The ribosomal A site-bound sense and stop codons are similarly positioned towards the A1823–A1824 dinucleotide of the 18S ribosomal RNA

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Abstract Positioning of the mRNA codon towards the 18S ribosomal RNA in the A site of human 80S ribosomes has been studied applying short mRNA analogs containing either the stop codon UAA or the sense codon UCA with a perfluoroaryl azide group at the uridine residue. Bound to the ribosomal A site, a modified codon crosslinks exclusively to the 40S subunits under mild UV irradiation. This result is inconsistent with the hypothesis [Ivanov et al. (2001) RNA 7, 1683–1692] which requires direct contact between the large rRNA and the stop codon of the mRNA as recognition step at translation termination. Both sense and stop codons crosslink to the same A1823/A1824 invariant dinucleotide in helix 44 of 18S rRNA. The data point to the resemblance between the ternary complexes formed at elongation (sense codon·aminoacyl-tRNA·AA dinucleotide of 18S rRNA) and termination (stop codon·eRF1·AA dinucleotide of 18S rRNA) steps of protein synthesis and support the view that eRF1 may be considered as a functional mimic of aminoacyl-tRNA.

Key words: Translation termination; Human ribosome; mRNA analog; Photocrosslinking; Ribosomal RNA

1. Introduction

Understanding eukaryotic translation termination is less advanced than other steps of protein synthesis (reviewed in [1–5]). The ribosomal complexes modeling various states of the ribosomes during the termination have so far not been crystallized. For better understanding a termination mechanism, one has to know whether any differences can be revealed in the arrangements of sense versus stop codons of mRNAs at the ribosomal A site towards the ribosomal components. To date, even for the better studied bacterial ribosomes, there is no definite answer to this question. The 4-thiouridine residue in the first position of the UAA stop codon in the A site of bacterial ribosomes crosslinks to the C1407 residue of the 16S rRNA [6]. Crosslinking in the 1385–1420 region of 16S rRNA containing C1407 was found for all s^4UGAN and s^4UAAN stop signals [7]. With the s^4U residue in the first position of the A site-bound sense codon, the crosslinked nucleotide was identified in the rRNA as the C1402/C1407 site [8]. Recent X-ray crystallographic data on the location of the mRNA and tRNAs within the bacterial 30S ribosomal subunit showed that the first nucleotide of the A site-bound sense codon is located close to C1401 of the 16S rRNA [9].

It was found that class 1 release factors (RFs) (in particular RF2) promoting hydrolysis of peptidyl-tRNA bound to the P site contacts the A site-bound stop codon and does not affect its position with respect to the 16S rRNA (reviewed in [2]). At present, nothing is known concerning interaction between stop codons and rRNA in the A site of eukaryotic ribosomes. For these ribosomes, it was shown that eRF1 contacts the A site-bound stop codon [10,11] and the site of eRF1 involved in this contact has been determined [12]. However, little is known regarding the placement of the A site-bound stop codon towards the rRNAs and possible effects of eRF1. The A1491G, U1495C, U912C and G886A replacements in yeast 18S rRNA compensated the arbitrarily lethal Pro86Ala mutation in eRF1 [13]. Besides, the protein environment of a mRNA stop codon bound to the A site of human ribosomes was studied with the use of short mRNA analogs composed of sense and nonsense codons and bearing a perfluoroaryl azide group on the first nucleotide of the stop codon [11].

In addition to class 1 RFs, another group of proteins called class 2 RFs (RF3 and eRF3 in prokaryotes and eukaryotes, respectively) is involved in termination and post-termination steps of translation. Class 2 RFs are GTPases [14–16] which activity depends on the class 1 RFs [14,17,18]. Bacterial RF3 is a GTP-GDP exchange factor responsible for the release of RF1/RF2 from the ribosome after completion of the translation termination reaction [17,18], while the biological role of eRF3 remains obscure although possibly its function is similar to prokaryotic RF3 [1].

