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Research paper

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Konstantin Bulygin [a,b], Alexey Malygin [a], Codjo Hountondji [b], Dmitri Graifer [a], Galina Karpova [a,*]

[a] Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, pr. Lavrentieva, 8, 630090 Novosibirsk, Russia
[b] UPMC Univ Paris 06, Equipe de Photobiologie Moléculaire, Tour 32 (4ème Etage, couloir 32-33), Case Courrier 60, 4 place Jussieu, F-75252 Paris Cedex 05, France

Keywords:
80S ribosomes tRNA binding sites
CCA-end localization
4-Thiouridine photocross-linking
28S rRNA
Hydroxyl radical footprinting

A B S T R A C T

Nucleotides of 28S rRNA involved in binding of the human 80S ribosome with acceptor ends of the A site and the P site tRNAs were determined using two complementary approaches, namely, cross-linking with application of $tRNA^{Asp}$ analogues substituted with 4-thiouridine in position 75 or 76 and hydroxyl radical footprinting with the use of the full sized tRNA and the tRNA deprived of the 3′-terminal trinucleotide CCA. In general, these 28S rRNA nucleotides are located in ribosomal regions homologous to the A, P and E sites of the prokaryotic 50S subunit. However, none of the approaches used discovered interactions of the apex of the large rRNA helix 80 with the acceptor end of the P site tRNA typical with prokaryotic ribosomes. Application of the results obtained to available atomic models of 50S and 60S subunits led us to a conclusion that the A site tRNA is actually present in both A/A and A/P states and the P site tRNA in the P/P and P/E states. Thus, the present study gives a biochemical confirmation of the data on the structure and dynamics of the mammalian ribosomal pretranslocation complex obtained with application of cryo-electron microscopy and single-molecule FRET [Budkevich et al., 2011]. Moreover, in our study, particular sets of 28S rRNA nucleotides involved in oscillations of tRNAs CCA-termini between their alternative locations in the mammalian 80S ribosome are revealed.

1. Introduction

One of the key events of the cell life is protein synthesis that is conducted on ribosomes, very complicated ribonucleoproteins, whose function is translation of genetic information incoming as sequences of trinucleotides-codons of mRNAs into the polypeptide chains of proteins. Amino acid residues for the protein synthesis are transported to ribosomes by short (75–80 nt long) transfer RNAs that share a common L-shaped spatial structure and contain the universally conserved trinucleotide CCA at the 3′-termini. Each tRNA is specific to the single amino acid that is attached to the ribose of the 3′-terminal adenosine via a complex ester bond. The ribosome possesses aminoacyl (A) site for incoming aminoacyl-tRNA and peptidyl (P) for tRNA with a nascent polypeptide chain; peptide bond is formed when α-amino group of the aminoacyl-tRNA bound in A site attacks C atom of the C-terminal amino acid of the nascent peptidyl moiety of the peptidyl-tRNA in the P site. Catalysis of the peptide bond formation is performed at the ribosomal peptidyl transferase centre (PTC) located on the large ribosomal subunit. Structures of the eubacterial and archaeal PTCs are well known primarily from X-ray crystallographic data on complexes of 70S ribosomes with tRNAs (e.g., see Refs. [1–3]) and on complexes of 50S ribosomal subunits with model substrates imitating CCA-arm of aminoacyl-tRNAs (e.g., see Ref. [4]). According to these data, in the eubacterial 50S subunit 3′-terminal nucleotide (A76) of the P site tRNA contacts 23S rRNA nucleotides in positions 2585 [1], 2451 [2,3] and 2063 [3] (here and then *Escherichia coli* numbering is used), while its A75 contacts 2252 [1–3], 2251 [2,3], 2602 [1,3] and 2063 [3]. As for the A site tRNA, A76 contacts nucleotide 2452 both in eubacterial [1] and archaeal [4] 50S subunits, while the contact with nucleotide 2494 was reported with the eubacterial subunits [1], and the contacts with nucleotides 2506 and 2583 were found with archaeal subunits [4]; A75 interacts with 23S rRNA nucleotide 2553 both in the eubacterial [1] and archaeal [4] subunits, whereas the contacts with nucleotides 2452 and 2494 were observed in the eubacterial subunit, and with 2507 and 2573 with the archaeal one [4]. All these made it possible to investigate in detail the mechanism of the peptidyl transfer (for a review, see Ref. [5]).

The main feature of the PTC of the 50S subunit is that it is composed solely of the 23S rRNA nucleotides, which had led to the commonly accepted idea that the ribosome is a ribozyme [5–8].

* Corresponding author. Tel.: +7 383 363 5140; fax: +7 383 363 5153.
E-mail address: karpova@niboch.nsc.ru (G. Karpova).

