRF, GTP, and either a tetranucleotide encompassing one of the three termination codons or a copolymer such as poly(U,G,A). The eRF1 activity is GTP independent (7), and the requirement for GTP in the original RF assay (12) was presumably due to the presence of eRF3 in the partially purified eRF1 preparation (10).

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It is known that the context 5′ and more significantly 3′ of the termination codon influences the efficiency of termination and suppression (reviewed in 1,13). In addition, the nature of the last two amino acids of the growing peptide chain affects the efficiency of suppression and possibly also of termination (2,14). In view of the drawbacks of the original RF assay, an assay system was developed that circumvents the limitations outlined above. Pure eRF1 was used, and a natural mRNA was the source of termination signal: it contains a naturally-occurring suppressible termination codon that served to evaluate readthrough, as opposed to release of fMet from fMet-tRNA.

A suppressible UAG codon is present in the genome of beet necrotic yellow vein furovirus (BNYVV). RNA2 of the tetrapartite RNA genome of this plant virus, is 4612 nucleotides (nt) long excluding the polyA tail (15). It contains towards its 5′ terminus, the open reading frame (ORF) for the viral CP of 22 kDa that terminates with a UAG codon. This is followed in-frame by a readthrough domain of 54 kDa. Readthrough of the UAG codon at the end of the CP ORF, leads to the synthesis of a 75 kDa protein (calculated size). Readthrough is detected *in vitro* (16) and *in vivo* (17). The readthrough protein is required for transmission of the virus by its fungus vector (18). It is also involved in virus assembly (19) and is present in trace amounts in purified virus particles (20).

Starting from a construct containing the cDNA corresponding to the BNYVV CP ORF and the readthrough domain, transcripts were synthesized *in vitro* and used as templates for *in vitro* translation studies in the absence or presence of exogenous eRF1. The results demonstrate that eRF1 prevents readthrough, that this occurs also when the UAG codon is replaced by UGA or UAA, and that eRF1 competes with suppressor tRNAs (tRNAsu$^+$) for all three termination codons.

MATERIALS AND METHODS

Constructs

The starting wild-type plasmid pB218 (designated here pB218-TAG) was used for the transcription experiments; it includes the fragment corresponding to BNYVV RNA2, from nt 1 to 2715 cloned between the T7 and T3 promoters of a Blue Scribe vector (21). This insert contains an *Nco*I and an *Mlu*I restriction site. The resulting BNYVV RNA fragment contains the 5′ untranslated region, the CP and readthrough domains, as well as 495 nt following the termination codon at the end of the readthrough domain.

Mutated cDNAs of pB218-TAG in which the TAG codon of the CP was replaced by TGA or TAA, were obtained by the polymerase chain reaction (PCR). Primer 1 was: *5′*CAAATTACCATGGACA-CCTGTTCAAGGTAGAACCAGTCCATCCGGACAA**TGA**C-AATTAGCTGC*3′*. It is homologous to nt 660–722 of viral RNA2 except for the TGA (bold) which replaced the TAG in pB218-TAG, and the introduction of a T residue in place of a C residue to form the *BspE*1 site (underlined) for screening purposes, and it contains an *Nco*I site (underlined). Primer 2 was identical to primer 1 except that the TGA in primer 1 was replaced by TAA. Primer 3 was *5′*CTGTAGCACGCGTGTGCAGC*3′*. It is complementary to nt 1130–1150 of viral RNA2, and contains an *Mlu*I restriction site (underlined). An additional mutated cDNA derived from pB218-TAG was produced. In this cDNA, the wild-type TAG codon was maintained, but the C residue following the TAG, was replaced by a G residue, yielding pB218-TAGG. This was achieved with primer 4 which was identical to primer 1 except that the TGAC was replaced by TAGG. Primers 1, 2 or 4 were used in conjunction with primer 3.