Recently, a hypothesis has been proposed that two hairpins of the rRNA of the large subunit recognize stop codons directly by their complementary 'anticodon-like' loops [19]. Although crosslinking studies revealed no contacts between mRNA stop codon and the rRNA of the large subunit at the A site of bacterial ribosomes [6], it seemed reasonable to test this hypothesis also on mammalian ribosomes.

Here, we have compared positioning of the A site-bound sense and stop codons toward the large and small subunit rRNAs and studied the effects of the eRFs on the interaction

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of the A site-bound stop codon with 18S rRNA. We have applied derivatives of the UUCUAAA heptaribonucleotide (containing the UUC codon for Phe and the stop signal UAAA) and UUCUCAA heptaribonucleotide (with UCA codon for Ser instead of the stop codon), that bore a perfluoroaryl azide group at the fourth nucleotide.

2. Materials and methods

2.1. Materials

$tRNA^{Phe}$ (1300 pmol/A_{260} 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 into 80S ribosomes were performed as described [20]. The full-length human eRF1 carrying a His_6-tag at the N-terminus was expressed, purified and assayed as described [21,22]. Human eRF3 was purified as described [23].

2.2. Photoreactive mRNA analogs

Synthesis of derivatives of the heptaribonucleotide UUCUAAA or UUCUCAA that carry the 2-(4-azido-2,3,5,6-tetrafluorobenzoyl)-aminoethyl group on the C5 atom of the fourth nucleotide (azido-mRNAs) was performed as described [24]. The photoactivatable moiety was coupled to the aliphatic amino group by treatment with the *N*-oxysuccinimide ester of *p*-azidotetrafluorobenzoic acid as described [24]. Before use, azido-mRNAs were 5′-end-labeled with [γ-^{32}P]ATP (specific activity about 1000 Ci/mmol) by T4 polynucleotide kinase purchased from Promega.

2.3. Complexes and crosslinking procedures

Preparation of the complexes of 80S ribosomes with $tRNA^{Phe}$, azido-mRNAs and eRFs, crosslinking and dissociation of the crosslinked ribosomes into 40S and 60S subunits were performed as described [11]. Analysis of crosslinking to RNAs, determination of regions of the 18S rRNA that contained the crosslinked mRNA analogs and of the crosslinked 18S rRNA nucleotides were carried out as described [25].

3. Results

The A site location of a modified UAA stop codon or a UCA sense codon in the 80S ribosomal complexes with azido-pUUCUAAA or azido-pUUCUCAA was achieved by

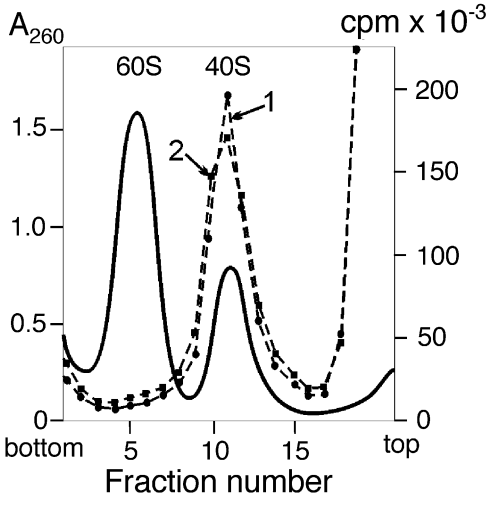

Fig. 1. Crosslinking of [5′-^{32}P]azido-pUUCUAAA to the ribosomal subunits. The 80S ribosomal complexes were obtained in the presence of $tRNA^{Phe}$, irradiated and sedimented in sucrose density gradient (10–30%) under conditions of dissociation of 80S ribosomes into the 40S and 60S subunits. Solid line, optical density (typical profile); dotted lines, radioactivity. 1, without eRFs; 2, with eRF1.