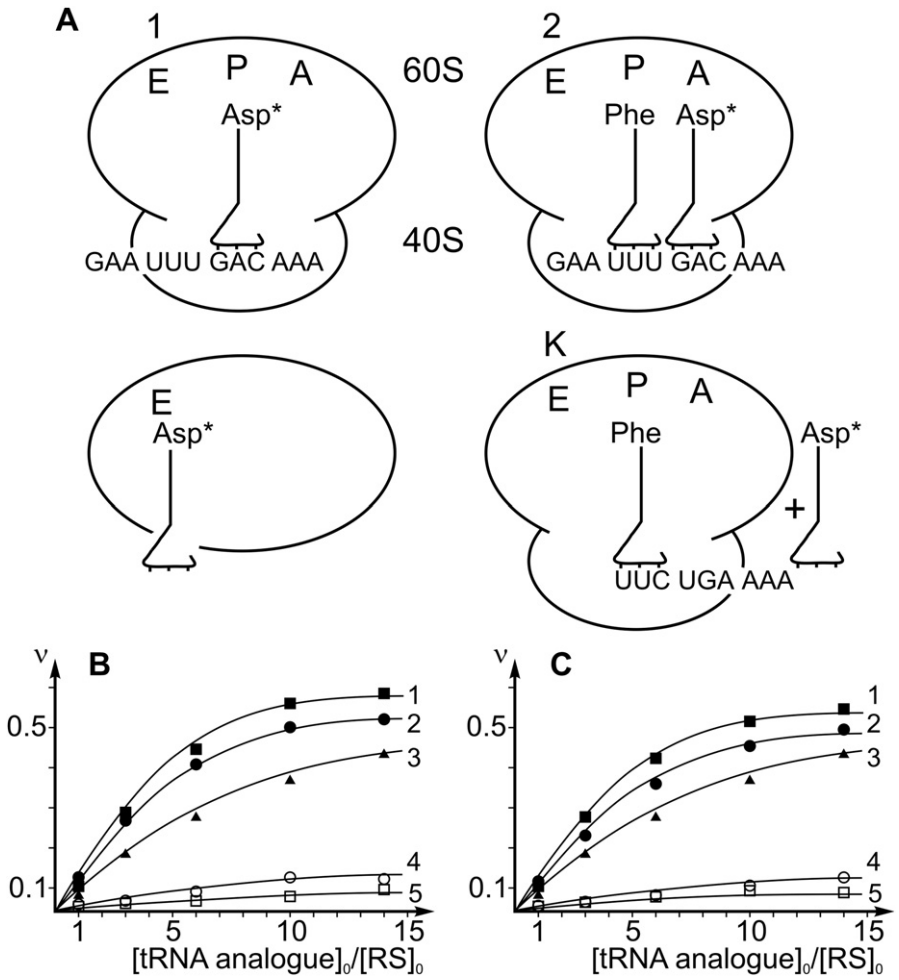

Fig. 1. Types of complexes of 80S ribosomes with $tRNA^{Asp}$ analogues (A), and isotherms of binding of $tRNA^{Asp}[^{32}P]ps^4U76$ (B) and $tRNA^{Asp}[^{32}P]ps^4U75$ (C) to the ribosomes. (A) Complex K (control) corresponds to binding of the tRNA analogues to 80S ribosomes preincubated with $tRNA^{Phe}$ and a short mRNA analogue containing UUC followed by the UGA stop-codon instead of the GAC triplet that was targeted to the A site in complexes 2. (B and C) 1 and 2, binding of tRNA analogues to 80S ribosomes; 3, binding of $tRNA^{Asp}$ to 60S subunits; 4, binding of tRNA analogues in complexes K; 5, binding of tRNA analogues to 60S subunits; ν, ratio of tRNA or tRNA analogue bound to the 80S ribosomes or 60S subunits (mol/mol). Designation $[\ ]_0$ means the initial molar concentration. s^4U-containing $tRNA^{Asp}$ analogues are marked with asterisks.

The rRNA fragments forming the PTC belong to the most conserved parts of large subunit rRNA; moreover, all nucleotides of the PTC are invariant (e.g., see www.rna.icmb.utexas.edu), which had been a basis for the widespread view that the PTC structure is very similar in all kingdoms. But actual extent of these similarities remains so far unknown because of the lack of direct experimental data on the fine structure of the eukaryotic PTC. Eukaryotic 80S ribosomes are larger and more complicated than their 70S eubacterial counterparts; late studies on deciphering of the structures of ribosomes from lower eukaryotes by X-ray crystallography [9,10] did not provide direct information on the PTC structure and the interactions of the CCA-termini of ribosome-bound tRNAs since the crystallized complexes did not contain PTC substrates. A recent study of structure and dynamics of the mammalian ribosomal pretranslocation complex carried out with application of cryo-electron microscopy (cryo-EM) and single-molecule FRET [11] showed the existence of strong distinctions in the structural and energetic features of bacterial and mammalian ribosomes. However, the mentioned study did not produce direct information on ribosomal interactions of the CCA ends of the bound tRNA molecules that is of principal importance, especially taking into account that peptide bond formation requires proper positioning of acceptor ends of the A site and the P site tRNAs on the ribosome.

Currently available experimental data on 80S ribosomal components that form the environment of the 3′-terminus of tRNA have been obtained by means of a site-directed cross-linking approach utilizing $tRNA^{Asp}$ analogues bearing either 4-thiouridine residue attached to the 3′-terminal adenosine [12] or 2′,3′-dialdehyde group at the 3′-terminus prepared with the use of periodate oxidation of the 3′-terminal ribose [13]. These data are fragmentary since the former study did not provide information on intimate contacts of the CCA-end, and the latter study utilized cross-linker targeted only to proteins.

Here we present a comprehensive study of large subunit rRNA nucleotides that participate in the interactions with the CCA ends of the A site and the P site tRNAs in the human 80S ribosome. To reveal intimate contacts of nucleotide bases of the tRNA acceptor ends, we used $tRNA^{Asp}$ analogues that bore in positions 75 or 76 s^4U residue capable to form zero-length cross-links. Besides, nucleotides of the 28S rRNA involved in accommodation of CCA termini of the ribosome-bound tRNAs to the PTC were determined by means of hydroxyl radical footprinting; in the latter case, conclusions on the nucleotides participating in binding of tRNA CCA termini were made based on the comparison of the data with 28S rRNA isolated from the ribosomal complexes containing full size $tRNA^{Asp}$ and the results obtained with $tRNA^{Asp}$ lacking in the CCA end.

2. Methods and materials

2.1. Ribosomes, tRNAs and mRNAs

40S and 60S ribosomal subunits containing intact rRNAs were isolated from unfrozen human placenta according to Ref. [14]. Prior to use, the subunits were re-activated by incubation in binding buffer A (50 mM Tris—HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$ and 0.5 mM EDTA) at 37 °C for 10 min. 80S ribosomes were obtained by association of re-activated 40S and 60S subunits taken in equimolar ratios. Activity of the ribosomes in the poly(U)-directed binding of [^{14}C]Phe-tRNA^{Phe} was 70%. tRNA^{Phe} (1300 pmol/A_{260} unit) from *E. coli* was kindly provided by Dr. V.I. Katunin (B.P. Konstantinov's St. Petersburg Institute of Nuclear Physics, Gatchina, Russia). Yeast tRNA^{Asp} transcript was obtained by *in vitro* T7 transcription [15]. Short mRNA analogues were purchased from Sigma—Aldrich.

2.2. Preparation of modified tRNA analogues

Photoactivatable tRNA analogues bearing a 4-thiouridine (s^4U) residue at position 75 or 76 (tRNA^{Asp}[^{32}P]ps^{4}U75 or tRNA^{Asp}[^{32}P]ps^{4}U76, respectively) were prepared in two steps. At first, tRNA^{Asp} transcript was shortened from the 3′-end by one or two nucleotides as previously described [16], then truncated tRNA derivatives were ligated with [5′-^{32}P]ps^{4}Up [12] and 3′-dephosphorylated. tRNA lacking CCA-end was prepared by the sequential removal of 3′-terminal nucleotides from the tRNA^{Asp} transcript as previously described [16].

