

# Positioning of the mRNA stop signal with respect to polypeptide chain release factors and ribosomal proteins in 80S ribosomes

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**Abstract** To study positioning of the mRNA stop signal with respect to polypeptide chain release factors (RFs) and ribosomal components within human 80S ribosomes, photoreactive mRNA analogs were applied. Derivatives of the UUCUAAA heptaribonucleotide containing the UUC codon for Phe and the stop signal UAAA, which bore a perfluoroaryl azido group at either the fourth nucleotide or the 3'-terminal phosphate, were synthesized. The UUC codon was directed to the ribosomal P site by the cognate tRNA<sup>Phe</sup>, targeting the UAA stop codon to the A site. Mild UV irradiation of the ternary complexes consisting of the 80S ribosome, the mRNA analog and tRNA resulted in tRNA-dependent crosslinking of the mRNA analogs to the 40S ribosomal proteins and the 18S rRNA. mRNA analogs with the photoreactive group at the fourth uridine (the first base of the stop codon) crosslinked mainly to protein S15 (and much less to S2). For the 3'-modified mRNA analog, the major crosslinking target was protein S2, while protein S15 was much less crosslinked. Crosslinking of eukaryotic (e) RF1 was entirely dependent on the presence of a stop signal in the mRNA analog. eRF3 in the presence of eRF1 did not crosslink, but decreased the yield of eRF1 crosslinking. We conclude that (i) proteins S15 and S2 of the 40S ribosomal subunit are located near the A site-bound codon; (ii) eRF1 can induce spatial rearrangement of the 80S ribosome leading to movement of protein L4 of the 60S ribosomal subunit closer to the codon located at the A site; (iii) within the 80S ribosome, eRF3 in the presence of eRF1 does not contact the stop codon at the A site and is probably located mostly (if not entirely) on the 60S subunit. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Translation termination; Human ribosome; mRNA analog; Photocrosslinking; Eukaryotic release factor 1; Eukaryotic release factor 3

## 1. Introduction

Termination of translation requires positioning of the peptidyl-tRNA at the ribosomal P site and of the termination (stop, nonsense) codon at the A site. Hydrolysis of the peptidyl-tRNA is promoted by class 1 polypeptide chain release factors (RF) at the peptidyl transferase center (reviewed in [1]). It has been demonstrated experimentally for both pro-

karyotes [2] and eukaryotes [3] that specificity of decoding of stop codons is associated with RFs rather than with the ribosome. The primary structures of many prokaryotic (RF1 and RF2), eukaryotic (eRF1), archaeal (aRF1) and mitochondrial (mtRF) class 1 RFs are known. RF1, RF2 and mtRF are structurally closely related and form one group, whereas eRF1 and aRF1 are also related and form another group which differs considerably from the first group [4–7]. Despite these pronounced dissimilarities, all class 1 RFs share a common tripeptide GGQ which is functionally important since mutations of the amino acid residues in or near this tripeptide cause complete or partial loss of the RF activity in vitro [8–10]. Mutations of the GGQ in yeast eRF1 are lethal in vivo [7]. In the crystal structure of human eRF1 (Fig. 1A), the GGQ tripeptide is located at the tip of the middle (M) domain [7]. This location in the three-dimensional structure of eRF1 together with its universality and functional importance supports the hypothesis [8] that GGQ is involved in promoting peptidyl-tRNA hydrolysis at the peptidyl transferase center located on the 50S/60S ribosomal subunit.

The second functionally essential site of class 1 RFs responsible for recognition of stop codons should be remote from the 'catalytic' site and positioned near the 30S/40S and 50S/60S interface in the ribosomal particle. Mutagenesis of yeast eRF1 followed by in vivo genetic assays points to the N domain [7] as a region of eRF1 where the termination codon recognition site (TCRS) is located [11]. The N-terminal domain (domain N) of human eRF1 contains a conserved NIKS motif (positions 61–64 for the human eRF1), common to both Eukarya and Archaea [6,7] (Fig. 1A).

