The First Position of a Codon Placed in the A Site of the Human 80S Ribosome Contacts Nucleotide C1696 of the 18S rRNA as Well as Proteins S2, S3, S3a, S30, and S15†

Konstantin Bulygin,[‡,§] Laurent Chavatte,[‡,⊥] Ludmila Frolova,[∥] Galina Karpova,[§] and Alain Favre*[,‡]

Institut Jacques Monod, UMR 7592 CNRS−Universites Paris 7−Paris 6, 2 place Jussieu Tour 43, 75251 Paris Cedex 05, France, Institute of Chemical Biology and Fundamental Medicine, Siberian branch of The Russian Academy of Sciences, pr. Lavrentieva 8, 630090 Novosibirsk, Russia, Engelhardt Institute of Molecular Biology, The Russian Academy of Sciences, Vavilova 32, Moscow 119991, Moscow, Russia

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ABSTRACT: Messenger RNA analogues (42-mers) containing a GAC codon (aspartic acid) in the middle of their sequence followed by a s[4]UGA stop codon were used to identify the components of the human ribosomal A site in direct contact with the photoactivatable 4-thiouridine (s[4]U) residue. We compared the behavior of the nonphased ribosome−mRNA complex, (−)tRNA^{Asp}, to the one of the phased complex, (+)tRNA^{Asp}, in the absence and in the presence of eRF1, the eukaryotic class 1 translation termination factor of human origin. The patterns of cross-links obtained for the three complexes were similar to those previously reported for rabbit ribosomes [Chavatte, L., et al. (2001) *Eur. J. Biochem. 268*, 2896−2904]. Cross-links involving proteins S2, S3, S3a, and S30 were poorly dependent on the presence of tRNA^{Asp} and eRF1. Cross-linking to nucleotide C1696 of 18S rRNA occurred in all complexes, but its yield was at least two times higher in the phased complex with an empty A site than in the nonphased complex or when the A site was occupied by eRF1. In contrast, protein S15 cross-linked only in the phased complex in the absence of eRF1. The data obtained point to notable differences in organization of the decoding site between mammalian and prokaryotic ribosomes and to large internal mobility of the components of the tRNA (eRF1)-free A site.

One of the key steps of translation is the interaction of mRNA[1] codons with cognate tRNA anticodons on the ribosome because this ensures fidelity of protein synthesis. At present, the structure of the decoding site where this interaction takes place is known in detail for prokaryotic ribosomes from high-resolution X-ray crystallographic data (*1, 2*). Crystals of eukaryotic ribosomal subunits suitable for X-ray analyses still remain unavailable. One of methods applicable to study the positioning of mRNA on both pro- and eukaryotic (including mammalian) ribosomes is site-directed cross-linking (*3*−*5*). To date, the nature of the contacts between the components of the A, P, and E sites of human ribosome has been determined using short mRNA analogues, that is, derivatized oligonucleotides in which the nature and site of attachment of the (photo)reactive probe were varied (*6*−*11*). A comparison of these results with X-ray crystallographic data of prokaryotic ribosomes led to the conclusion that the mRNA codons in these sites are surrounded by nucleotides located in the same positions of the conserved parts of the small subunit rRNA secondary structure (*9, 11*). Yet, dramatic differences in the protein environment of the mRNA codons at the P and E sites of bacterial and mammalian ribosomes were observed. For example, in the case of the human ribosome, protein S26, which has no homologue among prokaryotic ribosomal proteins, was located close to the P and E site bound mRNA codons (*10*).

Positioning of codons bearing a probe attached by a 10 Å linker to their first nucleotide in the human 80S ribosome A site revealed that the environment at the codon with respect to the 18S rRNA and r-proteins (rPs) does not differ whether the codon is sense or nonsense (*12, 13*). In these assays, the release factor eRF1, alone or together with eRF3, did not affect the arrangement of the A site bound stop codon on the ribosome. These data pointed to a resemblance between the ternary complexes formed at the elongation and termination steps of protein synthesis and supported the view that eRF1 may be considered as a functional mimic of aminoacyl-tRNA. A refined picture emerges if one uses a "zero length"

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* Corresponding author. Phone: (33) 1 44 27 42 24. Fax: (33) 1 44 27 57 16. E-mail: favre@ijm.jussieu.fr.

‡ Institut Jacques Monod.

⊥ Present address: Cleveland Clinic Foundation, 9500 Euclid Ave., no. NC-10, Cleveland, OH 44195

∥ Engelhardt Institute of Molecular Biology.

§ Institute of Chemical Biology and Fundamental Medicines.

[1] Abbreviations: mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; tRNA^{Asp}, aspartate-specific tRNA transcript; tRNA^{fMet}, formylmethionine-specific initiator tRNA; eRF1, eukaryotic release factor 1; s[4]U, 4-thiouridine; rPs, ribosomal proteins; TP40, total proteins from 40S subunits; RNase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

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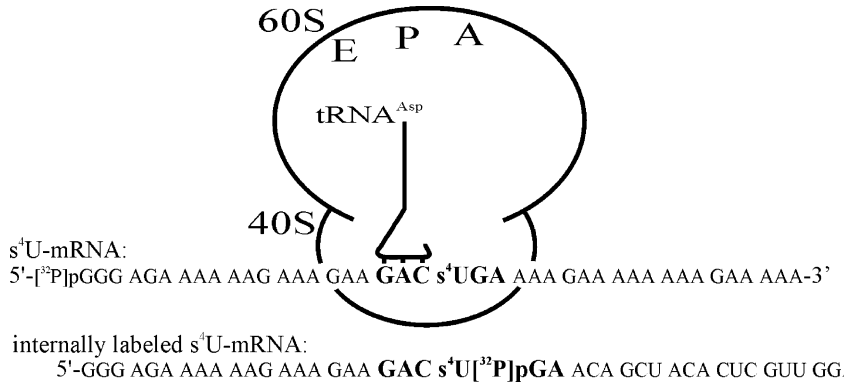

FIGURE 1: 80S ribosomal complex 2 and sequences of mRNA analogues.

photo-cross-linker such as 4-thiouridine (s^4U), a close analogue of uridine that provides information on the ribosomal components in direct contact with this nucleotide (*4*, *14*). Using 42-mer mRNAs containing a unique s^4U residue placed at the first position of the codon located in the A site of the rabbit ribosome, it was shown (*15*−*17*) that (i) the same pattern of cross-links with rPs and 18S RNA is obtained whether the codon is stop or sense when the A site is free and (ii) when the A site is programmed by a stop codon (or the cryptic stop codon UGG), eRF1 binds more tightly to this site than in the presence of sense codons and contacts the s^4U residue by a conserved sequence of its N-terminal domain.