2.3. Ribosomal complexes and cross-linking procedure

80S ribosomal complexes containing modified tRNA^{Asp} analogues (Fig. 1A) were obtained in buffer A at 20 °C. Ternary complexes 1 (with the tRNA analogues in the P site) were obtained by incubation of 80S ribosomes (0.5 μM) with a tRNA analogue (5 μM) and the dodecaribonucleotide GAA UUU GAC AAA (5 μM) as mRNA analogue for 40 min. Complexes 2 (with tRNA analogues in the A site) were obtained in two steps. First, a ternary complex of 80S ribosomes with tRNA^{Phe} in the P site and the mRNA analogue was obtained as described above; then, this complex was supplied with a tRNA^{Asp} analogue (5 μM) and incubation continued for 40 min. In control experiments, a tRNA^{Asp} analogue (5 μM) was added to the ternary complex of 80S ribosomes obtained by analogy as complex 2 (see above) but with the nonaribonucleotide UUC UGA AAA as mRNA. Extent of tRNA analogues binding was examined by nitrocellulose filtration technique using reaction mixtures (10 μl each) that contained 5 pmol of 80S ribosomes, 50 pmol of mRNA, 50 pmol of tRNA^{Phe} (for complex 2) and increasing concentrations of labelled tRNA analogues. Before use, filters (pore size 0.45 μm, Millipore) were incubated in 0.5 M KOH for 25 min at 25 °C to decrease non-specific adsorption of tRNA analogues. The radioactivity retained on the filters was measured by Cherenkov counting. Cross-linking of tRNA analogues to the ribosomes was carried out by mild UV-irradiation (wavelength >290 nm) of the complexes as described in Ref. [17].

2.4. Analysis of cross-linked ribosomal proteins

After irradiation, the reaction mixtures were treated with sodium dodecyl sulphate (SDS) and EDTA to dissociate ribosomal complexes and to disassemble ribosomes to the rRNA and ribosomal proteins, then the proteins were resolved by standard polyacrylamide gel electrophoresis in the presence of SDS (SDS PAGE), the gels were coomassie stained to develop bands of ribosomal proteins, dried and autoradiographed [18].

2.5. Analysis of cross-linked 28S rRNA

After irradiation, total RNA was isolated from the cross-linked 80S ribosomes by phenol deproteination and analysed by electrophoresis in 1% agarose [12]; the gels were dried, exposed onto a Kodak phosphorimager screen and quantified using the program Quantity One (BioRad, USA). Regions of the 28S rRNA containing cross-linked tRNA analogues were examined by hydrolysis of the cross-linked rRNA with RNase H (BioLabs) in the presence of deoxy 20-mers complementary to various sequences of the human 28S rRNA (Proligo, France) with subsequent separation of the resulting labelled RNA fragments by denaturing PAGE as described in Ref. [12]. Cross-linked nucleotides of 28S rRNA were established by reverse transcription with AMV reverse transcriptase using cross-linked rRNA as a template and appropriate 5′-^{32}P-labelled primers, and the resulting labelled products were separated by denaturing PAGE [19]. Each reaction mixture contained about 1 pmol of the cross-linked or control 28S rRNA and the same amount of the 5′-^{32}P-labelled primer. After reverse transcription, the samples were treated as described in Ref. [19]. Numbering of human 28S rRNA was according to the sequence taken from http://www.ncbi.nlm.nih.gov/nuccore/m11167.

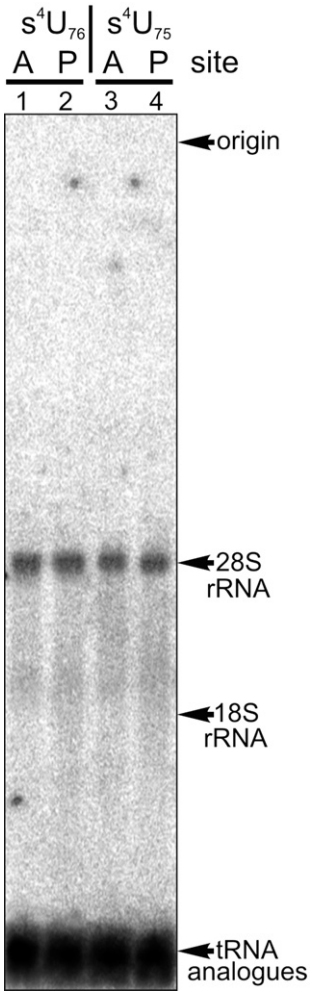

Fig. 2. Electrophoretic analysis in 1% agarose of tRNA^{Asp}[^{32}P]ps^{4}U76 (lanes 1 and 2) or tRNA^{Asp}[^{32}P]ps^{4}U75 (lanes 3 and 4) cross-linked to rRNA in ribosomal complexes 2 (lanes 1 and 3) and 1 (lanes 2 and 4) (Fig. 1A). Autoradiogram of the gel. The positions of rRNAs are marked as they are seen in the ethidium bromide stained gel.

2.6. Hydroxyl-radical probing

Hydroxyl radical cleavage of 28S rRNA in 60S ribosomal subunits within the 80S ribosomal complexes containing tRNA and mRNA analogues (see above) was carried out according to Ref. [19]; positions of the resulting cleavages in the 28S rRNA were determined by AMV reverse transcriptase analysis with following quantification of the results as described in Ref. [19].

3. Results

3.1. tRNA analogues, their binding and cross-linking to 80S ribosomes

In this study, full sized and truncated $tRNA^{Asp}$ transcripts were used. To obtain the tRNA transcripts lacking in one, two or three 3′-terminal nucleotides ($tRNA^{Asp}\Delta A$, $tRNA^{Asp}\Delta CA$ and $tRNA^{Asp}\Delta CCA$, respectively), 3′-terminal nucleotides of the transcript were removed stepwise by combining the periodate, lysine and alkaline phosphatase treatments. The truncated derivatives were isolated by denaturing 10% PAGE and were homogeneous products (purity >98%) of the expected lengths; all of them were able to recover their CCA-termini in the enzymatic reaction with tRNA-nucleotidyl transferase 1 resulting in formation of the full-sized $tRNA^{Asp}$ transcript (data not shown). Ligation of $tRNA^{Asp}\Delta A$ and $tRNA^{Asp}\Delta CA$ with [5′-^{32}P]ps^4Up (with following 3′-dephosphorylation) led to the formation of photoactivatable tRNA analogues ($tRNA^{Asp}$[^{32}P]ps^4U76 or $tRNA^{Asp}$[^{32}P]ps^4U75, respectively). The design of ribosomal complexes for the cross-linking and footprinting experiments and the way of formation of these complexes were based on the well-known fact that tRNA binds first to the P site [20–22]. Both s^4U substituted $tRNA^{Asp}$ analogues were able to effectively bind to the 80S ribosomal A and P sites when the respective site was occupied with the GAC triplet coding for Asp (Fig. 1A, complexes 1 and 2, see corresponding binding curves in Fig. 1B and C). The binding of the $tRNA^{Asp}$ analogues was codon-specific since it was negligible in the control complex K with codon UUC and $tRNA^{Phe}$ at the P site and stop codon UGA at the A site. For all that, both tRNA analogues had a reduced capability of binding to the isolated 60S subunits (Fig. 1B and C) that is generally assumed as the E site binding [20,23,24]. These results are consistent with the known observations that 3′-terminal adenosine of tRNA strongly contributes to the affinity of deacylated tRNA to the E site [25,26].