Cloned *Pfu* DNA polymerase (Stratagene) was used for PCR reactions according to the supplier's recommendations. The PCR products were inserted into the *Nco*I and *Mlu*I restriction sites of pB218-TAG. The sequence of the resulting constructs pB218-TGA, pB218-TAA and pB218-TAGG was verified by the dideoxynucleotide chain termination method (22) using the USB sequencing kit.

mRNAs synthesized by *in vitro* transcription

Conditions were as described (23). After linearization of the wild-type and mutated plasmids by *Bam*HI, transcription was performed with T7 RNA polymerase (BRL) and m^{7}GpppG (Pharmacia), and the integrity, size and concentration of the transcripts estimated. The wild-type transcript is designated tB218-UAG and the mutated transcripts tB218-UGA, tB218-UAA and tB218-UAGG.

Release factors and suppressor tRNAs

Escherichia coli-expressed *X.laevis* and human His-tagged eRF1 were purified as described (6). The factors were tested for RF activity using the fMet release assay (6,12). *Escherichia coli*-expressed *X.laevis* His-tagged eRF3 was purified and assayed as described (7).

Suppressor tRNAs were partially purified from human 293 cells transiently expressing plasmids ptRNAam, ptRNAop or ptRNAoc which contain respectively the amber, opal or ochre derivatives of a human tRNA^{Ser} gene (24). Plasmid ptRNAwt contains the wild-type version of this gene. These plasmids (24) were modified as described (25). The 293 cells at 50% confluency were transfected with 15 µg of plasmid DNA per 100 mm plate and 48 h after transfection total cellular RNAs were extracted as described (26). The RNA samples were electrophoresed in 7 M urea–10% acrylamide gels. After ethidium bromide staining, the RNA band containing the tRNA^{Ser} isoacceptors, localized by previous Northern blot hybridization with a probe specific for human tRNA^{Ser}, were excised from the gel, electroeluted in 0.5× TBE (4.5 mM Tris base, 4.5 mM boric acid and 0.1 mM EDTA) and concentrated by ethanol precipitation. They are referred to as the tRNAsu$^+$ (tRNAam, tRNAop and tRNAoc) and to the wild-type tRNAsu$^-$. Their concentration was estimated on a urea–acrylamide gel by comparison with pure tRNA^{Met} used as standard.

Translation

The mRNAs (0.2 µg) were translated for 1 h at 25°C using 530 kBq of Pro-Mix [^{35}S]methionine+cysteine (>37 TBq/mmol; Amersham) in 10 µl containing 5 µl of home-made rabbit reticulocyte lysate as described (27). The translation products were analyzed on 12.5% acrylamide–0.1% bisacrylamide–0.1% SDS gels (modified from ref. 28). The gels were then fixed, dried and submitted to autoradiography.

RESULTS

Capped tB218-UAG containing the BNYVV CP ORF and the readthrough domain was added to a reticulocyte lysate without or

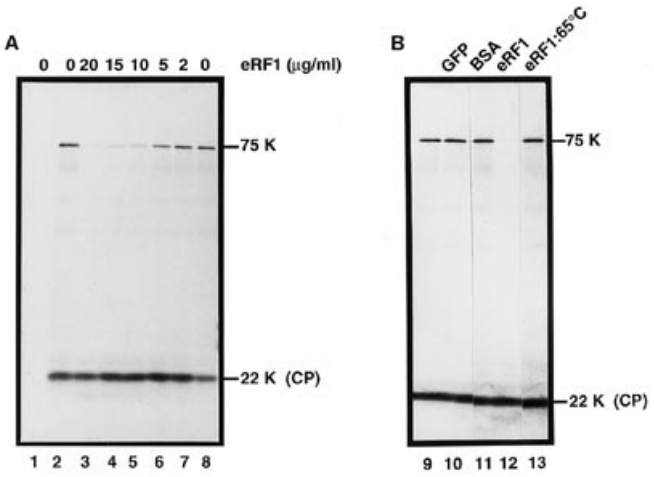

Figure 1. Analysis by SDS–PAGE of the effect of *X.laevis* eRF1 on readthrough *in vitro* of the suppressible UAG codon of the BNYVV CP in tB218-UAG. (**A**) Effect of increasing eRF1 concentrations. Lane 1: without mRNA. Lanes 2–8: with tB218-UAG as mRNA. Lanes 3–7: with decreasing eRF1 concentrations as indicated above the lanes. Lane 8: eRF1 was replaced by the equivalent volume of buffer used for dilution of the factor. (**B**) Effect of different proteins and of heated eRF1 on readthrough. Lane 9: as lane 2. Lanes 10–13: with 20 µg/ml of His-tagged GFP, BSA, eRF1, or eRF1 heated for 10 min at 65°C. In both panels, the positions of the CP (22 kDa) and of the readthrough protein (75 kDa) are indicated. Lanes 9–13 are from the same gel.