In the work cited above, the nature of the ribosomal components in contact with the s^4U residue was not determined. Here, we have characterized the corresponding cross-links: the first position of a codon in the A site of the human ribosome contacts C1696 of the 18S rRNA located in helix 28, which forms the 40S subunit neck, and a number of rPs were identified.

EXPERIMENTAL PROCEDURES

Ribosomes, Yeast $tRNA^{Asp}$, and eRF1. Ribosomal 40S and 60S subunits were isolated from human placenta (*18*). The subunits were first reactivated by incubation in buffer A (50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol) at 37 °C for 10 min and then mixed at a 40S/60S ratio of 1:1.3 (assuming that 1 A_{260} unit corresponds to 50 pmol of 40S or 25 pmol of 60S subunits (*19*)). Yeast $tRNA^{Asp}$ was obtained by in vitro T7 transcription (*15*). The full-length human eRF1 carrying a His_6-tag at the N terminus was expressed in *Escherichia coli*, purified, characterized, and assayed as described previously (*20*, *21*).

Preparation of mRNA Analogues. Messenger RNA analogues (s^4U-mRNA and biotinylated s^4U-mRNA) with the sequence 5′-[^{32}P]pGGG AGA AAA AAG AAA GAA **GAC s^4UGA** AAA GAA AAA AAA GAA AAA-3′ were prepared by in vitro T7 transcription from synthetic DNA template and 5′-end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase according to the procedures previously described (*15*). The ribonucleotide triphosphate mixture was composed of ATP, CTP, GTP, and s^4UTP (Ambion) instead of UTP. Replacement of UTP by s^4UTP introduced the s^4U probe in the single position available. For synthesis of biotinylated s^4U-mRNA used to purify cross-linked rPs for mass spectra analysis, biotin-11-ATP (Perkin-Elmer) was added to a transcription mixture in a molar biotin-11-ATP/ATP ratio of 1:20. Taking into account the fact that efficiency of incorporation of biotin-11-ATP and ATP are nearly the same (according to the manufacturer's protocol) in T7 transcription and that s^4U-mRNA has 30 adenine residues (Figure 1), one can conclude that every biotinylated mRNA molecule (Bio-s^4U-mRNA) contains from 1 to 1.5 randomly distributed biotin residues.

The internally labeled s^4U-mRNA used to identify cross-linked rPs with the sequence 5′-GGG AGA AAA AAG AAA GAA **GAC s^4U[^{32}P]pGA** ACA GCU ACA CUC GUU GGA-3′, was prepared as previously described (*16*).

Cross-Linking Procedure. Reaction mixtures (20 μL), containing 0.2 μM reassociated 80S ribosomes, 0.1 μM ^{32}P-labeled s^4U-mRNA, 3 μM $tRNA^{Asp}$ (where specified), and 3 μM eRF1 (where specified) in buffer A, were incubated for 1 h at 37 °C (*15*). When biotinylated s^4U-mRNA was used instead of s^4U-mRNA, 0.1% Triton X-100 was added to the reaction mixture. Each sample was irradiated in a siliconized glass capillary for 30 min at 4 °C. The light source was a HBO 150 W superpressure mercury lamp providing near-ultraviolet light (>320 nm) placed 5 cm from the sample. Shorter wavelengths were cut off using an MTO J320A filter (Specivex). Dissociation of irradiated 80S ribosomes into their subunits was carried out as described (*6*).

Analysis of Cross-Linked 18S rRNA. Ribosomal RNAs, tRNA, and mRNA were recovered from reaction mixtures by phenol deproteination as described (*22*). Cross-linking to 18S rRNA was analyzed by 5% PAGE followed by phosphorimaging. Determination of the 18S rRNA region containing the site(s) of cross-linking to mRNA was performed using specific cleavage of the cross-linked 18S rRNA with RNase H (BioLabs) in the presence of deoxy 20-mers complementary to various rRNA sequences (*23*). The RNA fragments obtained were characterized and purified by 5% PAGE. The cross-linked nucleotide in the 18S rRNA was identified by a primer extension approach (*24*). Fragment 1641−1869 excised from the modified 18S rRNA (after RNase H treatment) was used as a template for reverse transcriptase.

Analysis of the Modified Proteins. Irradiated reaction mixtures containing 80S ribosomes, mRNA analogues, and (where specified) $tRNA^{Asp}$ and eRF1 (see section Cross-Linking Procedure) were diluted with Laemmli sample buffer and resolved by 10% or 15% SDS−PAGE as indicated (*25*). Each mRNA−rP cross-link was digested with micrococcal nuclease (BioLabs), thus removing the mRNA but leaving

a modified 3′ phosphate nucleoside attached to the rPs. Analysis of cross-linked proteins was performed according to Madjar et al. (*26*).

To separate the cross-linked rPs from un-cross-linked rPs, a biotin−streptavidin technique was used. The irradiated reaction mixture (750 μL) containing 150 pmol of 80S ribosomes was diluted with 250 μL of denaturing solution (1.2% SDS, 20 mM EDTA, and 6 mM DTT) and incubated for 10 min at 55 °C. Then, 30 μL of streptavidin−agarose (Sigma) (prewashed with 500 μL of water) was added, and the mixture was incubated for 30 min at room temperature under gentle agitation. After centrifugation (3000 rpm, 10 s), the supernatant was removed. The precipitate was washed 3 times with 500 μL of 20 mM Tris-HCl, pH 7.5, containing 2% SDS and 3 times with 500 μL of water. Finally, the beads were incubated with 10 μL of RNase T1 mixture (4 U of T1 (Ambion) in buffer A) for 30 min at 37 °C to recover modified rPs. The supernatant was analyzed by 15% SDS−PAGE, and the gel was stained with Coomassie brilliant blue. The band corresponding to the 19-kDa rP was excised and analyzed by mass spectrometry. Mass spectra were obtained on a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems) equipped with a pulsed nitrogen laser (337 nm, 3 ns pulse). The operating parameters for reflectron include accelerating voltage (20 kV), grid voltage (75%), guide wire voltage (0.005%), and 100 laser shots per spectrum. Angiotensin I, des-Arg^1-bradykinin, Glu^1-fibrinopeptide B, and neurotensine were used for external calibration. Monoisotopic masses were used with deviation for mass assignment within ±0.5 Da. Samples were digested with trypsin (15 mg/mL) at 37 °C overnight before analysis. MALDI-TOF spectra were analyzed with the program PeptIdent (http://au.expasy.org/tools/peptident.html).