Irradiation of the ribosomal complexes with mild UV-light resulted in cross-linking of the tRNA analogues solely to the 28S rRNA (Fig. 2). Quantification of the results presented in Fig. 2 showed that with both tRNA analogues in complexes 1 and 2 the extent of cross-linking was similar and equal to about 1% of amount

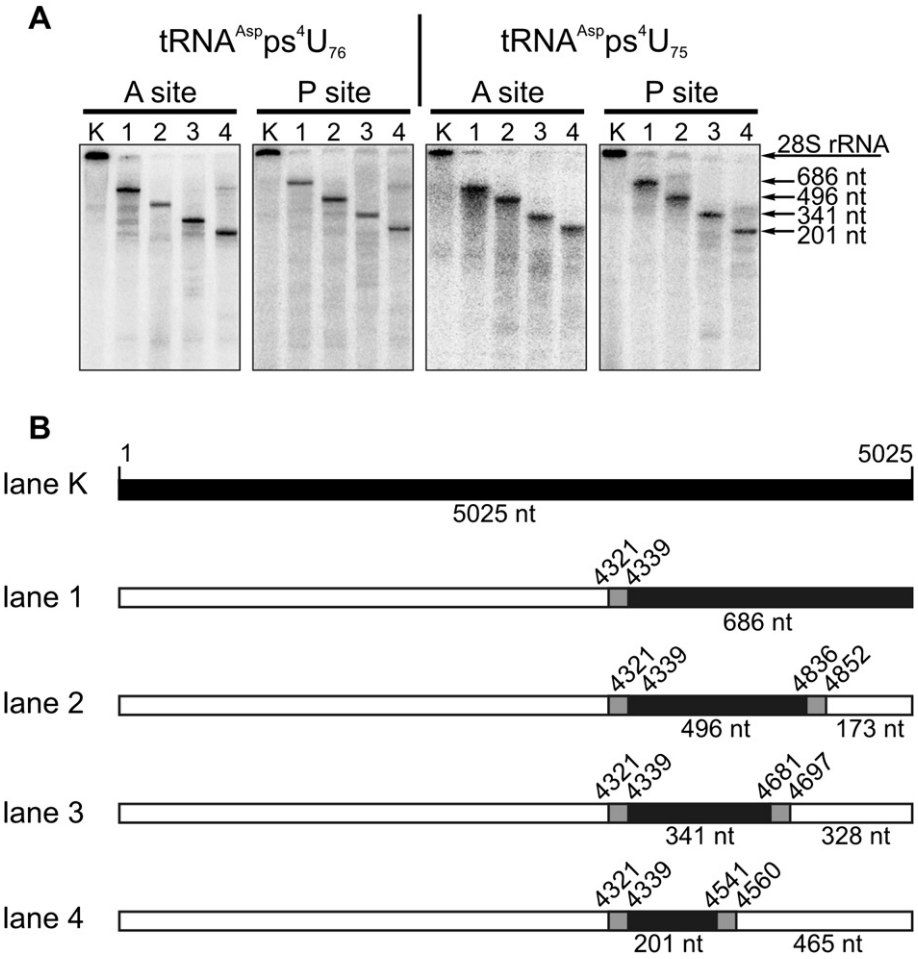

Fig. 3. Analysis of RNase H digests of 28S rRNA cross-linked to the labelled $tRNA^{Asp}$ analogues in ribosomal complexes in the presence of deoxy-oligomers (single one or pairs) complementary to sequences 4321–4339, 4541–4560, 4681–4697 and 4836–4852 of the 28S rRNA by denaturing 5% PAGE. (A) Autoradiograms of the gels. Lanes K, control (rRNA isolated from the cross-linked complexes and treated with RNase H without deoxy-oligomers). (B) Schematic representation of the hydrolysis of 28S rRNA with RNase H. Sequences complementary to deoxy-oligomers are shown in grey, rRNA fragments containing nucleotides cross-linked to the labelled tRNA analogues are black; the lengths of fragments are indicated, the length of the cross-linked tRNA analogues having been subtracted.