Class 2 termination factors, eRF3/RF3, are known to be GTPases [12–14]. The human eRF1 and eRF3 interact through their C-termini [15] (Fig. 1A) and this mutual binding is critical for the eRF3 GTPase activity within the ribosome [12,16]. This property can be used to detect binding of eRF1 to eRF3 and to the ribosome [10]. The N and M domains of eRF1 are required to activate the eRF3 GTPase, although they are not involved in eRF3 binding. This implies that eRF1 binding to the ribosome is a prerequisite to allow activation of the eRF3 GTPase.

To investigate the positioning of the ribosomal components and protein factors associated with the ribosome during the translation process, the crosslinking approach has been widely used (reviewed in [17–19]). In particular, crosslinking between prokaryotic class 1 RFs and short mRNAs was demonstrated, although the specificity of this reaction appeared to be low

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with respect to the nucleotide composition of the codons [19]. For human eRF1, high specificity of crosslinking was achieved when the stop codon was placed immediately after a sense codon within a 42-mer mRNA phased within the ribosome due to the presence of the cognate tRNA targeted to the sense codon on the ribosomal P site [20].

Here, we have extended this approach using short mRNA analogs composed of a sense and a nonsense codon and applying another type of crosslinking group. These mRNAs were derivatives of heptaribonucleotides (Fig. 1C) and consisted of the triplet UUC encoding Phe and the adjacent termination tetraplet UAAA (or control tetraplet UCAA) bearing a perfluoroaryl azido group at the fourth or seventh nucleotide residue (positions +1 and +4 of the termination tetraplet, respectively). We show that modified nucleotides at either position +1 or +4 are able to crosslink with eRF1 and crosslinking is specific for mRNA analogs containing a stop codon. The main targets of crosslinking of the mRNA with ribosomal proteins have been determined.

## 2. Materials and methods

### 2.1. Materials

tRNA<sup>Phe</sup> (1300 pmol/A<sub>260</sub> unit) was a kind gift from Dr. T. Shapkina (Konstantinov's St. Petersburg Institute of Nuclear Physics, Gatchina, Russia). Isolation of the 40S and 60S ribosomal subunits from unfrozen human placenta and their association in 80S ribosomes were performed as described earlier [21]. The full-length human eRF1 carrying a His<sub>6</sub> tag at the N-terminus was expressed, purified and assayed as described [4,16]. Human eRF3 expressed in a baculovirus system was purified as described [22].

### 2.2. Photoreactive mRNA analogs

Synthesis of a derivative of the heptaribonucleotide UUCUAAA which carries the 2-(4-azido-2,3,5,6-tetrafluorobenzoyl)-aminoethyl group at the C5 atom of the fourth nucleotide was performed as described [23]. To introduce a photoreactive group at the 3'-phosphate position, a modified oligomer UUCUAAA-p-OCH<sub>2</sub>-CH(OH)-CH<sub>2</sub>-NH<sub>2</sub> was synthesized by the solid phase H-phosphonate procedure [24] using the modified polymer 3'-Amino-Modifier C3 CpG 500 (Glen Research, USA). The modified heptaribonucleotide was purified by ion exchange and reverse phase HPLC with subsequent precipitation as lithium salt. The photoactivatable moiety was coupled to the aliphatic amino group by treatment with the *N*-oxysuccinimide ester of *p*-azidotetrafluorobenzoic acid as described elsewhere [23]. Before use, the mRNA analogs were 5'-end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP (specific activity about 1000 Ci/mmol) by T4 polynucleotide kinase purchased from Promega.