RESULTS

mRNA Analogues, 80S Ribosomal Complexes, and Cross-Linking Procedures. Synthetic analogues of mRNA (either 5′- or internally ^{32}P-labeled) containing a single s^{4}U residue in the selected position (Figure 1) were used in photo-cross-linking experiments with human ribosomes. These analogues are purine-rich to minimize the formation of secondary structure and self-association. In their middle part, they contain a GAC codon cognate to tRNA^{Asp} followed by a stop codon s^{4}UGA. In separate experiments, an mRNA analogue was used bearing 1−1.5 biotin residues per chain (Bio-s^{4}U-mRNA, Figure 1), randomly introduced by in vitro T7 transcription in the presence of biotin-11-ATP.

In all three complexes studied here, ribosomes were in 2-fold excess over mRNA to ensure full mRNA binding. Complex 1 "unphased" was obtained by incubating 80S ribosomes and the mRNA analogue, complex 2 "phased" (Figure 1) was formed by addition of tRNA^{Asp} transcript to complex 1, while complex 3 was generated by addition of both tRNA^{Asp} and eRF1 to complex 1. Incubation of the complete mixture was performed at 37 °C in the presence of 10 mM Mg^{2+}. Addition of tRNA^{Asp} targets the GAC codon to the P site since tRNAs have much higher affinity for this site than for the A or E sites (*27*, *28*). Thus, in complexes 2 and 3, the s^{4}UGA codon was located in the A site placing the s^{4}U in position +4 with respect to the first nucleotide of the P-site bound codon. Whereas the A site is empty in complex 2, it is almost fully occupied by eRF1 in complex 3. Assuming that human and rabbit ribosomes behave similarly and owing to the dissociation constant (~0.1 μM) previously determined in the latter case (*17*), 90%−95% of the ribosomal A sites should contain eRF1 under the conditions used here for the formation of complex 3. Earlier, it was shown that mild UV irradiation of complexes of rabbit 80S ribosomes with the same s^{4}U-mRNAs results in cross-linking of the thiouridine residue to eRF1 (in complex 3) and to components of the ribosomal mRNA-binding center (in complexes 1, 2, and 3). In the latter case, both 18S rRNA and ribosomal proteins were modified (*15*). To examine cross-linking of the 5′-[^{32}P]-labeled mRNA analogue to the ribosomal subunits, the irradiated 80S ribosomal complexes were dissociated, and the subunits were separated on a sucrose density gradient (not shown). As anticipated, the ^{32}P label was associated only with 40S subunits in all three complexes.

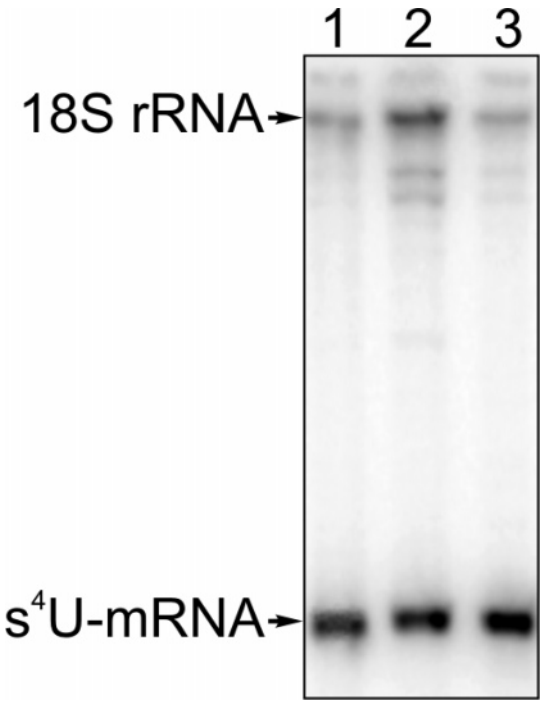

FIGURE 2: Analysis of the cross-linking of ^{32}P-labeled s^{4}U-mRNA to 18S rRNA in complexes 1, 2, and 3 (lanes 1−3, respectively) by 5% PAGE. Positions of 18S rRNA and s^{4}U-mRNA are indicated.

Cross-Linking of mRNA Analogues to 18S Ribosomal RNA. To analyze cross-linking to 18S rRNA, 5′-^{32}P-labeled s^{4}U-mRNA was used. Analysis of RNA isolated from the irradiated complexes by denaturing 5% PAGE (Figure 2) showed that radioactivity associated with the 18S rRNA was about 2.3%, 5.0%, and 1.7% for complexes 1, 2, and 3, respectively, relative to the total radioactivity within each lane. Taking into account the 2-fold excess of ribosomes over mRNA analogue used here, the actual yield of the modified 18S rRNA was two times lower than indicated above.

To determine the nature and position of 18S RNA residues involved in the cross-link(s), the rRNA was isolated from the irradiated complexes and digested with RNase H in the presence of deoxyoligomers (20 nt) complementary to various regions. The resulting rRNA fragments were separated by denaturing PAGE. "Stains all" staining showed their location on the gel, and autoradiography indicated which