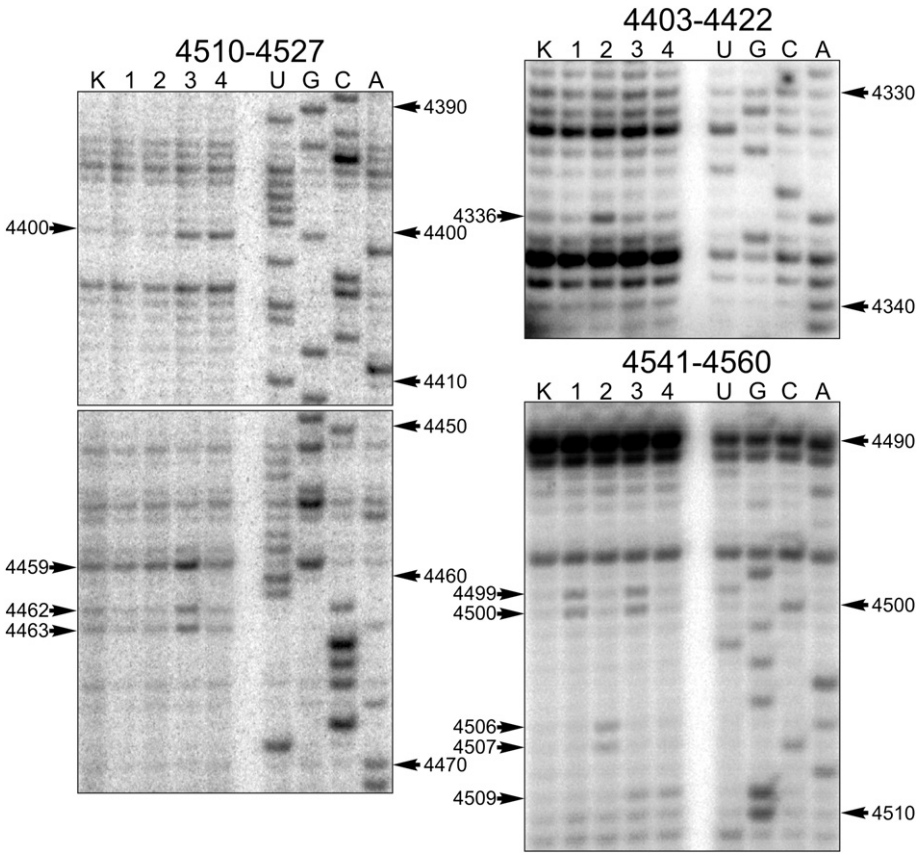

Fig. 4. Identification of cross-linking sites of the tRNA analogues in 28S rRNA by reverse transcription and subsequent analysis of the resulting products by denaturing 8% PAGE. Autoradiograms of gels obtained with [5′-^{32}P] labelled primers complementary to positions 4403–4422, 4510–4527 and 4541–4560 of the 28S rRNA. Lanes 1 and 3 are primer extensions on 28S rRNA recovered from irradiated complexes 2, lanes 2 and 4 – from complexes 1 (Fig. 1A). Lanes 1 and 2 correspond to tRNA^{Asp}[^{32}P]ps^{4}U76, and lanes 3 and 4 to tRNA^{Asp}[^{32}P]ps^{4}U75. Lanes U, G, C and A are sequencing of the 28S rRNA isolated from human placenta tissue. Lanes K, primer extension on a control 28S rRNA isolated from ribosomes treated the same as with the tRNA analogues. Positions of the reverse transcription stops caused by the cross-links are indicated. With each primer, only those gel fragments are presented that contain stops caused by the cross-links.

of tRNA analogue bound to the ribosome. No cross-links to ribosomal proteins were found (data not shown).

3.2. Identification of cross-linked 28S rRNA nucleotides

To determine fragments of 28S rRNA cross-linked to the tRNA analogues, total RNA isolated from irradiated ribosomal complexes was treated with RNase H in the presence of one or two oligodeoxyribonucleotides complementary to various parts of human 28S rRNA. The digests were resolved by PAGE followed by autoradiography of the gels (Fig. 3). It is seen that, with both tRNA analogues in complexes 1 and 2, the same fragment is cross-linked. When the rRNA was digested in the presence of a cDNA complementary to the rRNA sequence 4321–4339, the label comigrated with the smaller 3′-fragment of 28S rRNA (the larger one does not leave the origin and does not migrate in PAGE, Fig. 3A, lanes 1), thus, cross-link took place within 28S rRNA positions 4321–5025. More precise localization of the cross-link on the 28S rRNA was made with the use of pairs of deoxy-oligomers (Fig. 3, lanes 2–4). Comparison of the results presented in these lanes led to

Table 1
Comparison of nucleotides of 28S rRNA cross-linked to tRNA analogues in 80S ribosomal complexes and nucleotides of 23S rRNA interacting with the respective tRNA nucleotides in 50S subunits.

Position of s^{4}U in tRNA analogue	P site[a]			A site[a]		
	28S rRNA nucleotides cross-linked in complex 1[a]	Positions of 23S rRNA nucleotides[b] contacting the respective tRNA nucleotide in the 50S subunit		28S rRNA nucleotides cross-linked in complex 2[a]	Positions of 23S rRNA nucleotides[b] contacting the respective tRNA nucleotide in the 50S subunit	
		X ray data	Cross-linking		X ray data	Cross-linking
76	C4335 (C2427), G4505 (G2599), A4506 (A2600)	2063 [3]; 2451 [2,3,29,30]; 2585 [1]	2585, 2506 (major); 2069 (minor); 2452 (only without mRNA) [32]	G4498 (G2592), U4499 (U2593)	2452 [1,4]; 2494 [1]; 2506, 2583 [4]; 2584, 2585 [29]	2585, 2506 (major); 1926 (minor) [32]
75	U4399 (U2493), A4508 (A2602)	2063 [3]; 2251 [2,3,29,30]; 2252 [1–3,30]; 2602 [1,3]	Not studied	U4399 (U2493), U4458 (U2552), U4461 (U2555), C4462 (C2556), A4508 (A2602), G4498 (G2592), U4499 (U2593)	2452, 2494 [1]; 2507, 2573 [4]; 2553 [1,4,29]	Not studied

[a] In brackets, *E. coli* numbering is presented.
[b] *E. coli* numbering.