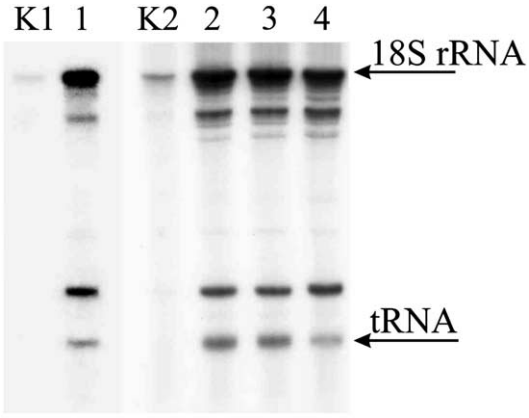

Fig. 2. Radioautographic analysis of crosslinking of 5′-^{32}P-labeled azido-mRNAs to 18S rRNA and tRNA by electrophoresis in 5% polyacrylamide gels. The RNA isolated from 80S ribosomal complexes of azido-pUUCUCAA (left part) and azido-pUUCUAAA (right part) with (lanes 1–4) and without (lanes K1 and K2) $tRNA^{Phe}$. Lanes 3 and 4 correspond to the complexes obtained in the presence of eRF1 and or eRF3+eRF1, respectively. The positions of 18S rRNA and tRNA are marked as they were seen in the gels stained with 'Stains All'.

$tRNA^{Phe}$ targeting the cognate UUC codon to the P site. In these complexes, the modified uridine was situated in the +4 position of the mRNA with respect to the first nucleotide of the P site-bound codon. Without tRNA, binding of the mRNA analogs was negligible. Mild UV irradiation of the complexes obtained with $tRNA^{Phe}$ resulted in crosslinking of the labeled azido-mRNAs exclusively to the 40S subunits; no radioactivity was detected in the fractions containing 60S subunits (data for azido-pUUCUAAA are presented in Fig. 1).

Analysis of the RNA isolated from the crosslinked complexes by denaturing PAGE (Fig. 2) showed a strong modification of 18S rRNA and a weak crosslinking to tRNA. Besides, crosslinking to partially degraded 18S rRNA fragments (not detectable in the stained gels) was noticed. Modification of tRNA was decreased in the presence of eRF3 together with eRF1 (Fig. 2, lane 4). Crosslinking to 18S rRNA was almost completely dependent on the presence of $tRNA^{Phe}$ and was hardly detectable in binary mixtures of azido-mRNAs with 80S ribosomes (Fig. 2, lanes K1 and K2). Evidently, this was due to the negligible binding of azido-mRNAs to ribosomes without tRNA. eRF1 alone or together with eRF3 did not affect the crosslinking of azido-mRNAs to 18S rRNA in the presence of $tRNA^{Phe}$.

To determine the sequences in the 18S rRNA containing the crosslinked nucleotides, 18S rRNA isolated from the irradiated complexes was digested with RNase H in the presence of deoxy-oligomers complementary to various fragments of 18S rRNA in parallel experiments (see scheme in Fig. 3). The hydrolytic fragments of the 18S rRNA were separated by PAGE under denaturing conditions. Part of the rRNA that contained the crosslinked ^{32}P-labeled mRNA analog was detected by autoradiography (Fig. 3). The analysis revealed that the crosslinking sites were located within the 1812–1831 fragment of 18S rRNA for all complexes. This conclusion was based on the absence of ^{32}P label in the lanes corresponding to the hydrolysis of the crosslinked 18S rRNA in the presence of deoxy-oligomer complementary to positions 1812–1831 (Fig. 3, lanes 4). The results of RNase H digestion in the

