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Nucleotides of 18S rRNA surrounding mRNA codons at the human ribosomal A, P, and E sites: A crosslinking study with mRNA analogs carrying an aryl azide group at either the uracil or the guanine residue

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ABSTRACT

The 18S rRNA environment of the mRNA at the decoding site of human 80S ribosomes has been studied by crosslinking with derivatives of hexaribonucleotide UUUGUU (comprising Phe and Val codons) that carried a perfluorophenylazide group either at the N7 atom of the guanine or at the C5 atom of the 5'-terminal uracil residue. The location of the codons on the ribosome at A, P, or E sites has been adjusted by the cognate tRNAs. Three types of complexes have been obtained for each type derivative, namely, (1) codon UUU and Phe-tRNA^{Phe} at the P site (codon GUU at the A site), (2) codon UUU and tRNA^{Phe} at the P site and PheVal-tRNA^{Val} at the A site, and (3) codon GUU and Val-tRNA^{Val} at the P site (codon UUU at the E site). This allowed the placement of modified nucleotides of the mRNA analog at positions –3, +1, or +4 on the ribosome. Mild UV irradiation resulted in tRNA-dependent crosslinking of the mRNA analogs to the 18S rRNA. Nucleotide G961 crosslinked to mRNA position –3, nucleotide G1207 to position +1, and A1823 together with A1824 to position +4. All of these nucleotides are located in the most strongly conserved regions of the small subunit RNA structure, and correspond to nucleotides G693, G926, G1491, and A1492 of bacterial 16S rRNA. Three of them (with the exception of G1491) had been found earlier at the 70S ribosomal decoding site. The similarities and differences between the 16S and 18S rRNA decoding sites are discussed.

Keywords: 18S ribosomal RNA; decoding site; human ribosomes; mRNA analogs; photocrosslinking

INTRODUCTION

The interaction of mRNA codons with anticodons of the correct (cognate) tRNAs is one of the key steps of protein synthesis on the ribosome (translation). Data on the arrangement of mRNA at the site of codon-anticodon interactions (the decoding site) are of great importance for understanding the molecular mechanisms of translation. Significant progress in studying the mRNA binding center has been achieved with the use of site-directed crosslinking. This approach is based on the application of mRNA analogs that bear chemi-

cally reactive groups or photoreactive groups at defined positions in the RNA sequence. Numerous data on nucleotides of ribosomal RNA and ribosomal proteins crosslinked with mRNA analogs have been accumulated within the past 10 years, both for prokaryotic (mostly *Escherichia coli*; for a review, see Baranov et al., 1999) and eukaryotic (in particular, human; e.g., see Malygin et al., 1994; Bulygin et al., 1997a, 1997b; Matasova et al., 1998) ribosomes. Comparison of the crosslinking data obtained with the same synthetic mRNAs (about 50 nt long) substituted randomly with 4-thiouridines revealed significant differences in interaction of mRNA with 70S and 80S ribosome (Graifer et al., 1994a). For human 80S ribosomes, the only major target for crosslinking, U630, was found (Graifer et al., 1994a) in contrast to the experiments with *E. coli* 70S ribosomes, where up to 12 mRNA-16S rRNA cross-

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links have been determined (Bhangu & Wollenzien, 1992). The general conclusions on the arrangement of the mRNA on the ribosome may be drawn from the sum of data obtained with the use of mRNA analogs of various natures, because results of the crosslinking may depend both on the position of the modified mRNA nucleotide on the ribosome and on the nature of the nucleotide and type of the crosslinking group (Vladimirov et al., 1990; Malygin et al., 1994; Bulygin et al., 1997b; Sergiev et al., 1997). Data obtained with the use of mRNA analogs that carry photoreactive groups at the positions not involved in the Watson–Crick base pairing (e.g., C5 atom of uracil or N7 atom of guanine) are especially important. Such analogs allow the study of the ribosomal environment of the mRNA nucleotides directly involved in the codon–anticodon interactions on the ribosome.