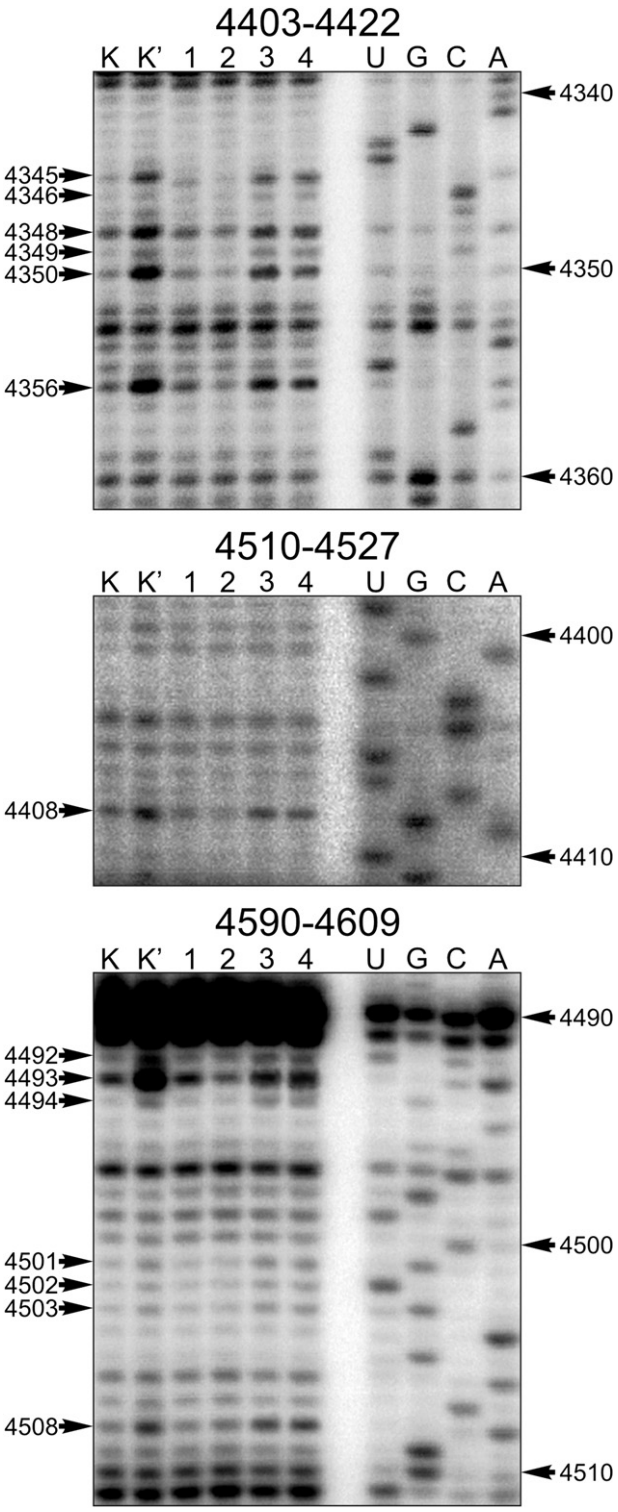

Fig. 5. Analysis of positioning of CCA-ends of tRNA relative to 28S rRNA by hydroxyl radical footprinting with subsequent identification of nucleotides protected by tRNA CCA termini by reverse transcription. Autoradiograms of gels after separation of reverse transcription products obtained with [5′-^{32}P] labelled primers complementary to positions 4403–4422, 4510–4527 and 4590–4609 of the 28S rRNA. Lanes 1 and 3 are primer extensions on 28S rRNA recovered from complexes 2 and lines 2 and 4 from complexes 1 (Fig. 1A). Lanes 1 and 2 correspond to the complexes with full-sized tRNA^{Asp} transcript, and lanes 3 and 4 to the complexes with the truncated analogue tRNA^{Asp}ΔCCA. Lanes U, G, C and A are sequencing of the 28S rRNA isolated from human placenta tissue. Lane K, primer extension on 28S rRNA isolated from untreated ribosomes; lane K′, the same on 28S rRNA isolated from ribosomes treated with hydroxyl radicals. Positions of the reverse transcription stops caused by the hydroxyl radical cleavages are indicated on the left. With each primer, only those gel fragments are presented that contain stops corresponding to hydroxyl radical cleavages dependent on the presence of the intact CCA end in the tRNA^{Asp} analogue.

a conclusion that the cross-linking site of the tRNA analogues was within positions 4321–4560 of domain V of the 28S rRNA. Nucleotides of 28S rRNA cross-linked to the tRNA analogues were identified by reverse transcription with a primer chosen on the basis of the results on RNase H digestion. The cross-linking site is generally assumed to be at the level of the nucleotide 5′ of the primer extension stop site. The results of primer extension analysis are presented in Fig. 4 and summarized in Table 1. It is seen that cross-linking sites of the tRNA analogue bearing s^{4}U in position 76 bound at the P and the A site are different, while with the analogue containing s^{4}U75 both cross-links observed at the P site are present at the A site as well. In addition, two A site-specific ones (G4498 and U4499) are the same as with the A site bound tRNA^{Asp}ps^{4}U76.

3.3. Hydroxyl radical footprinting of CCA termini of tRNAs bound at the A and the P sites

To find 28S rRNA nucleotides involved in binding of CCA ends of tRNA molecules at the 80S ribosomal A and P sites, we carried out hydroxyl radical footprinting of the rRNA in complexes 1 and 2 (Fig. 1A) obtained with full-sized tRNA^{Asp} transcript and with the transcript deprived of the CCA end (tRNA^{Asp}ΔCCA). Comparison of hydroxyl radical-induced cleavages of the 28S rRNA observed with full-sized tRNA and tRNA^{Asp}ΔCCA made it possible to reveal rRNA nucleotides whose accessibilities to hydroxyl radical attack depended on the presence of CCA ends in the A site and the P site bound tRNAs. First, we examined region 4321–4560 where sites of cross-linking of tRNA analogues containing s^{4}U in position 75 or 76 are located (Fig. 5). One can see that the sets of 28S rRNA nucleotides protected by CCA termini of the A site and the P site bound tRNAs are the same. Positions of these nucleotides on the rRNA secondary structure are summarized in Table 2 and shown in Fig. 6 together with positions of nucleotides cross-linked to the s^{4}U substituted tRNA^{Asp} analogues. Then, we examined reverse transcription on primers complementary to 28S rRNA fragments 3906–3925 and 4282–4301 to monitor 28S rRNA regions around nucleotides C3878 and G4157 corresponding to 23S rRNA nucleotides C2063 and G2251, respectively (*E. coli* numbering), that are the closest neighbours of C75 of tRNA bound at the prokaryotic ribosomal P site according to the data of X-ray crystallography [2,3]. However, no protections of 28S rRNA nucleotides that could be caused by the tRNA CCA-ends were observed (the respective autoradiograms are not shown).

4. Discussion

Here, with the use of zero-length cross-linking, we determined nucleotides of 28S rRNA whose bases make intimate contacts with nucleotides in positions 75 or 76 of tRNA bound at the A or the P site of human 80S ribosomes. Applying hydroxyl radical footprinting, we revealed 28S rRNA nucleotides involved in accommodation of CCA ends to the PTC region of the ribosome. These approaches, when applied for studying bacterial PTC, provided reliable results that turned out to be in a good general agreement with later X-ray crystallography data (Tables 1 and 2). To compare ribosomal structures involved in the accommodation of tRNA CCA ends to the prokaryotic and mammalian ribosomes, we applied our results to the X-ray derived model of the archaeal 50S subunit [28] (Fig. 7). To do that, on the model we marked the conserved 23S rRNA nucleotides corresponding to 28S rRNA ones cross-linked to or protected

Table 2
Comparison of nucleotides of 28S rRNA protected by CCA-ends of tRNAs bound at the A site and the P site of human 80S ribosome and nucleotides of 23S rRNA interacting with CCA-ends of tRNAs in 50S subunits according to chemical footprinting data (the respective X-ray data are presented in Table 1).