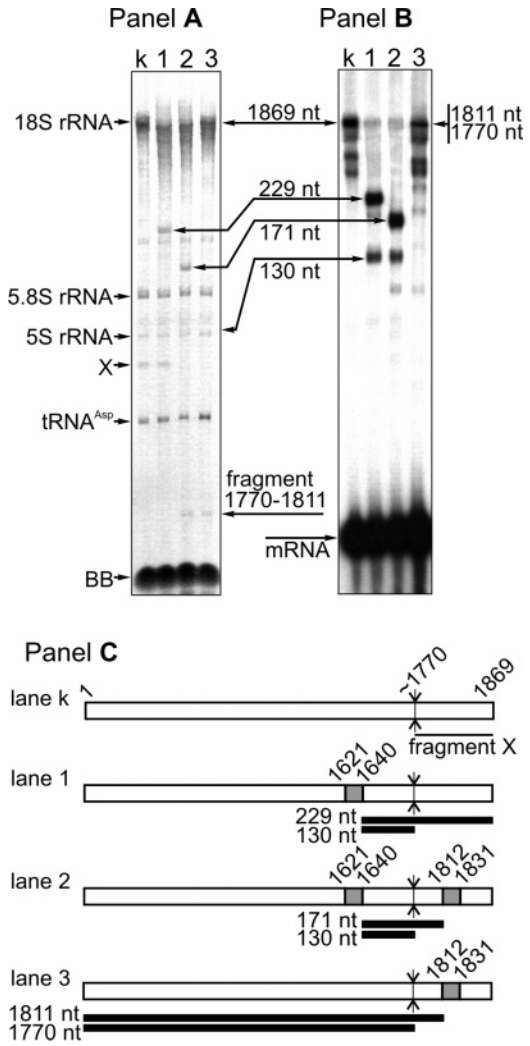

FIGURE 3: RNase H digestions of 18S rRNA cross-linked to s^{4}U-mRNA in complex 2 in the presence of deoxyoligomers complementary to various 18S rRNA regions (lanes 1−3). Lanes k contains 18S rRNA treated with RNase H without deoxyoligomers. Positions of 18S rRNA, 5.8S rRNA, 5S rRNA, tRNA, and 18S rRNA fragments (the expected lengths are marked; the length of the cross-linked mRNA analogue has been subtracted) are indicated. BB indicates Bromophenol Blue dye. Panel A contains a 5% gel stained with "Stains all"; panel B contains an autoradiogram of the gel; panel C contains a scheme of the hydrolysis of cross-linked 18S rRNA. Black bars represent ^{32}P-labeled 18S rRNA fragments. The same results were obtained for complexes 1 and 3 (respective gels and autoradiograms are not shown).

rRNA region was cross-linked to the [^{32}P]-labeled mRNA analogue (Figure 3). This approach proved fruitful because it allowed detection of the cross-linked rRNA fragment(s) even when the yield of modification was low. Digestion of 18S rRNA (isolated from complex 2) with RNase H in the presence of a deoxyoligomer complementary to the 1621−1640 18S rRNA sequence showed that the site(s) of cross-linking lies within a 229 nt long (1641−1869) 3′-terminal fragment (Figure 3, lane 1). A specific cut at position 1811 using deoxyoligomer (1812−1831) showed that only the 5′ rRNA part (1−1811) retained the radioactive label (Figure 3, lane 3). Digestion in the presence of both deoxyoligomers used above led to a single 170 nt fragment (1641−1811) as shown Figure 3 (lane 2). It is known that position 1770 located near the apical loop of helix 44 of 18S rRNA is very sensitive to placental tissue endonuclease digestion probably

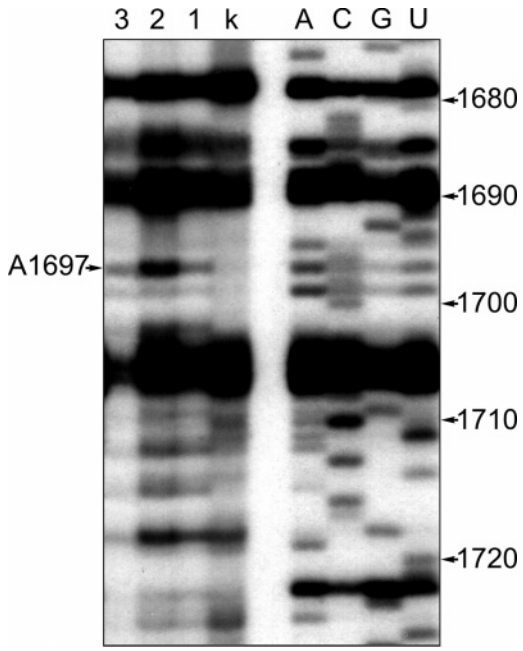

FIGURE 4: Identification of 18S rRNA cross-link sites to s^{4}U-mRNA by reverse transcription. Extension of 5′-^{32}P-labeled primer (complementary to 18S rRNA positions 1812−1831) on the 229 nt 18S rRNA fragment (1641−1869) obtained after RNase H digestion of 18S rRNA (see Experimental Procedures) recovered from irradiated complexes 1, 2, and 3 (lanes 1−3, respectively). Lane k contains the same for 18S rRNA fragment 1641−1869 recovered from irradiated 80S ribosomes without s^{4}U-mRNA. Lanes A, C, G, and U contain sequencing of the 18S rRNA isolated from placenta tissue. Positions of the reverse transcription stop (A1697) and 18S rRNA nucleotides (on the right) are indicated.

as a consequence of exposure at the surface of the 40S subunit. In isolated 40S subunits, the 18S rRNA is partially cleaved at this position (*9*, *11*) generating a ∼100 nt long fragment, which is clearly visible on the stained gel (Figure 3A, lanes k and 1), while no corresponding radioactive X-mRNA cross-link (∼140 nt) could be detected. It can thus be safely concluded that the site(s) of cross-linking is (are) located within the 1641−1770 fragment of the 18S rRNA (Figure 3B, lanes 1 and 2), and this statement extends to complexes 1 and 3 (data not shown).

The site of cross-link on 18S rRNA was determined by a primer extension approach. Due to the low yield of cross-linked rRNA with respect to total 18S rRNA extracted from irradiated complexes, no stops were detected using a primer complementary to positions 1812−1831 (not shown). Primer extension was thus performed on a fragment obtained after cleavage at position 1641 (see above). The corresponding radioactive fragment (271 nt), consisting of unlabeled 18S rRNA fragment (229 nt) and 5′ end-labeled s^{4}U-mRNA (42 nt), migrates more slowly than the unlabeled fragment (229 nt) (compare lanes 1 in panels A and B of Figure 3). It was excised and eluted from the gel. Using a primer complementary to sequence 1812−1831 showed that whatever the complex considered, primer extension was blocked at a single position, A1697 of 18S rRNA (Figure 4) yielding a strong signal for complex 2 and much weaker signals for complexes 1 and 3. The cross-linking site is generally assumed to be 5′ to the primer extension stop pointing to C1696 located in helix 28 as the single cross-linked nucleotide of 18S rRNA.