### 2.3. Complexes and crosslinking procedures

Complexes of 80S ribosomes with tRNA<sup>Phe</sup> and mRNA analogs were obtained by incubation of these components (at  $5.4 \times 10^{-7}$  M,  $2.6 \times 10^{-6}$  M and  $4.5 \times 10^{-6}$  M concentrations, respectively) for 40 min in buffer A containing 13 mM MgCl<sub>2</sub>, 120 mM KCl, 0.6 mM EDTA and 20 mM Tris-HCl, pH 7.5 at room temperature. Typically, the reaction mixtures for irradiation contained 30 pmol of 80S ribosomes. Then eRF1 or both eRF1 and eRF3 were added where specified in a seven-fold molar excess each with respect to the 80S ribosomes. In the experiments with eRF3, GTP was added to 1 mM concentration. The reaction mixtures were again incubated for 40 min at room temperature. Binding of the labeled mRNA analogs to 80S ribosomes was examined by the nitrocellulose filtration technique as described elsewhere [25]. Irradiation of complexes and dissociation of 80S ribosomes into the subunits was carried out as described [23].

If purified, the irradiated complexes were centrifuged at 21 500 rpm for 17.5 h at 4°C in a 15–30% sucrose density gradient containing buffer A (Beckman L8M, SW-40 rotor), the peaks of 80S ribosomes were collected, ethanol-precipitated, and the pellets dissolved in 50  $\mu$ l of buffer B (2% SDS, 1% 2-mercaptoethanol, 20% glycerol, 60 mM Tris-HCl, pH 7.5 and 0.05% bromophenol blue), incubated 10 min at

60°C and analyzed by SDS-PAGE on a 15% gel. Analysis of the crosslinked 40S subunits was carried out similarly. For analysis of the irradiated reaction mixtures (without purification to separate the ribosomes from unbound ligands), 2  $\mu$ l of the mixture was added to 13  $\mu$ l of buffer B followed by the treatment described above. The probes for SDS-PAGE contained typically about 5 pmol of cross-linked 80S ribosomes or 40S subunits. To identify crosslinked proteins, the radioautograms were superimposed on the respective stained gels and the positions of the radioactive bands with respect to those of unmodified proteins were determined. For protease digestions, samples were treated after irradiation with 80  $\mu$ g of proteinase K for 1 h at 37°C.

## 3. Results

### 3.1. Binding and crosslinking of mRNA analogs to 80S ribosomes

The photocrosslinking mRNA analogs applied here to study interaction of the termination signal with polypeptide chain release factors (eRF1 and eRF3) in human 80S ribosomal complexes are schematized in Fig. 1B,C. A perfluoroaryl azido group is attached either at the C5 atom of the fourth nucleotide (reagents A and B) or at the 3'-terminal phosphate of the last adenosine residue (reagent C). Reagent C is used here for the first time for crosslinking reactions within ribosomal complexes. Placement of the UUC codon of the mRNA analogs at the ribosomal P site (see Fig. 1B) was achieved by addition of tRNA<sup>Phe</sup>.

Factor-free ('non-enzymatic') binding of the derivatives to human 80S ribosomes almost completely depends on the presence of tRNA<sup>Phe</sup> under the conditions used here (13 mM MgCl<sub>2</sub> and room temperature). Typically, binding is about 0.7 and 0.05 mol of mRNA analog per mol of 80S with and without tRNA<sup>Phe</sup>, respectively. Crosslinking of all photoreactive mRNA analogs to the ribosomal components is significantly enhanced in the presence of tRNA<sup>Phe</sup> (Fig. 2A–C, compare lanes 1 and 2) indicating that crosslinking indeed occurs in complexes in which codon–anticodon interaction at the P site takes place.