Here, we present the first crosslinking study on human ribosomes with the use of two short mRNA analogs that carry a *p*-azido-tetrafluorobenzamide group attached via a spacer to either the C5 atom of the uracil residue, or the N7 atom of the guanine residue. Both mRNA analogs contain the same hexaribonucleotide moiety, UUUGUU, which bears triplet UUU coding for phenylalanine and GUU coding for valine. Codons of the mRNA analogs were placed at the ribosomal A, P, or E sites by the interaction with the respective cognate tRNA molecules. This allowed the study of model ribosomal complexes in which the crosslinking group of the mRNA analog was placed at the first nucleotide of the codon located at the A, P, or E site.

RESULTS

Design of photoreactive mRNA analogs

The structures of the mRNA analogs used in this study are shown in Figure 1. (azidoU)UUUGUU (derivative I) and UUU(azidoG)UU (derivative II) were synthesized using different approaches. In the case of the derivative I, 5'-terminal 5-bromouridine was incorporated into the oligoribonucleotide chain in the course of its chemical synthesis, and then the $\text{—NHCH}_2\text{CH}_2\text{NH}_2$ group was substituted for the Br atom (Repkova et al., 1999). Finally, perfluoroaryl azide was selectively attached to the aliphatic amino group at the uracil residue by a reaction with *N*-hydroxysuccinimide ester of *p*-azido-tetrafluorobenzoic acid. In the case of derivative II, we applied an approach that had been used earlier for preferential introduction of aryl azide groups at the guanines in tRNA (Graifer et al., 1989). At the first step, UUUGUU was alkylated with 4-(*N*-2-chloroethyl-*N*-methylamino)benzylamine, which is known to modify preferentially guanines at the N7 atoms but does not react with uridines (see Knorre et al., 1985). Then, a perfluoroaryl azide group was coupled to the aliphatic

FIGURE 1. Structures of photoreactive derivatives of hexaribonucleotide UUUGUU used in this study. The lengths of the modifying groups of the derivatives I and II are 11 Å and 14 Å, respectively.

amino group introduced at the guanine as in the case of derivative I.

Preparation of the complexes and crosslinks

Three types of complexes of the mRNA analogs with 80S ribosomes and the cognate tRNAs are shown in Figure 2. The complexes were obtained nonenzymatically as previously described for the derivative of pUUUGUU, which carried an aryl azide group at the 5'-phosphate (Bulygin et al., 1997a). Data on binding and crosslinking of derivatives I and II to 80S ribosomes are summarized in Table 1. It is seen that both derivatives basically do not either bind or crosslink to human ribosomes without tRNAs. tRNAs had different stimulatory effects on binding of the mRNA analogs to 80S ribosomes (Table 1, compare complexes 1 and 3). tRNA^{Phe} stimulated binding of the mRNA analogs approximately five times more strongly than tRNA^{Val}; this effect was the same for both derivatives (see Table 1). Complexes of 80S ribosomes with 5'-³²P-labeled mRNA analogs and the cognate tRNAs were irradiated with mild UV light, then dissociated, and the ribosomal sub-

FIGURE 2. Types of complexes of UUUGUU derivatives with human 80S ribosomes used for crosslinking experiments. The E-, P-, and A- tRNA binding sites are indicated. All mRNA positions on the ribosome where photoreactive groups were located for crosslinking are marked.

units were separated by sucrose density gradient. In all cases, labeling of 60S subunits was negligible (<0.01 mol/mol 60S), so in Table 1 only crosslinking to 40S subunits is presented. Efficiency of crosslinking to the 40S subunits was high and slightly dependent on both the type of the derivative and the type of the complex. In all complexes, about a half or even more of an mRNA analog being bound to 80S ribosomes became crosslinked to the 40S subunit. The crosslinking was almost completely tRNA dependent, being negligible without tRNAs (see Table 1). The distribution of the crosslinking (analyzed by centrifugation in SDS-containing sucrose density gradients) between 18S rRNA and proteins depended more on the type of the complex than on the derivative type (see Table 1). In this study, crosslinking to RNA was examined.