28S rRNA nucleotides protected by the CCA-end of the P site or the A site tRNA[a]	Positions of 23S rRNA nucleotides[b] interacting with CCA-end of tRNA in the 50S subunit	
	P site	A site
4344–4349 (2438–2443), 4355 (2449), 4407 (2501), 4491–4493 (2585–2587), 4500–4502 (2594–2596), 4507 (2601)	2252, 2253, 2506, 2584, 2585 [24,31]	2553 [31]

[a] In brackets, *E. coli* numbering is presented.
[b] *E. coli* numbering.

by acceptor ends of the tRNA analogues (Fig. 6). On the same model we indicated the 50S subunit components interacting with CCA termini of the A site, the P site and the E site tRNAs (Table 1 and Refs. [2,3,28]). To compare our cross-linking and footprinting results with data on the eukaryotic PTC region, we applied them to the recently reported X-ray derived model of the yeast 80S ribosome [9] (Fig. 7A and B). Since the model was obtained in the absence of tRNA, we marked locations of ribosomal components corresponding to those forming the 50S ribosomal subunit A, P and E sites (see above) on this model. One can see that, in general, the arrangements of ribosomal components corresponding to A, P and E sites are very similar in the archaeal and yeast large subunits (to facilitate the figure, we presented the footprinting results only at the 60S model as an example, Fig. 7B).

Fig. 7 shows that targets of cross-linking of the A site s^4U-substituted tRNA analogues are located in the 60S ribosomal regions corresponding to both the A site and the P site positions of the tRNA acceptor ends in the archaeal 50S subunit. These imply that in the 80S ribosome the A site tRNA is present at both A/A and A/P states. Then, 28S rRNA nucleotides cross-linked to the P site tRNA analogues are located in positions corresponding to 23S rRNA regions interacting with both the P site and the E site CCA-termini positions in the archaeal 50S subunit, indicating P/P and P/E states of the tRNA in the human ribosome. These results are in a good agreement with recently published cryo-EM and single-molecule FRET data on tRNA positioning in the mammalian pretranslocational complex [11] and with the data on cross-linking of the oxidized 3′-terminal ribose of the P site tRNA analogues to mammalian 60S ribosomal protein (rp) L36AL [13,27]. This protein belongs to the L44e protein family and was suggested to be a component of the 60S ribosomal E site [13,27] by analogy to rpL44e that is known to contact CCA-terminus of the E site tRNA in archaeal 50S subunit [28]. According to the data of Ref. [11], CCA end of the P site tRNA oscillates between the classical P/P and the hybrid P/E positions. However, tRNA analogues used in our study are almost deprived of the ability to bind at the classical E site requiring an intact CCA end (see Section 3.1) as are the tRNA analogues with oxidized 3′-terminal ribose applied in Refs. [13,27]. To explain cross-linking of tRNA analogues with modified CCA ends at the E site region, in Ref. [27] it was suggested that the modified tRNA might bind first to the classical P/P site before it flips spontaneously into the P/E site, where it is sampled by the crosslinking with Lys 53 of rpL36AL [27]. Similar explanation is reasonable with cross-linking of P-site $tRNA^{Asp}ps^4U76$ to C4335 that belongs to E-site region as is Lys 53 of rpL36AL (Lys 55 of rpL44e, Fig. 7) that is capable of contacting ribose of 3′-terminal adenosine of the P site tRNA [27]. Notably, cross-linking to C4335 takes place only with $tRNA^{Asp}ps^4U76$ but not with $tRNA^{Asp}ps^4U75$, which could occur because of the lack of contacts of the large rRNA with a nucleotide in tRNA position 75. Indeed, in the archaeal 50S subunit nucleotides 74 and 75 of the E site tRNA contact only rpL44e but not the 23S rRNA [28].

In the footprinting experiments, we deduce nucleotides of the 28S rRNA participating in binding of the CCA ends from the comparison of footprints with the full sized and the truncated tRNA transcripts bound to the ribosome. In contrast to cross-linking that can trap an unstable state of tRNA in the complex (as discussed

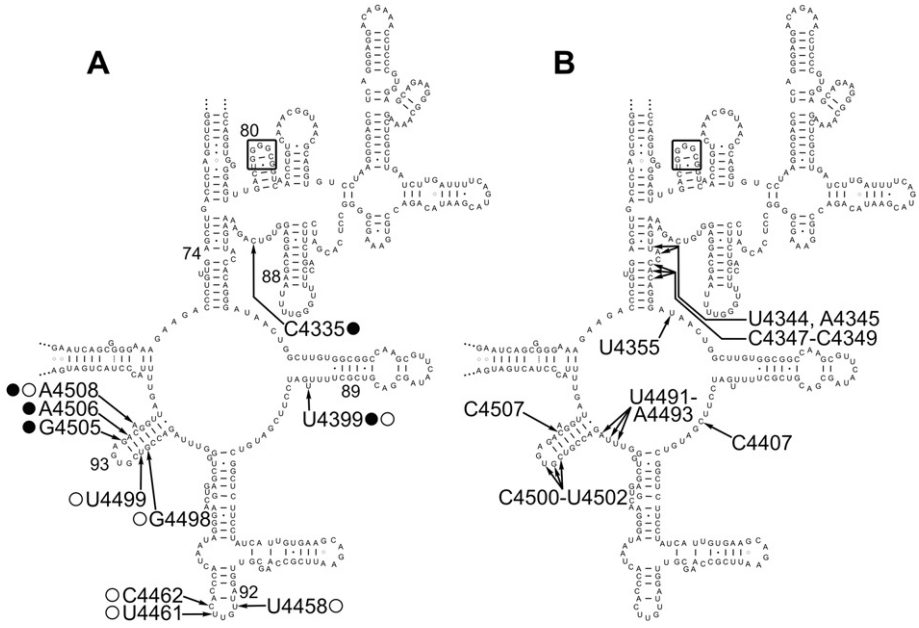

Fig. 6. Locations of 28S rRNA nucleotides that neighbour CCA end of tRNA according to the data of this study on the secondary structure of a fragment of human 28S rRNA domain V (http://www.rna.ccbb.utexas.edu/). Helices are numbered as in Ref. [2]. Nucleotides cross-linked to the A site and the P site tRNA analogues are shown with blank and filled circles, respectively (A); nucleotides protected by CCA-ends of tRNA according to the data of hydroxyl radical footprinting are indicated by arrows (B). The apex of helix 80 containing nucleotides 2251 and 2252 (*E. coli* numbering) contacting CCA terminus of the P site tRNA in the 50S subunit [1–3,24,29–31] is boxed.