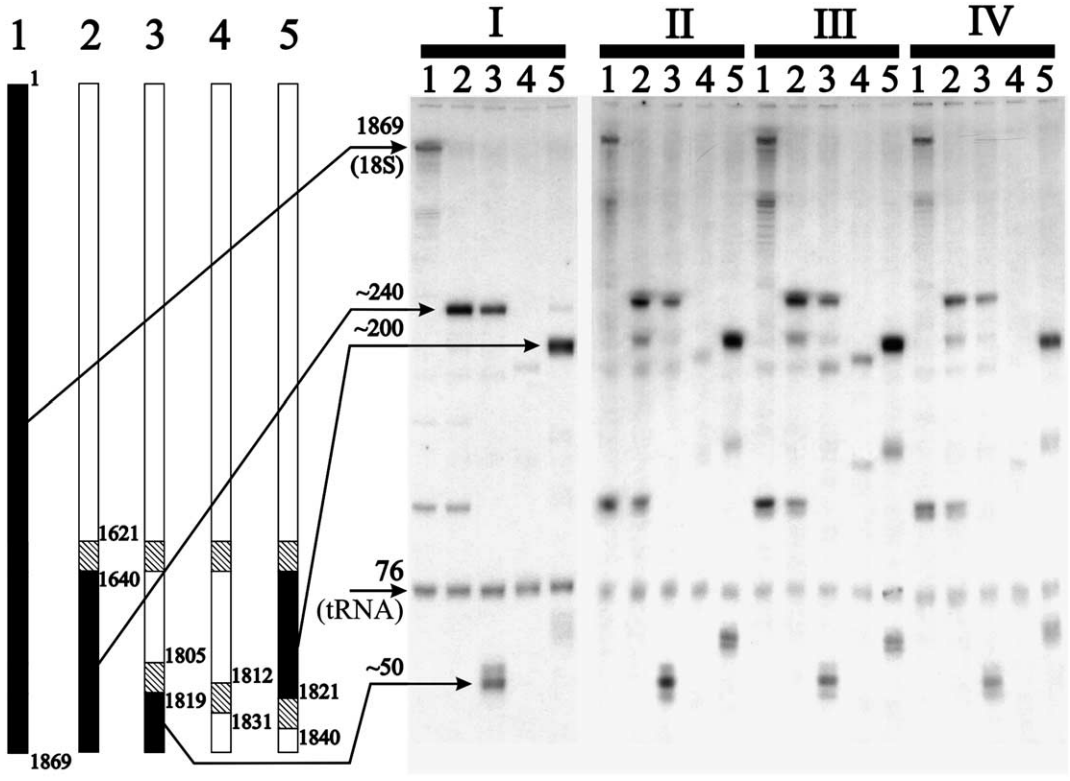

Fig. 3. Ribonuclease H digests of 18S rRNA crosslinked to 5′-labeled azido-mRNAs. Ternary complexes of 80S ribosomes with $tRNA^{Phe}$ and either azido-pUUCUCAA (panel I) or azido-pUUCUAAA (panel II); 80S ribosomal complexes with azido-pUUCUAAA and $tRNA^{Phe}$ obtained in the presence of eRF1 (panel III), and in the presence of eRF1 together with eRF3 (panel IV). The image is from autoradiograms of the gels. Lanes '1' are control rRNA treated with ribonuclease H but without deoxy-oligomers. Positions of 18S rRNA and the excised fragments (the expected lengths are marked, lengths of the crosslinked mRNA analogs have been subtracted) are indicated. Scheme of the hydrolysis of crosslinked 18S rRNA is given on the left. Fragments of 18S rRNA complementary to the deoxy-oligomers are shaded, fragments containing the crosslinked labeled mRNA analog are black. Numbers 1–5 on the scheme correspond to the lanes 1–5 on panels I–IV.

presence of other deoxy-oligomers (Fig. 3, lanes 2, 3 and 5) are consistent with this location for the crosslinking site.

Nucleotides of 18S rRNA crosslinked to azido-mRNAs were identified by a primer extension approach; the primer was chosen on the basis of RNase H treatment data. Since the crosslinking site is generally assumed to be 5′ to the primer extension stop or pause site, crosslinked nucleotides of 18S rRNA are A1823 and A1824 (Fig. 4) for complexes of both mRNA analogs formed in the presence of $tRNA^{Phe}$. For azido-pUUCUAAA, the primer extension pattern was not affected by eRF1 or by a mixture of eRF3 and eRF1. Note that 18S rRNA isolated directly from placenta tissue and without crosslinking was used for the sequencing. This is why several stops seen at the top of lanes 1–4 and K are absent in the sequencing lanes (Fig. 4).