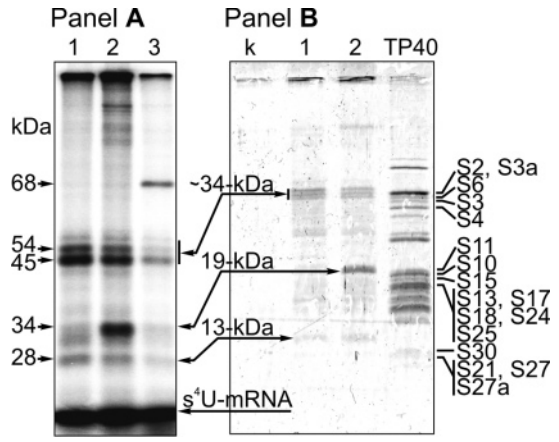

FIGURE 5: Analysis of ribosomal proteins cross-linked to s^{4}U-mRNA. Panel A shows the cross-linking patterns of irradiated complexes 1, 2 and 3 (lanes 1, 2, and 3, respectively) in an autoradiogram of the gel after separation of cross-linked proteins by 10% SDS−PAGE. Panel B shows the silver-stained gel after separation of cross-linked proteins by 15% SDS−PAGE. Proteins were affinity purified by the biotin−streptavidin technique (before electrophoresis, the RNA moieties of the cross-linked proteins were hydrolyzed by RNase T1). The molecular masses of the cross-linking products are provided on the left (panel A). Positions of some ribosomal proteins are indicated on the right (panel B) according to refs *45* and *46*). The position of s^{4}U-mRNA and the molecular masses of cross-linked proteins after subtraction of the mass of the covalently attached s^{4}U-mRNA are indicated in the middle. Lane k contains the same products as lane 2, but standard s^{4}U-mRNA was used instead of biotinylated s^{4}U-mRNA. Lane TP40 contains total 40S ribosomal protein.

Nature of the Cross-Linked Ribosomal Proteins (rPs). Analysis by SDS−PAGE of the mRNA-rP cross-links obtained with human ribosomes yielded patterns very similar to those previously reported for rabbit ribosomes (compare panel A in Figure 5 (this study) and Figure 4 (*17*)). It clearly shows the 34 kDa cross-link characteristic of complex 2 and the 68 kDa cross-link (mRNA−eRF1) formed exclusively in complex 3, together with efficient quenching of cross-link formation in the latter complex. Total amount of radioactivity associated with rPs in complex 3 was more than 4 times less than that in complex 2 and radioactivity corresponding to the 34 kDa cross-link in complex 3 was reduced about 20 times as compared with that in complex 2 (compare lanes 2 and 3 in Figure 5A). Radioactivity associated with rPs in complex 2 was about 2.2% for 54 kDa, 7.2% for 45 kDa, 7.1% for 34 kDa, and 1.6% for 28 kDa cross-links of the total radioactivity within the lane 2 (Figure 5A), whereas for 34 kDa cross-link in complex 1, it was 0.1% (Figure 5A, lane 1). In complexes 1 and 2 (Figure 5), one can notice the absence of "weak" 68 and 23 kDa cross-links occasionally observed with some rabbit ribosome preparations and presumably due to contaminant nonribosomal proteins (*15*, *17*).

To determine the nature of the cross-linked rPs, we used biotinylated mRNAs and verified first that the biotin residues modified neither the pattern of the cross-links nor their yields (data not shown). After dissociation of the irradiated complexes in Laemmli buffer, the biotin-containing mRNAs were immobilized on streptavidine−agarose (in parallel experiments) and thoroughly washed to remove non-covalently bound proteins and rRNA. Digestion by RNase T1 liberates proteins attached to a small mRNA oligonucleotide

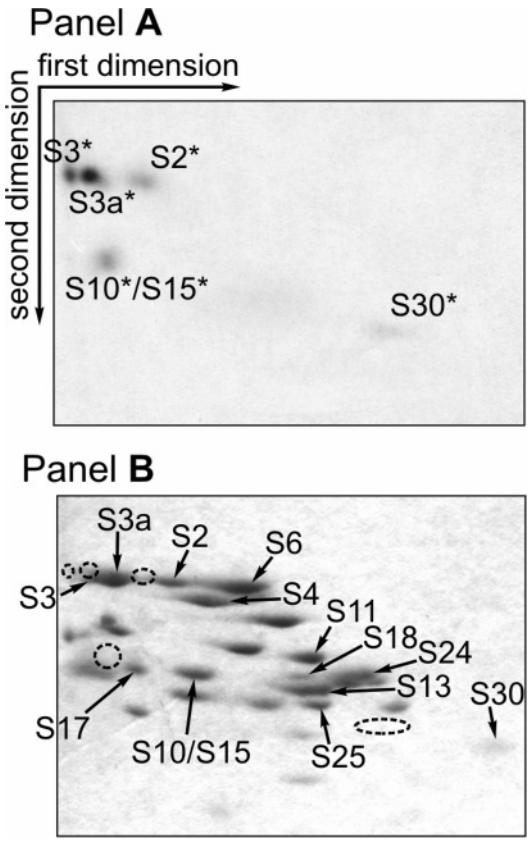

FIGURE 6: Analysis of modified proteins cross-linked in complex 2 with internally labeled mRNA after micrococcal nuclease digestion by 2D-electrophoresis. Panel A shows an autoradiogram of the gel; panel B shows the Coomassie stained gel after separation of total 40S ribosomal proteins. Positions of some ribosomal proteins are indicated according to ref *26*. Locations of radioactive spots corresponding to the cross-linked proteins (panel A) are marked with dotted lines on panel B.

ACXG, where X stands for modified s^{4}U. These rPs were resolved by SDS−PAGE (Figure 5B). The correspondence between the mRNA−rP cross-links and the free modified rPs is shown Figure 5. The small RNA fragment (∼1.4 kDa) that remains attached to the modified rPs decreases their mobilities in a predictable way (*29*−*31*). Accordingly (Figure 5B, compare lanes 1, 2, and TP40), proteins S2, S3a, S6, S3 and S4 are candidates for the modified 34 kDa rP doublet. The 19 kDa rPs may correspond to S10, S11, S13, S15, S17, S18, S24, and S25. Finally, candidates for the 13 kDa modified rP are S21, S27, S27a, or S30.