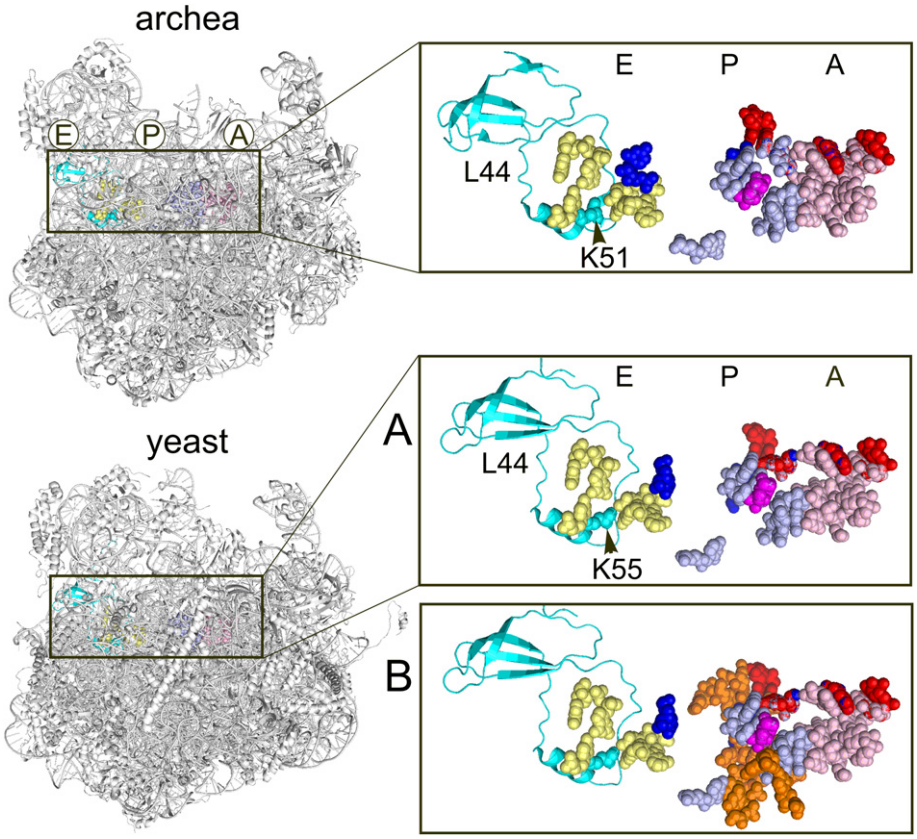

Fig. 7. Visualization of the results obtained in this study on the 3D structures of *H. marismortui* 50S subunits (PDB entry 1QVF [28]) and of *S. cerevisiae* 60S subunits (PDB entry 3U5D [9]). Nucleotides of the large rRNA located at A, P and E sites (see Table 1 and Refs. [2,3,28]) are coloured in pink, light-violet and yellow, respectively. Cross-linking data are given in red and blue (with the A and the P site tRNA analogues, respectively), and the footprinting data are presented in orange (B). Nucleotides of large rRNA corresponding to G2251 and G2252 in the *E. coli* 23S rRNA are coloured in magenta. Ribosomal protein L44e (homologous to human L36AL) is shown in light green. Positions of lysines corresponding to K53 in the human rpL36AL are indicated by arrows.

above concerning the P/E state) footprinting provides an averaged pattern of the tRNA states. This is the most probable reason why the footprinting failed to detect the E site nucleotides involved in accommodation of the CCA terminus of the P site tRNA to 60S subunit, i.e., the P/E state of the P site tRNA. Indeed, as one can see from Fig. 7A and B, 28S rRNA nucleotides protected by CCA-end of the P site tRNA are centred only at the region of the P site. On the other hand, the E site nucleotides could not be detected because the acceptor end of the P site tRNA (at least, nucleotides in positions 74 and 75) interacts mainly with the E site protein L36AL/L44e rather than with rRNA [27], which resembles the respective interaction of the E site tRNA with archaeal 50S subunit (as already mentioned in the preceding paragraph [28]). Then, footprinting shows that sets of protections with the A site and the P site tRNAs are the same (Table 2 and Fig. 7). Therefore, with the A site tRNA we do not observe the A/A state but only the A/P state. Thus, as with the P/E state, we detected the A/A state by cross-linking but not by the footprinting (Fig. 7). The only possible explanation for these results is that A/A state is unstable and therefore could be trapped only by cross-linking. Therefore, we can state that cross-linking and footprinting approaches used here provided information complementary to each other. Evidently, the former approach is suitable to fix interactions, which involve tRNA bases, while the latter reveals also interactions implicating the ribose-phosphate backbone. In particular, a cluster of 28S nucleotides in the lower part of the P site region was detected only by footprinting (Fig. 7B), leading us to a suggestion that the respective 28S rRNA nucleotides interact with ribose-phosphate backbone rather than with bases of tRNA acceptor ends.

An unexpected finding in this study is the lack of both cross-links and footprints in the region of the tip of helix 80 of the large rRNA (Fig. 6) whose nucleotides (2251 and 2252 in *E. coli* numbering) contact CCA-terminus of the P site tRNA in the 50S subunit according to X-ray data obtained in various groups [2,3,29,30] (Table 1). Contacts of the P site CCA terminus with the tip of H80 had been revealed also in earlier footprinting studies with bacterial ribosomes [24,31] and therefore it seems unlikely that the respective contacts in the 60S subunit were missed because of limitations of the approaches used in this study. It should be noted here that in our previous study with the use of $tRNA^{Asp}$ analogue bearing s^4U residue attached to the 3′-terminal adenosine as an additional nucleotide [12], we also have not detected cross-linking in the region of H80. Taking all these into account, we have a ground to suggest that helix 80 is involved in the binding of the P site CCA termini only to 50S subunits but not to 60S subunits. However, the detailed comparative analysis of the X-ray models of archaeal and yeast large ribosomal subunits discussed in this study reveals that orientations of 23S rRNA nucleotides G2251 and G2252 at the tip of H80 in the 50S subunit and of the respective large rRNA nucleotides in the 60S subunit are similar as well as contacts of these nucleotides with other ribosomal components. Thus, the available data on the structure of 60S subunits from lower eukaryotes lead to a conclusion that the lack of interactions of the P site tRNA acceptor end with H80 does not relate to different structural organization of H80 in the 60S and 50S subunits. We suggest that the discussed distinction reflects different dynamic properties of mammalian and prokaryotic ribosomal complexes reported in Ref. [11], and these differences

apparently are the reason why 28S rRNA nucleotides cross-linked to the CCA-ends or protected by them in major do not exactly correspond to the 23S rRNA nucleotides interacting with the CCA-ends in the 50S subunit (Tables 1 and 2).

Thus, our results provide a biochemical confirmation of the structural dynamics of mammalian ribosomal complexes and for the first time outline particular sets of 28S rRNA nucleotides contacting tRNAs CCA-termini in the course of their oscillations between the alternative locations in the mammalian 60S ribosomal subunit.

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (grant 11-04-00597 to G.K.) and by the Program "Molecular and Cellular Biology" of the Presidium of the Russian Academy of Sciences to G.K.

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