### 3.2. Crosslinking of mRNA analogs to ribosomal subunits and proteins

All radioactive bands moving faster than the rRNA band (Fig. 3) correspond to crosslinked proteins, since treatment of the irradiated 80S ribosomal complexes with proteinase K resulted in almost complete loss of these bands (not shown). Proteins are clearly preferential targets of the crosslinking reaction as compared to the rRNA (which was not subjected for further analysis in this study). The fastest migrating band (which is more distinctly seen in Fig. 2A,B) seems to correspond to products of proteolysis of some crosslinked proteins, since after purification of the irradiated 80S ribosomal complexes this band almost disappears (Fig. 3A,B). Crosslinking of the mRNA analogs to ribosomal proteins depends on the position of the modifying group in the mRNA analog (Figs. 2 and 3, compare lanes 2–4 in panels A and B with the same lanes in panel C) and, for reagent C, on the type of complex (Fig. 3C, compare lanes 2–4). In the latter case, this concerns crosslinking to the protein migrating between rRNA and eRF\* (Fig. 3C, lane 3). This radioactive band may be assigned to crosslinked protein L4 (or much less probably, to L3) based on the shift of this band towards unmodified L4 in the stained gel (Fig. 2D). Gel shifts for several high molecular weight ribosomal proteins crosslinked to RNA hexamers have

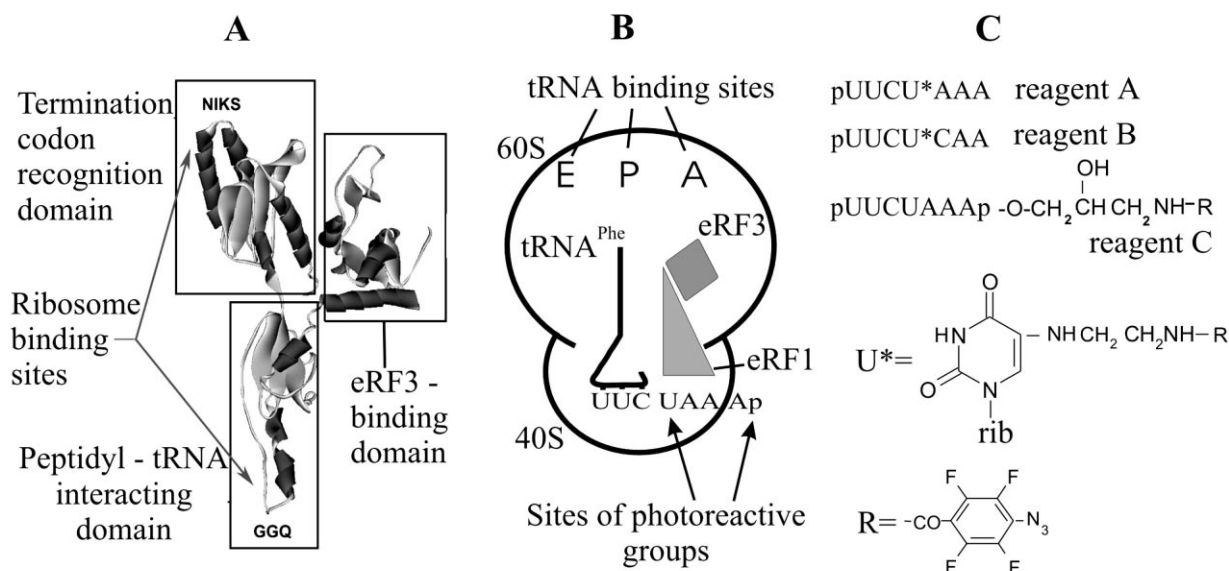


Fig. 1. Domain organization and three-dimensional structure of human eRF1 [7] (A). 80S ribosome complexed with mRNA analog, tRNA and translation termination factors (B). mRNA analogs used in this work (C). The lengths of the modifying groups of the mRNA analogs were about 11 Å.

been estimated earlier [26,27]. In the case of crosslinking to L3, the radioactive band would coincide with unmodified protein L4. Crosslinking to protein L4 is observed only with reagent C and is clearly detectable in the presence of eRF1 (Fig. 3C, lane 3), although without eRF1 or in the presence of both eRF1 and eRF3 no crosslinking is observed (Fig. 3C, lanes 2 and 4). Moreover, for reagent C, eRFs decrease yield of crosslinking to proteins that move in the lower part of the gel (Fig. 3C, compare lanes 2–4).