4. Discussion

Recently, we showed that derivatives of the UUCUAAA heptaribonucleotide that carry a perfluoroaryl azide group at the designed positions of the termination tetraplet UAAA are valuable tools for studying the vicinity of the A site-bound stop signal on human ribosomes. Binding of these mRNA analogs to 80S ribosomes is substantial only in the presence of $tRNA^{Phe}$ that directs the UUC codon to the P site and targets the modified stop signal to the A site. Modified nucleotides of the A site-bound stop signal could be specifically crosslinked to eRF1 [11].

Here, we found that crosslinking of the modified uridine at position +4 of the heptanucleotide to 18S rRNA is independent of the codon nature containing the modified nucleotide (sense or stop codon) (Fig. 4). Even more, the crosslinking is insensitive to the presence of eRF1 at the A site. In contrast, addition of eRF1 to the phased system obtained with a 42-mer containing the GAC triplet encoding Asp and s^4UGA as stop codon, decreased the yield of crosslinks of the mRNA to rRNA [10,12]. In the case of the UUCUAAA derivative, a flexible spacer separating the first nucleotide of the stop codon and the photoactivated group, allowed to produce crosslinks with the rRNA despite the presence of eRF1 in the A site. On the other hand, eRF1 and eRF3 together (but not eRF1 alone) partially shielded the stop codon from the crosslinking to the P site-bound $tRNA^{Phe}$ (Fig. 2). It is known that the C domain of eRF3 binds to the C domain of the A site-bound human eRF1 [26]. The latter resembles an L-shaped tRNA molecule in which the C domain mimics the elbow of tRNA while the conserved anticodon-like NIKS loop is in contact with the stop codon [12]. Our observation that eRF3 in complex with eRF1 partially protects the P site-bound tRNA from crosslinking to the modified stop codon bound in the A site indicates that at least part of the eRF3 (but not its C domain) is probably positioned between the NIKS loop of eRF1 and the P site-bound tRNA.

The invariant dinucleotide A1823/A1824 crosslinked to the modified nucleotides of azido-mRNAs is located in the 3′-terminal mini-domain of 18S rRNA in the upper part of

helix 44, one of the most highly conserved fragments of the secondary structure of the small subunit rRNA (Fig. 5), and corresponds to the A1492/A1493 dinucleotide of 16S rRNA [27,28]. These nucleotides are known to play a key role in the formation of the decoding site of bacterial ribosomes [9,29,30] and in recognition of correct codon–anticodon duplexes on the A site [31]. Surprisingly, A1492/A1493 of 16S rRNA have never been found at least to our knowledge in the decoding site of bacterial ribosomes by means of crosslinking of mRNA analogs containing 4-thiouridine in position +4. Evidently, this was due to hydrogen bonds of the A1492/A1493 nucleotides of 16S rRNA formed with nucleotides of the A site-bound codon via hydroxyl groups of the 1492 and 1493 riboses [31]. Therefore, 4-thiouridine interacting with these riboses was unable to contact adenines 1492 and 1493 and crosslinked to a 1398–1419 sequence of 16S rRNA [8] that is located directly across from the A1492/A1493 in the upper part of helix 44 (Fig. 5).

In the present study, a modifying group on uridine in position +4 could reach the A1823/A1824 dinucleotide of 18S rRNA due to a flexible spacer between the uridine residue and the perfluoroaryl azide group. Our results together with the data on bacterial ribosomes [7,8] imply that the upper part of helix 44 of the small subunit rRNA plays an universal role in the formation of the decoding site of bacterial and mammalian ribosomes and interacts with the A site-bound codon during the elongation and termination steps of translation.