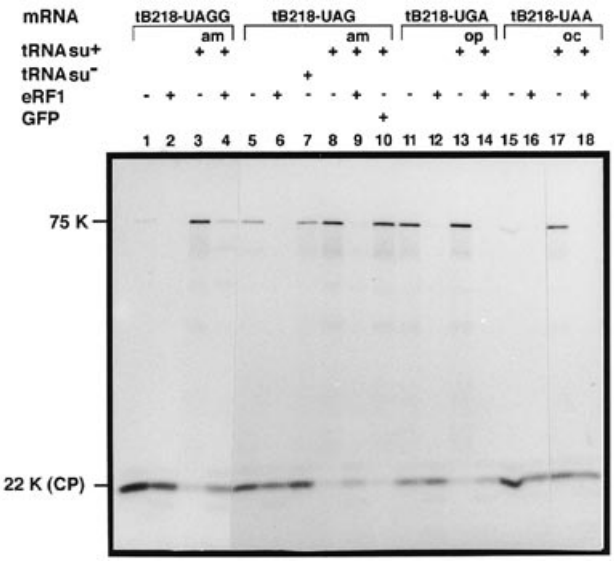

Figure 2. Analysis by SDS–PAGE of the competition between eRF1 and tRNAsu$^+$ using tB218-UAG, tB218-UGA, tB218-UAA and tB218-UAGG as mRNAs. Addition of eRF1 (20 µg/ml) and/or tRNAsu$^+$ (10 µg/ml) was as indicated above the lanes. In lane 10, GFP (20 µg/ml) replaced eRF1. All lanes are from the same gel, except for lanes 1–4 which are from another gel. The positions of the CP (22 kDa) and of the readthrough protein (75 kDa) are indicated.

with increasing *X.laevis* eRF1 concentrations. The resulting proteins were resolved by SDS–polyacrylamide gel eletrophoresis (SDS–PAGE). In the absence of exogenous eRF1, the CP and the readthrough protein of 75 kDa were easily detected (Fig. 1A). Synthesis of the 75 kDa protein reflects the presence in the lysate of (an) unidentified suppressor tRNA(s) recognizing the UAG codon. Addition of increasing eRF1 concentrations led to a progressive decrease in the synthesis of the 75 kDa protein; at 20 µg/ml of eRF1 (lane 3), readthrough was virtually abolished. Similar results were obtained with human eRF1 (not shown).

To verify that eRF1 was responsible for the decrease in readthrough, various controls were performed (Fig. 1B). When eRF1 was heated for 10 min at 65°C prior to addition to the translation system, it no longer affected readthrough (compare lanes 12 and 13). Neither bovine serum albumin (BSA; lane 11), nor His-tagged green fluorescent protein (GFP) prepared and isolated as was eRF1 (lane 10), affected readthrough.

To determine the specificity of the *X.laevis* eRF1 and examine the competition between eRF1 and the appropriate tRNAsu$^+$ for the three termination codons, the wild-type transcript tB218-UAG and the mutated counterparts tB218-UGA or -UAA were used as templates in translation experiments performed in the absence or presence of eRF1, and in the absence or presence of the tRNAsu$^+$. In addition, transcript tB218-UAGG in which the C residue following the UAG codon was mutated to a G residue, was translated in a reticulocyte lysate in the same conditions as those outlined above. In the absence of exogenous eRF1, readthrough was easily detected whatever the termination codon present at the end of the CP ORF (Fig. 2, lanes 5, 11 and 15), except in the case of tB218-UAGG where the level of readthough was very low (lane 1). Addition of 20 µg/ml of eRF1 decreased the level of readthrough which was virtually (lanes 6, 12 and 16) or even totally (lane 2) abolished. In the presence of the appropriate tRNAsu$^+$, but without eRF1, increase in the level of the readthrough protein was accompanied by a decrease of the yield of CP (lanes 3, 8, 13 and 17). In the presence of tRNAsu$^+$ and eRF1, the level of readthrough protein decreased whereas the level of the CP increased (lanes 4, 9, 14 and 18). Neither wild-type tRNAsu$^-$ alone (lane 7) nor GFP in the presence of tRNAsu$^+$ (lane 10) affected readthrough. Equally efficient competition was observed in the presence of tB218-UAG between eRF1 and pure serine accepting tRNAop from *Schizosaccharomyces pombe* (not shown).