To examine crosslinking of mRNA analogs to the ribosomal subunits, irradiated 80S ribosomal complexes were dissociated and the ribosomal subunits were separated in a sucrose density gradient. As anticipated, about 90% of the label was associated with the 40S subunits for all three mRNA analogs. The only weak band corresponding to crosslinked protein L4 was detected in the 60S subunit crosslinking pattern (data not

shown). On the 40S crosslinking patterns, both proteins and 18S rRNA are labeled (Fig. 4A,C). In general, the same proteins are crosslinked to reagents A and C. The positions of the two main bands, corresponding to the crosslinked 40S proteins, are very similar to those observed earlier for proteins S2 and S15 crosslinked to a derivative of UUUGUU with a photoreactive group at the guanosine residue. These proteins were labeled in various complexes, including those in which the crosslinking group was in the first nucleotide of the A site bound codon [26] as in the case of reagent A.

We suggest that the main bands of the 40S proteins crosslinked to reagents A and C correspond to S2 and S15 (Fig. 4). Protein S15 is preferentially labeled as compared to other proteins by the mRNA analog A with a crosslinking group at the first nucleotide of the UAAA stop signal (Fig. 4A), whereas for reagent C with a modifying group at the last

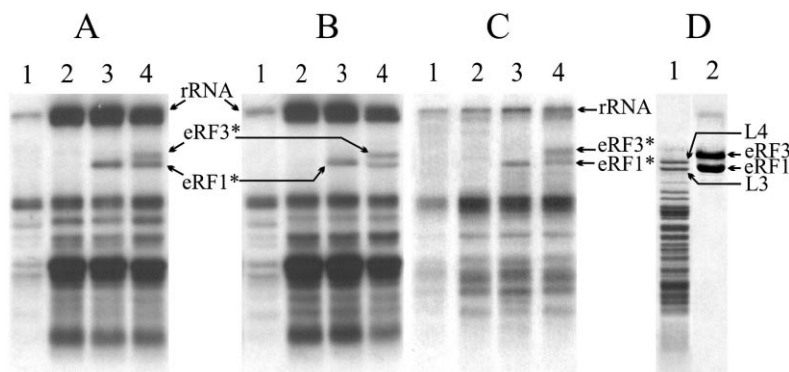


Fig. 2. SDS-PAGE analysis of irradiated reaction mixtures of reagents A, B and C (panels A, B and C, respectively). Radioautogram, exposure times were 17 h for A and B and 3 h for C. Lanes 1, reagent with 80S ribosomes; lanes 2, reagent with 80S ribosomes and tRNA<sup>Phe</sup>; lanes 3, reagent with 80S ribosomes, tRNA<sup>Phe</sup> and eRF1; lanes 4, the same with both eRF1 and eRF3. Bands corresponding to crosslinked rRNA and eRFs (marked as eRF1\* and eRF3\*) are indicated. D: Gel stained with Coomassie brilliant blue; lane 1, typical gel for 80S ribosomal complexes without eRFs; lane 2, eRF1 and eRF3 without ribosomes. Positions of proteins L3 and L4 on the stained gel are indicated according to [31].