Earlier, several hypotheses concerning the structural basis

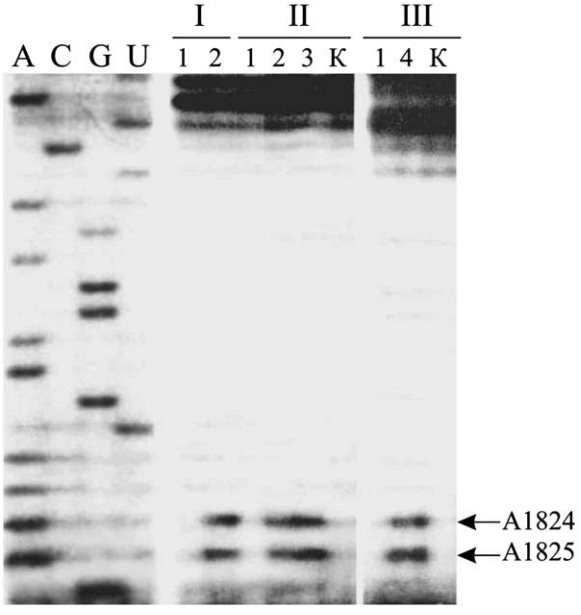

Fig. 4. Identification of 18S rRNA crosslinks sites to azido-mRNAs by reverse transcription. I, azido-pUUCUCAA; II and III, azido-pUUCUAAA. Extension of 5′-^{32}P-labeled primer (complementary to 18S rRNA positions 1830–1849) on the 18S rRNA isolated from the irradiated complexes. Lanes 1 correspond to binary mixtures of azido-mRNAs with 80S ribosomes; lanes 2, ternary complexes of 80S ribosomes with azido-mRNAs and tRNA^{Phe}; lanes 3 and 4, complexes obtained in the presence of eRF1 and eRF1 together with eRF3, respectively. Lanes K are control 18S rRNA samples isolated from 80S ribosomes irradiated without mRNAs and treated in the same way as the other samples. Lanes U, G, C, A are sequencing of the 18S rRNA isolated from placenta tissue. Positions of the reverse transcription stops caused by crosslinking are indicated.

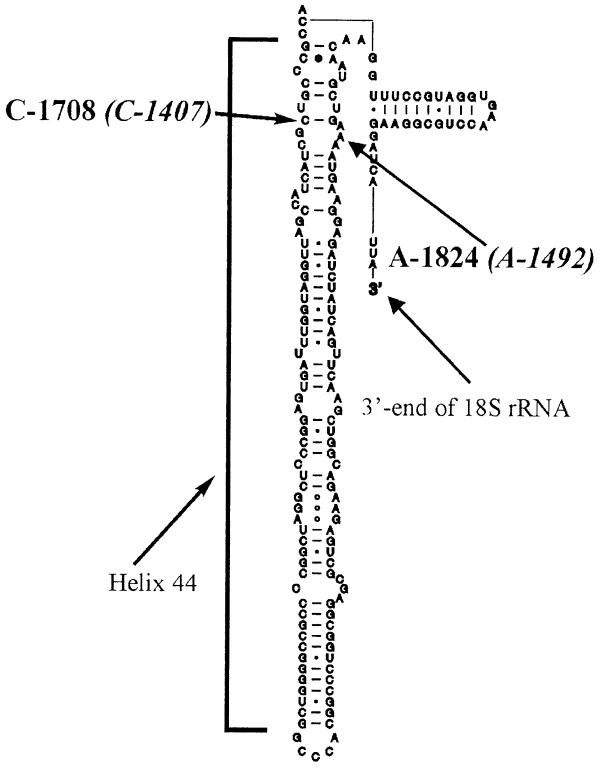

Fig. 5. Secondary structure of the 3′-terminal mini-domain of human 18S rRNA [27,28]. Nucleotides of *Escherichia coli* 16S rRNA corresponding to the marked nucleotides of 18S rRNA by their positions in the secondary structure are shown in italics and in brackets. Nucleotide C1708 of 18S rRNA corresponds to C1407 that had been found to be crosslinked to stop codon bound to the A site of bacterial ribosomes [6].