In a wheat germ translation system, and for the same amounts of *X.laevis* eRF1 and/or tRNAsu$^+$ as used in the reticulocyte lysate, similar responses were obtained, but the efficiency of termination triggered by eRF1 was lower than in the reticulocyte lysate (not shown).

In another series of experiments, the possible influence of eRF3 on readthrough efficiency was examined (Fig. 3) using tB218-UAG, either in the absence (lanes 1–7) or in the presence (lanes 8–14) of exogenous suppressor tRNAam. As expected, eRF3 alone did not affect the level of readthrough (lanes 6, 7, 13 and 14). When eRF3 was added with eRF1 to the incubation mixture in a 4-fold molar excess over eRF1, it barely enhanced the effect of eRF1 (lanes 5 and 12). However, when present in a much larger excess over eRF1, eRF3 enhanced the effect of eRF1 in triggering termination (lanes 4 and 11). It was verified that the buffer containing eRF3 was not responsible for this effect (not shown).

DISCUSSION

We have developed an assay system to evaluate eukaryotic termination of protein synthesis versus readthrough *in vitro* using pure eRF1, suppressor tRNA and a naturally-occurring suppressible UAG codon embedded in the genome of a plant RNA virus,

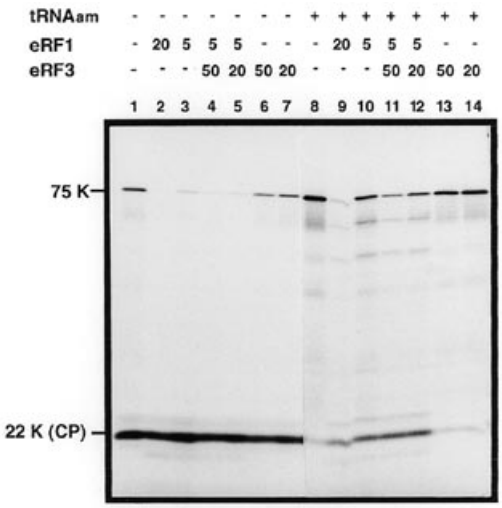

Figure 3. Analysis by SDS–PAGE of the effect of *X.laevis* eRF3 on eRF1 activity *in vitro*. Transcript tB218-UAG was used as mRNA throughout. Addition and concentrations (in µg/ml) of eRF1 and/or eRF3 were as indicated above the lanes. Where indicated, tRNAam (10 µg/ml) was included. All lanes are from the same gel. The positions of the CP (22 kDa) and of the readthrough protein (75 kDa) are indicated.

RNA2 of BNYVV. Suppression of the UAG codon located between the CP ORF and a 54 kDa domain on RNA2 leads to the synthesis of a readthrough product of 75 kDa that is indispensible for virus propagation.

In the present study, the capped transcript tB218-UAG containing the 5′-terminal 2715 nt of RNA2 and thus encompassing the entire 75 kDa ORF, was produced from pB218-TAG (21), and used as template for *in vitro* translation studies with a rabbit reticulocyte lysate to evaluate the effect of eRF1 and of suppressor tRNAam on the level of termination of translation. Transcripts tB218-UGA and tB218-UAA in which respectively the opal and ochre codon replaced the naturally-occurring amber codon were also tested in similar conditions using the corresponding tRNAsu$^+$. Analysis of the translation products synthesized revealed that all three types of transcript lead to the production of the CP and of the readthrough protein of 75 kDa and that the readthrough protein results from the presence of suppressor tRNAs in the lysate.