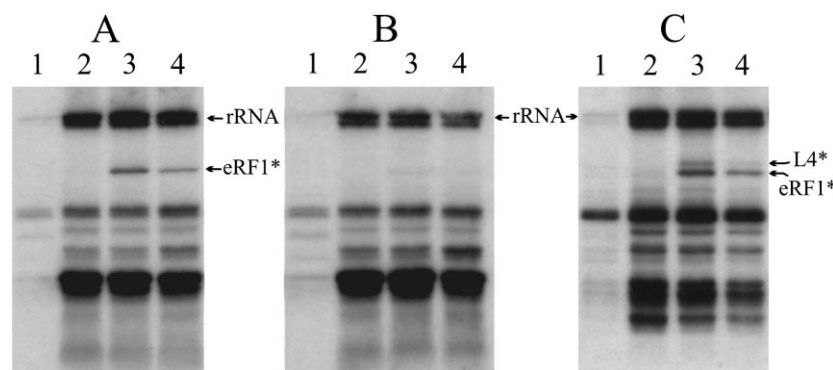


Fig. 3. SDS-PAGE analysis of irradiated ribosomal complexes separated by sucrose density gradient centrifugation from ligands not bound to ribosomes. Radioautogram, exposure times, 17 h. Panels A, B and C correspond to experiments with reagents A, B and C, respectively. Lanes 1–4, see legend to Fig. 2. Bands corresponding to crosslinked rRNA, protein L4 and eRF1 (marked as L4\* and eRF1\*) are indicated.

nucleotide of the UAAA stop signal, protein S2 is the main target of crosslinking (Fig. 4C). Besides these proteins, for reagent C one can observe a weak band above the crosslinked S2 (Fig. 4C, lanes 2–4). This band corresponds to crosslinking to a 40S subunit protein with a molecular mass of about 40 kDa (pr40). This protein has been found earlier in human 40S ribosomes [28]. As for the weak faster migrating radioactive band detected with reagent C (Fig. 4C, lanes 2–4), it may be assigned to crosslinked protein S20 based on the shift of this radioactive band towards unmodified S20 in the stained gel (Fig. 4, left lane), values of such shifts caused by crosslinked RNA hexamer have been estimated earlier for proteins S26 and S30 [26,27] that are positioned close to S20 in the gel, see Fig. 4. On the 40S crosslinking patterns (Fig. 4C, lanes 2–4), two bands corresponding to proteins moving more slowly than S15 visible on the 80S crosslinking pattern of Fig. 3C, lanes 2–4, are lost. These proteins do not originate from the 60S subunit, since only crosslinking to L4 is seen on the 60S crosslinking patterns. Probably, these crosslinked proteins were lost during isolation of the modified ribosomal subunits from the irradiated 80S complexes. It should be noted that some portion of the crosslinked S2, S15 and S20 proteins was

also lost during sucrose density gradient centrifugation (compare the relative intensities of the radioactive bands of rRNA and the proteins mentioned in lanes 2–4 in Fig. 4A,C, with the same lanes in Fig. 3A,C).

### 3.3. Crosslinking of mRNA analogs to eRFs

For all reagents (even for reagent B lacking a stop signal), crosslinking to both eRFs is easily detectable when the irradiated reaction mixtures are analyzed (Fig. 2 A–C, lanes 3 and 4). Crosslinking of both eRF1 and eRF3 occurs efficiently also in mixtures of the eRFs with the mRNA analogs without ribosomes and tRNAs (not shown). This crosslinking is insensitive to the sequence of the mRNA analog, and eRF3 crosslinks as well as eRF1. This is why we separated irradiated 80S ribosomal complexes from free (unbound) eRFs that crosslinked to the mRNA analogs without the ribosome before analysis of the crosslinked components. In the case of the separated 80S complexes, radioactive band corresponding to crosslinked eRF1 is clearly visible for only the A and C mRNA analogs (Fig. 3A,C, lanes 3). For the mRNA analog B bearing a sense UCA codon this band is hardly detectable (Fig. 3B, lane 3). Therefore, crosslinking is specific for ribo-