The cross-linked proteins were identified more precisely by two-dimensional (2D) gel electrophoresis (Figure 6). Internally labeled s^{4}U-mRNA was used for this purpose. After photo-cross-linking and treatment with micrococcal nuclease (*16*), a single nucleotide X[^{32}P]p (X stands for modified s^{4}U) remains attached to the cross-linked rPs. This slightly reduces protein mobilities in the first dimension, which is performed under alkaline conditions where basic proteins remain positively charged, an effect that is expected to be more pronounced for small proteins. In the second dimension (slightly acidic but in the presence of SDS), the proteins are resolved according to their masses and addition of a single nucleotide will have negligible effect. Taking into account the data of the 2D-PAGE (Figure 6) and of the SDS−PAGE (Figure 5B), one can conclude that proteins S2,

Table 1: MALDI Analysis of Peptides Obtained by Trypsin Digestion of the 19 kDa S15 Protein[a]

measured mass[b] (Da)	SwissProt/ TrEMBL mass[c] (Da)	mass difference[d] (Da)	matching peptide	position in protein S15
617.3291	617.4021	0.073	FIPLK	140−144
714.4137	714.3933	−0.0203	KFTYR	13−17
714.4137	714.4482	0.0345	RRLNR	42−46
745.4079	745.3727	−0.0351	AEVEQK	1−6
853.545	853.5254	−0.0195	KQHSLLK	51−57
1001.5484	1001.5626	0.0142	AEVEQKKK	1−8
1332.7768	1332.6879	−0.0888	HGRPGIGATHSSR	127−139

[a] Protein S15 Sequence (RS15_HUMAN entry name in SwissProt/ TrEMBL) is AEVEQKKKrt frKFTYRgvd ldqlldmsye qlmqlysarq rRRLNRglrr KQHSLLKrlr kakkeappme kpevvkthlr dmiilpemvg sm-vgvyngkt fnqveikpem ighylgefsi tykpvk HGRP GIGATHSSRF IPLK; peptides detected are indicated with underlined capital characters. [b] Determined by MALDI. [c] Predicted from SwissProt/TrEMBL database. [d] Calculated as SwissProt/TrEMBL mass minus measured mass.

S3, and S3a cross-linked to the s^{4}U-mRNA in all types of complexes. On the other hand, S30 can be identified unambiguously in the 2D gel (Figure 6) because it is the only protein located on the right of the labeled 13 kDa rP. Obviously, neither proteins S11, S13, S18, S24, nor S25 could be the 19 kDa rP, since the presence of an additional mononucleotide will be unable to trigger the strong shift observed in the second dimension of the 2D-PAGE (Figure 6B) (*31*). Protein S17 can be excluded since its shift relative to the 19 kDa protein in the first dimension of the 2D gel (Figure 6B) was smaller than expected from the SDS−PAGE (Figure 5B, lanes 2 and TP40). Hence the 19 kDa protein is either S10 or S15. MALDI-TOF mass spectral analysis of the 19kDa rP, isolated as described above using biotinylated s^{4}U-containing mRNA, allowed its unambiguous identification as protein S15 (see Table 1).

DISCUSSION

Phasing mRNA on the 80S Ribosome. Accurate phasing of the mRNA on the ribosome is achieved by addition of a tRNA able to target its cognate codon to the P site (*27*, *28*). Accordingly a given cross-link can be assigned to a position of the mRNA track only if it is stimulated by addition of the phasing tRNA, and in general no conclusion can be made for tRNA-independent cross-links. Successful phasing of mini-mRNAs (hexa- to heptamers) was achieved using tRNA^{Phe} (*E. coli*) since its addition strongly stimulates mRNA−ribosome complex formation (*12*, *13*). It was observed however that tRNA^{Val} (*E. coli*) is much less efficient than tRNA^{Phe} to trigger binding of the corresponding 13-mer mRNAs in contrast to the expected strength of codon−anticodon interaction (*11*). Thus distinct tRNAs do have widely different phasing abilities, and this is certainly related to their codon-independent binding to the P site (*28*). The lack of phasing (*15*) observed with 14-mer mRNAs (containing an As^{4}UG codon) upon addition of tRNA^{fMet} (*E. coli*) may essentially reflect the weak binding of this tRNA to the P site (*14*).

The 42-mer mRNA used in the present study binds efficiently to 80S ribosome in the absence of tRNA^{Asp}, and as stressed in Results, its behavior in the presence of ribosomes of human or rabbit origin is quasi-identical. Evidence for accurate phasing relies on the following facts: (i) The 34 kDa mRNA−rP corresponding to the 19 kDa rP (identified here as S15) is weakly detected, if at all, in complex 1, while it becomes one of the major cross-links in complex 2. In the latter complex, its formation is position-dependent (Table 2). (ii) In the presence of tRNA^{Asp} transcript, the mRNA associates with the ribosome to form a 1:1 complex with an apparent subnanomolar dissociation constant (*17*). (iii) Formation of an eRF1−mRNA cross-link is observed only when the A site is programmed by one of the stop codons or the "cryptic" stop codon UGG (*17*). A closely related approach to the one described here was performed with 51−54-mer mRNAs. With one partial exception, all mRNA−18S rRNA cross-links identified showed no tRNA dependence (*22*, *32*); there are several differences between these studies, aimed at identifying 18S rRNA residues located in the mRNA track, and our present work: (i) multiple s^{4}U substitutions may lead to a complex picture masking possible phasing; (ii) tRNA^{Gly} (*E. coli*) was used to phase these systems, and so far it is unknown whether this tRNA binds efficiently to the P site; (iii) the mRNAs were G- and C-rich (∼40% and 30%, respectively), which may lead to the formation of stable secondary structures possibly preventing the interaction of tRNA^{Gly} with its GGG codon; (iv) a 4-fold excess of mRNA over ribosomes was used possibly favoring multiple (partially folded) mRNA binding to the mRNA track. In summary, under the conditions generally used with mini-mRNAs appropriate choice of the phasing tRNA appears to be crucial. This condition is obviously true for longer mRNAs, which should be devoid of secondary structure and preferentially studied in the presence of excess ribosome. The mRNA−ribosome system proposed here thus appears to be an adequate reference system to study the role of tRNA in phasing mRNAs of various lengths and sequences on the 80S ribosome.