With all three types of transcript and in the experimental conditions used here, readthrough was virtually abolished in the presence of 20 µg/ml of *X.laevis* eRF1 that corresponds to three to four added eRF1 molecules per ribosome. This should in effect correspond to a large excess of eRF1 over termination codons, given that one molecule of eRF1 is normally required to terminate one polypeptide chain on polyribosomes. Nevertheless, the exact ratio between eRF1 and ribosome is difficult to evaluate, because the proportion of active eRF1 molecules in the preparation is not known.

The results obtained demonstrate that eRF1 specifically recognizes all three termination codons when contained in an mRNA, as it does also in the assay based on the release of fMet from fMet-tRNA in response to tetraplets containing termination codons (5,6). The assay described here presents the advantage that eRF1 interacts with termination codons embedded in an mRNA whose affinity towards ribosomes is much higher than that of tetraplet termination signals. Moreover, a natural protein is terminated as opposed to hydrolysis of fMet-tRNA which probably never occurs in a natural system.

The ability of eRF1 on its own to compete efficiently with tRNAsu$^+$ of various nonsense codons *in vitro* (this work) and *in vivo* (25) implies that association of eRF1 with eRF3 is probably not required for productive eRF1 binding to the ribosomal A site. Recent data on yeast eRF1 (Sup45p) mutants (29) agree with this interpretation. In these studies, reduction of the level of Sup45p in yeast cells caused the appearance of a suppressor phenotype, while the level of Sup35p (equivalent to eRF3) remained unaltered. The results presented here are also in line with those obtained with a *Salmonella typhimurium* strain (30) carrying a UGA suppressor allele in the gene encoding RF2 (a prokaryotic analog of eRF1). In these experiments, reduction in the level of RF2 was responsible for the suppressor phenotype. In view of all these findings (7,25,29,30; this work) the earlier data (31 and references therein) regarding the antisuppressor activity of eRF3 on its own requires further experiments.

Suppression of termination during translation is a strategy frequently encountered among animal and plant RNA viruses for the synthesis of their proteins (reviewed in 32–34). To date only suppressible UAG and UGA codons have been encountered. The codon context surrounding the suppressible amber codon in BNYVV RNA2, is identical to the one surrounding the suppressible amber codon in tobacco mosaic tobamovirus (TMV) RNA (35) over 12 nt (CAA**UAG**CAAUUA). In the case of TMV in which readthrough is required to produce the RNA-dependent RNA polymerase (36), it has been demonstrated that the two codons downstream of the UAG signal are important determinants of suppression *in vivo* (37), and *in vitro* (38). Moreover, it has been demonstrated that translational termination efficiency is strongly influenced by the base following the termination codon (39), A and G being preferred over C and U. Indeed, mutating the nucleotide following the suppressible UAG codon in BNYVV RNA2 from a C to a G resulted in barely detectable readthough protein as compared with the wild-type transcript. Thus it is not surprising that in the BNYVV and TMV RNAs in which readthrough is required, a C residue follows the termination codon, thereby presumably decreasing the possibility of termination at this position.

Addition of excess *X.laevis* eRF3 over eRF1 enhanced the release activity of eRF1. Alone, eRF3 had no effect on the efficiency of readthrough, and conversely, eRF1 alone was sufficient to inhibit readthrough. Earlier it was shown that even in the absence of eRF3 and GTP, eRF1 on its own was active in promoting termination codon-dependent fMet-tRNA hydrolysis (7). The finding described here, that eRF1 in the absence of exogenous eRF3 efficiently competes with endogenous and exogenous suppressor tRNA(s) implies that recognition of termination codons and peptidyl-tRNA hydrolysis are predominantly associated with eRF1. These experiments show the contribution of eRF3 in stimulating the antisuppressor activity of eRF1 though this effect is obviously of secondary importance. Our experiments indicate that in this *in vitro* translation system, eRF1 rather than eRF3 limits the efficiency of termination.

Readthrough of termination codons requires the positioning of a suppressor tRNA in the ribosomal A site. Our results indicate that exogenous eRF1 efficiently competes at the A site with endogenous and exogenous suppressor tRNA(s) for the termination codon in a natural mRNA·ribosome complex. This observation

emphasizes the functional resemblance between tRNAs and eRF1, and implies that eRF1 could also be structurally similar to aminoacyl-tRNA. This suggestion is in line with the recently proposed tRNA-protein mimicry concept (40) and its extension to RFs (2,41).

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