of stop codon recognition during termination were proposed (reviewed in [32]). Initially, bacterial RF2 was assumed to recognize the A site-bound stop codon directly (see [2]). Alternatively, it was suggested for prokaryotes that two fragments in 16S rRNA (in helices 34 and 44) together with the stop codon triplet form a unique complex that may be specifically recognized by RF2 [33]. If an analogous complex is formed between the A site-bound sense codon and 16S rRNA it should be distinguished by class 1 RFs. To achieve this discrimination, the spatial structure of the complexes should differ. However, at least in eukaryotes as shown here, the complexes look identical. In addition, there are no differences in the protein environment of the A site-bound sense and stop codons on mammalian ribosomes [11]. This implies that the specificity of stop codon recognition is brought by a class 1 RF, rather than by the ribosomal components. This is entirely consistent with the earlier conclusion that eRF1 and not the ribosome governs the stop codon recognition [34].

A hypothesis has been proposed that the A site-bound stop codon is recognized by the complementary ‘anticodons’ of two tRNA-like folds of the large subunit rRNA (namely, hairpin 69 of domain IV and hairpin 89 from domain V), and that RFs only assist in this recognition [19]. If interaction between the stop codon and the above-mentioned hairpins of 28S rRNA does occur, the conformationally flexible modifying group on the uridine of the A site-bound stop codon of azido-pUUCUAAA should reach the 28S rRNA. Our results show that a modified stop codon on the ribosomal A site is

unable to crosslink to the large subunit rRNA both in the presence or absence of eRF1 (Fig. 1). This implies that the 28S rRNA does not interact with the stop codon and, therefore, the hypothesis suggested in [19] most probably is not applicable at least for mammalian ribosomes.

As shown previously [10] and in this study, eRF1 does not prevent stop codon crosslinking to 18S rRNA, and 18S rRNA does not block the crosslinking of the stop codon to eRF1. In other words, the eRF1, the stop codon and the AA dinucleotide of 18S rRNA are positioned close to each other and potentially are able to form a ternary complex between the stop codon, eRF1 and the 18S rRNA fragment at the decoding site of the eukaryotic ribosome. This explains why mutations of both rRNA (see [13]) and eRF1 [35,36] cause alterations in stop codon recognition.

At the elongation step of protein synthesis, a ternary complex of prokaryotic ribosome composed of a codon–anticodon duplex between the mRNA and aminoacyl-tRNA associated with the conserved nucleotides of the 16S rRNA has been demonstrated (see [37]). Here, for mammalian ribosomes, we observed a close similarity between sense and stop codon interactions with the same AA dinucleotide of 18S rRNA. It was shown earlier that a modified guanosine residue at the first position of the A site-bound sense codon crosslinks to this 18S rRNA dinucleotide in the ribosomal complexes with a free A site or the A site occupied with aminoacyl-tRNA [24]. The resemblance or even identity of the interactions of the A site-bound stop and sense codons with their ligands indicates that the decoding site is not remarkably changed when aminoacyl-tRNA is substituted by eRF1. Previous data [10,11] and those presented here support the view [32] that the decoding site of the eukaryotic ribosome at the termination step of translation is likely to be composed of ternary complex between a stop codon, eRF1 and the AA dinucleotide of the 18S rRNA similarly to a prokaryotic decoding site. Consequently, eRF1 is in fact a functional mimic of aminoacyl-tRNA as suggested earlier [38,39].

Recent cryo-EM [40,41] and chemical probing [42] data on prokaryotic ribosomes charged with RF2 and a stop codon-containing mRNA are consistent with the view that the GGQ motif is located at the peptidyl transferase center of the ribosome as proposed earlier [43,44]. However, different nucleotides of a large subunit rRNA are implicated at elongation and termination steps of translation [45] indicating that tRNA mimicry seems to be functional rather than structural as discussed elsewhere [46].

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