The Eukaryotic 80S Ribosomal A Site. Characterizing a structure using (photo) cross-linking agents provides a partial picture because each probe will react only with accessible acceptor groups. For example, photoexcited s^{4}U should closely approach a putative acceptor group (3−4 Å) with a favorable orientation of the C4−S bond with respect to the acceptor bond (*14*, *33*). Here s^{4}U is an intrinsic part of the mRNA and will be considered as a "zero-length" cross-linker. The mini-RNAs equipped with (photo)reactive probes linked to a defined mRNA position by a flexible spacer (up to 14 Å) will be called "midrange" cross-linkers. Whatever the complexes considered (phased or unphased) and the probe used, a first salient feature is that cross-linking to the 60S subunit appears insignificant and is not stimulated by the presence of a stop codon in the A site (*13* and this work). This observation strongly disproves the hypothesis that direct contact between the large subunit rRNA and the A site bound codon may act as a signal for translation termination (*34*).

In phased complexes, the s^{4}U residue at position +4, s^{4}U-(+4), crossreacts (∼5%) with a single nucleotide C1696 of 18S rRNA yielding a cross-link not detected previously (*32*) for reasons discussed above. Midrange cross-linkers revealed that position +4 of mRNA can approach G1702 (*6*) and the A1823−1825 track (*9*, *13*, *35*) at distances lower than 14 Å, and our study indicates that it should transiently contact C1696 (see below). The data on cross-linking of 40S rPs to the zero-length or to midrange probes are summarized in Table 2 and are in good agreement, with the notable

Table 2: Ribosomal Proteins Crosslinked to Various Positions of the A Site Bound Codons[a]

		position			
MW, kDa[b]	nature	+4	+5	+6	+7
34	S2	+			
		⊕			⊕
	S3a	+	+[c]	+[c]	+[c]
		⊕		⊕	⊕
	S3	+			
		⊕[d]	⊕	⊕	⊕
19	S15	+	+	(+)	
		⊕	⊕	⊕	⊕
13	S30	(+)	+	+	+
		⊕			*e*

[a] + and (+) indicate zero-distance strong and weak cross-links to a given rP (this work and ref *16*); ⊕ indicates midrange (up to 14 Å) cross-links obtained with mini-mRNA derivatives (*7*, *11*, *30*, *44*). [b] Molecular masses according to ref *16*. [c] The data do not discriminate which of the three proteins (S2, S3a, S3) are effectively cross-linked. [d] Position +3 and +4. [e] Not detected.

exception of S30. All these cross-links are found with practically invariant yields whether the A site is occupied by a stop or sense codon (*12*, *13*, *16*, *31*), thereby showing that stop codons do not exhibit specific interactions with A site components. A significant feature is that $s^4U(+4)$ is able to cross-react with multiple targets (one nucleotide, five rPs). Formation of multiple cross-links was encountered previously in RNA structures every time s^4U was localized in or near flexible loops (*33*). An important conclusion is therefore that in complex 2, components of the 40S subunit A site and $s^4U(+4)$ are endowed with considerable conformational flexibility with respect to each other. Consistent with this view, high Mg^{2+} concentrations (>15 mM) expected to make the 80S structure tighter were shown to quench formation of the cross-links (*15*).

In complex 1, the s^4U residue is expected to occupy several positions, including unreactive ones. This explains why formation of s^4U induced cross-links occurs with roughly half-yield (relative to complex 2). However, S15 becomes unreactive (see Table 1 in ref *17*) suggesting that its "acceptor group" becomes accessible only as a consequence of a local conformational change triggered by occupancy of the P site by the phasing tRNA.

In complex 3 obtained upon saturation of complex 2 by eRF1 (Figures 2 and 5), most cross-links exhibit a more than 3 times reduced yield and formation of the S15 cross-link is abolished. This is likely due to restriction of internal motions of A site components. From the data available so far (*17*), the quenching effect appears to be independent of the nature of the A site codon (whether stop or sense). This suggests that restriction of internal motions impeding cross-link formation in the 40S subunit is mainly due to contacts of eRF1 with its 60S partner components of the A site and is only little influenced by eRF1−stop codon interactions.

Conservation of the Decoding Site between Prokaryotes and Eukaryotes. Crystallographic analysis of 70S prokaryotic ribosome and its 30S and 50S subunits has shown that all the major functional features consist of RNA elements and that rPs appear to serve as struts, linkers, and support (*1*, *2*, *36*−*39*). The decoding center is located between the body and head of the small subunit and consists essentially of 16S RNA elements that show remarkable conservation in agreement with the universality of the decoding process. It involves notably the upper part of helix H44, together with other conserved elements from distinct 16S RNA domains. The upper bulge of H44 and notably nucleotides 1492 and 1493 are components of the A site and are involved in stabilization of cognate codon−tRNA anticodon interaction by forming H-bonds with the 2′-OH groups of the A codon (*40*). The P site involves the upper part of H44 and notably C1400 and U1498 (*1*, *2*). The cross-linking data obtained with mini-mRNA derivatives (*6*, *9*, *13*, *35*) show that there is close resemblance in organization of the decoding center in bacterial and mammalian ribosomes (Figure 7).

A first notable difference however is that in the 80S ribosome several rPs yield zero-distance cross-links to positions +4, +5, and +6 of the mRNA (Table 2) while in the corresponding prokaryotic system these positions are unreactive toward rPs (*4*). Only S12 is located close to the A site in the 30S subunit (*1*, *2*) and its nonreactivity could be due to lack of a suitable acceptor group. Among the "reactive" rPs found in the 80S ribosome, S3a and S30 have no prokaryotic counterparts in contrast to S2, S3, and S15 (*41*). In the 40S subunit, the position of proteins S2 and S3 in the vicinity of the decoding site are very similar to those of their homologues (S5, S3) in the 30S subunit (*42*). S19, the prokaryotic homologue of S15, has a core located in the head and a tail that extends toward the decoding site but remains at a large (~40 Å) minimal distance of the A site codon (*1*, *2*, *37*). Our finding that S2, S3, and S15 cross-react indicates that they contain chain expansions bringing some of their residues in close proximity to the bases of the A site codon. In any case, our data suggest that rPs play an increased role in the eukaryotic A site as compared to the corresponding prokaryotic site.