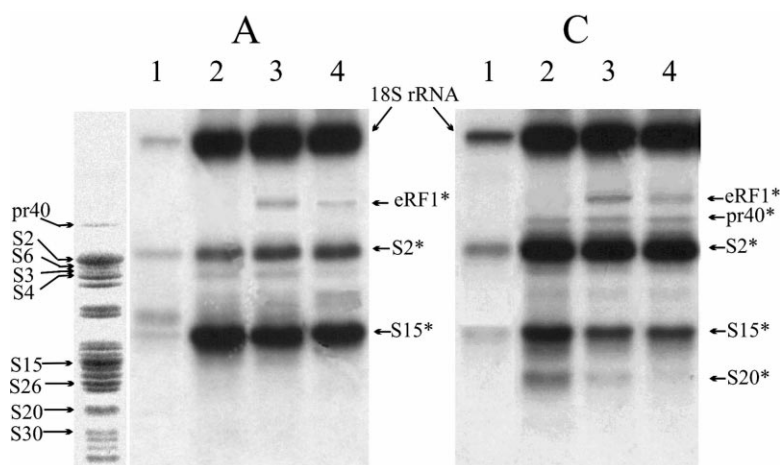


Fig. 4. SDS-PAGE analysis of 40S subunits isolated from the irradiated complexes obtained with reagents A and C (panels are designated as respective reagents). Lanes 1–4, see legend to Fig. 2. Radioautogram, exposure times, 48 h. Bands corresponding to crosslinked ribosomal proteins and eRF1 (marked with asterisks) and rRNA are indicated. On the left, typical gel stained by Coomassie brilliant blue; positions of 40S proteins on this gel are marked according to [32].

somal complexes with mRNA analogs carrying a stop signal. eRF3 significantly decreases the yield of eRF1 crosslinking to mRNA analogs, but itself does not crosslink (Fig. 3A,C, compare lanes 3 and 4). We have studied crosslinking of eRF3 only in the presence of eRF1 since eRF3 is functionally active as a GTPase only when complexed to eRF1. Interestingly, on the 40S crosslinking patterns, weak bands corresponding to crosslinked eRF1 (Fig. 4, lanes 3 and 4) are visible. This implies that the mRNA analog is so tightly bound at the ribosomal decoding site, that some portion of the mRNA analog crosslinked to eRF1 remains bound to the 40S subunit even after separation of the 80S ribosomes into the subunits.

#### 4. Discussion

Short mRNA analogs barely bind to human 80S ribosomes without tRNA cognate to one of the codons of the mRNA. The cognate tRNA and its codon bind at the P site (for references see [18]). Occupation of the P site governs the specific phasing of the mRNA analog on the ribosome and directs the stop signal to the A site (Fig. 1B).

Ribosomal proteins S2 and S15 that crosslink to modifying groups at both the first and the last nucleotides of the UAAA stop signal were found earlier to crosslink to derivatives of the mRNA analog UUUGUU bearing an aryl azido group on the guanosine residue [26]. The yield of crosslink did not depend on whether the modified GUU codon was located at the ribosomal A or P site, and the extent of crosslinking to S15 was higher than to S2 [26] as in the case of crosslinking from position +1 of the stop signal in this study. This implies that the protein environment of the first nucleotide of the A site bound codon is similar at the elongation and the termination steps of translation. Based on earlier data on crosslinking of human 80S ribosomes to short mRNA analogs bearing various modifying groups at the 5'-terminal phosphate, protein S2 was suggested to be located in the vicinity of the mRNA sequence between the first nucleotide of the E site bound codon and the first nucleotide of the P site bound codon on the elongating ribosome [18]. Crosslinking of protein S2 to the last modified nucleotide of the mRNA stop signal UAAA (Fig. 4B) implies that S2 is probably located in a central position in the region of the E, P and A sites, as are proteins S3 and S3a [18]. The location of many ribosomal proteins within prokaryotic ribosomes has recently been determined (e.g. see [29]). For eukaryotic ribosomal proteins these remain largely unknown. It seems somewhat useless to compare ribosomal proteins of bacteria and higher eukaryotes because the extent of homology between them is as low as about 20–30% [30].

The yield of crosslinking from position +1 with ribosomal components hardly depends on eRF1 (Figs. 2A and 3A, compare lanes 2 and 3). In contrast, addition of eRF1 to the phased system obtained with a 42-mer containing the GAC triplet coding for Asp and s<sup>4</sup>UGA as stop codon, dramatically decreased the yield of crosslinks of the mRNA to both rRNA and ribosomal proteins [20]. This implies that eRF1 strongly shields the ribosomal components from direct contact with the stop codon, whereas the UUCUAAA derivatives with flexible spacers separating nucleotides of the stop signal UAAA and the photoactivated group produce crosslinks with the ribosomal components despite the presence of eRF1 on the A site.

Small but clearly detectable alterations within the ribosome

caused by binding of eRF1 at the A site are seen for the UUCUAAA derivative with the crosslinking group in the 3'-terminal nucleotide. In particular, crosslinking of this mRNA analog to protein L4 is observed only in the presence of eRF1 (Fig. 3C, compare lanes 2 and 3). Since crosslinking of this protein does not occur in the 80S ribosomal complex without eRF1 (Fig. 3C, lane 2), it may suggest that L4 is not located near the decoding site of the elongating ribosome. Probably, binding of eRF1 causes a conformational change in the decoding site bringing protein L4 in a vicinity of the mRNA stop signal. The fact that eRF3 prevents crosslinking to protein L4 (Fig. 3C, lane 4) indicates that the factor either shields the protein against contact with the crosslinking group in the stop signal, or causes a conformational change opposite to the one caused by eRF1, moving protein L4 away from the mRNA stop signal (as in the elongating ribosomes). eRFs also decrease the yield of crosslinking of reagent C to protein S15 and to another weakly labeled protein, most probably S20 (Fig. 4C, lanes 2–4). This may be due either to a direct protection effect of eRF1 or to conformational alterations of the ribosome induced by eRF1–eRF3 binding placing these proteins away from the crosslinking group on the mRNA stop signal.

Crosslinking of eRF1 to mRNA analogs is highly specific for stop signal containing mRNA sequences only in 80S complexes. Without ribosomes, eRF1 does not specifically recognize the mRNA sequence. Crosslinks to eRF1 from both positions +1 and +4 of the stop signal are reduced in the presence of eRF3, whereas for s<sup>4</sup>UGA-containing 42-mer, eRF3 hardly affects the yield of the mRNA–eRF1 crosslink [20]. This latter result was indeed anticipated since eRF3 binds to the C-terminal domain of eRF1 [15] that is not involved in interaction with the stop codon [16]. On the other hand, a flexible spacer in the modifying groups of the UUCUAAA derivatives could enable aryl azido groups to attack not only a region of the N-terminal domain of eRF1 that probably determines the specificity of interaction with the stop codon in mRNA [11], but also another region of N domain neighboring the C domain in the spatial structure of eRF1. This region of eRF1 can be shielded by eRF3 against crosslinking to UUCUAAA derivatives. However, this interpretation requires verification by structural analysis. Alternatively, binding of eRF3 to eRF1 may induce reduction of the strength of eRF1 binding to the 40S subunit bearing the stop signal that in turn will diminish the yield of crosslink. This interpretation is consistent with the observation that RF3, a prokaryotic analog of eRF3, triggers the release of RF1/RF2 from the ribosome [13]. Finally, binding of eRF3 to eRF1 may cause conformational changes that bring crosslinking groups attached to the UAAA stop signal close to amino acid residues of eRF1 that are poor targets for the crosslinking from a chemical point of view. It is likely that eRF3 itself does not contact the stop codon at the A site and is probably located mostly (if not entirely) on the 60S subunit.

The results of this study clearly indicate that an approach based on the use of short mRNA analogs bearing aryl azido groups attached to various nucleotides at or near the stop codon makes it possible to obtain information on the mutual orientation of the mRNA stop signal and components of the 80S ribosome at the termination step of translation. Even minor rearrangements of the mRNA stop signal with respect to the ribosome caused by eRFs are clearly detectable.

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