A second more subtle difference concerns the formation of mRNA−rRNA cross-links. We show here that in 80S ribosomes, $s^4U(+4)$ crossreacts exclusively with C1696 in helix H28 while in 70S ribosomes $s^4U(+4)$ cross-links to the 1402−1415 region of 16S rRNA, most likely to nucleotide 1402 (*43*). On the other hand, C1395, the conserved 16S rRNA nucleotide corresponding to C1696 in helix 28 is the target of downstream mRNA positions from +6 to +9 (*43*). H28 is the only RNA helix connecting the body and head of the 30S subunit and appears to behave as a flexible hinge in relation with the "switch" helix H27, which packs near the upper part of helix H44 (*36*). Comparison of the path of either a 6-mer or a 36-mer mRNA in crystallographic structures of 70S ribosome containing tRNAs in both the A and P site (*1*, *2*) shows that while the P site mRNA codons are similarly positioned, the mRNA A site codons adopt different conformations and orientations with respect to the H44 large bulge. Hence in solution and in the absence of tRNA in the A site, large motions of components of the A site and mRNA codons are expected to occur. First, transient winding of helix H28 around its axis will expose C1395 in a position suitable for cross-link formation. Second, the mRNA bases have to orient so as to face C1395, and this will be possibly accompanied by an upstream sliding of mRNA positions +7 to +9 for the corresponding bases to contact C1395. In the 80S ribosome, the conserved bulge of helix 28 (Figure 7), including residue C1696, may well serve

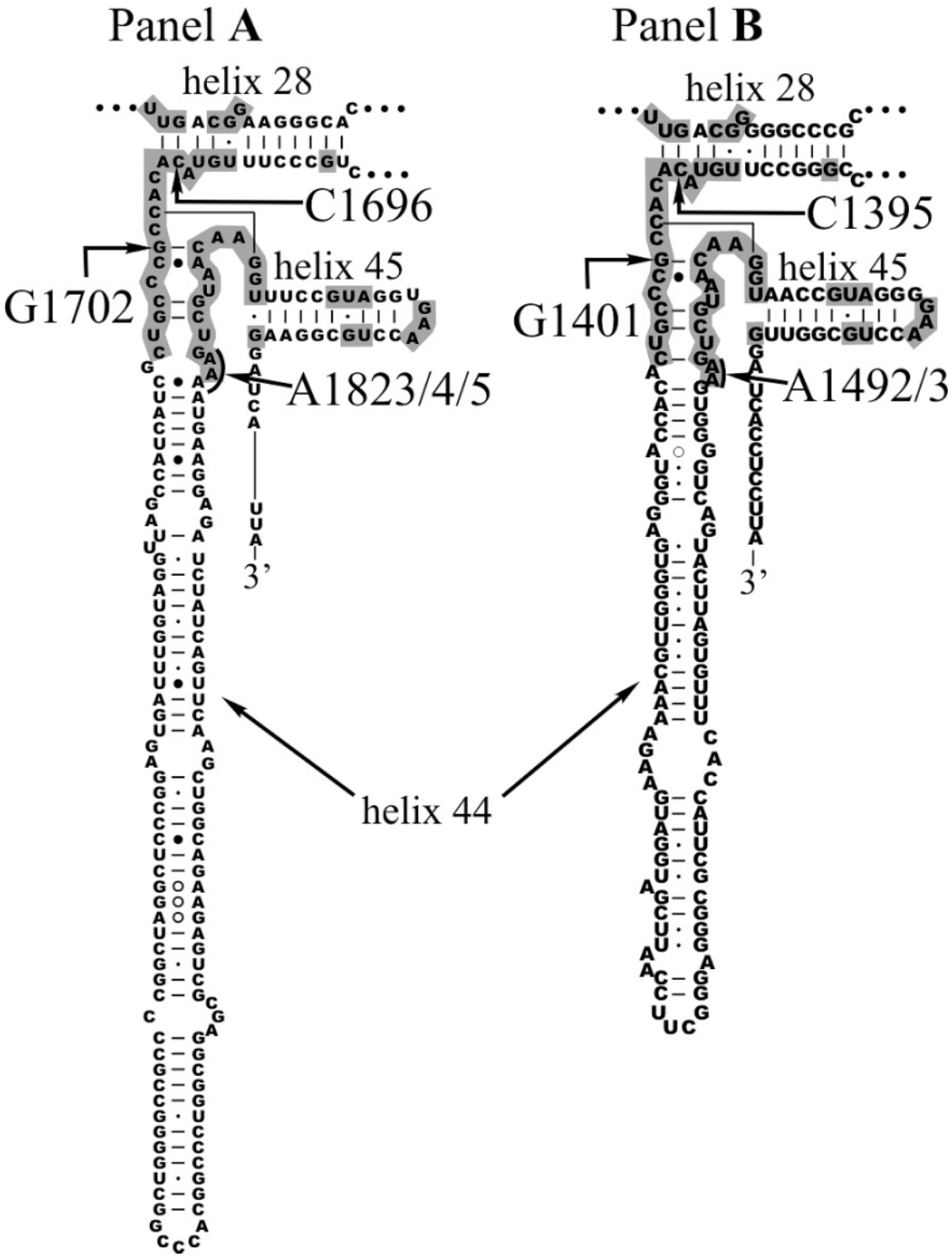

FIGURE 7: Secondary structures of the 3′-terminal mini-domains of human 18S rRNA (panel A) and *E. coli* 16S rRNA (panel B) [http://www.rna.icmb.utexas.edu/]. Nucleotides of 18S rRNA cross-linked to mRNA position +4 (panel A) and the corresponding nucleotides of 16S rRNA (panel B) are indicated. Strictly conserved regions are shaded.

as a hinge between head and body of the 40S subunit. Formation of the +4 to C1696 cross-link does not seem to be related to translation termination because in the rabbit system $s^4U(+4)$ yields the same pattern of cross-links whether inserted within a stop or sense codon (*15*−*17*). Rather it points to subtle differences in organization and internal motions of A site components in 80S and 70S ribosomes. Analysis of the cross-links generated on 18S rRNA by positions +4, +5, and +6 as a function of their nucleotide context and in the absence or presence of tRNA (or eRF1) in the A site will refine the picture. In this respect, we have shown that in complex 2 positions +5 to +7 cross-link to the same conserved 3′ terminal part (nucleotides 1641−1770) of 18S rRNA as position +4 does (work in progress). Whether the behavior of 80S ribosomes reflects the higher protein content of its decoding site, 18S RNA expansions (relative to 16S rRNA), or both is an open question. It is likely that the differences noticed above may

reflect a fine-tuning of tRNA anticodon−codon interactions by the ribosome.

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