Analysis of total RNA isolated from the irradiated complexes is presented in Figure 3. The position of tRNA was identified in the stained gels in those samples containing tRNA (stained gels are not shown). Derivative I crosslinked to 18S rRNA in complexes 1 and 2 to a similar extent (Fig. 3A, lanes 1 and 2). The crosslinking to tRNA was less effective (as compared to 18S rRNA)

and was also similar in both complexes. In complex 3, the crosslinking of derivative I to 18S rRNA was weaker than in complexes 1 and 2, and the crosslinking to tRNA was negligible (Fig. 3A, lane 3). Derivative II crosslinked to 18S rRNA in complex 1 significantly more strongly than in complex 2. In complex 3, the crosslinking was at the background level (Fig. 3B). In both cases, minor crosslinking to fragments of partially degraded 18S rRNA (which were not detectable in the stained gels) was seen. Differences in the crosslinking patterns for derivatives I and II reflect the different location of the respective crosslinking sites in 18S rRNA (see below). Crosslinking of derivative II to the tRNA molecules was detectable in all complexes; in complex 2, the crosslinking was higher than in complexes 1 and 3 (Fig. 3B). Without tRNAs, crosslinking to rRNA was barely detectable for both mRNA analogs (Fig. 3, lanes 4).

Identification of crosslinked nucleotides of 18S rRNA

To determine sequences of 18S rRNA containing crosslinked nucleotides, 18S rRNA isolated from irradiated complexes was digested with RNase H in the presence of deoxy-20-mers (cDNAs) complementary to various sequences of 18S rRNA in parallel experiments. The cDNAs were chosen on the basis of our previous results on crosslinking of mRNA analogs to human ribosomes (Malygin et al., 1994; Bulygin et al., 1997a). Fragments of the rRNA resulting from the hydrolysis were separated by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. Staining the gels allowed the detection of both parts of the rRNA cleaved in the presence of a definite cDNA, and radioautography of the gels indicated which part of the rRNA contained the crosslinked [32 P]-labeled mRNA analog. Comparison of the results obtained with various cDNAs for each crosslinked 18S rRNA sample allowed the identification of relatively short sequence intervals that contained the crosslinking site. Data on the analysis of crosslinked 18S rRNA isolated from irradiated complexes are given in Figures 4 and 5. For (azidoU)UUUGUU in complex 1, the crosslinking site is located within 18S rRNA positions 1196–1216, because in the presence of cDNA 1197–1216 neither fragment of 18S rRNA that remained after the hydrolysis contained the crosslinked 32 P-labeled mRNA analog (Fig. 4A, lane 2). Results with other cDNAs seen in the neighboring lanes are consistent with this identification. The same results were obtained for complex 2 (data not shown). In complex 3, the crosslinking site of derivative I is located within 18S rRNA region 840–1081, as may be easily estimated from the data presented in Figure 4B.

For [5'- 32 P]pUUU(azidoG)UU in complex 2, the crosslinking site was initially found in the 3'-terminal region (about 230 nt long) of 18S rRNA, as RNase H digestion

TABLE 1. Binding and crosslinking of 5'-³²P-labeled mRNA analogs to human ribosomes.

Type of complex ^a	mRNA analog ^b	Binding (mol of mRNA analog per mol of 80S ribosomes)	Crosslinking (mol of mRNA analog per mol of 40S subunits)	Distribution of cross-linking between 18S rRNA and proteins % ^c	
				Proteins	18S rRNA
-tRNA	I	0.02 ± 0.01	0.03 ± 0.01	n.d. ^d	n.d.
1	I	0.78 ± 0.04	0.52 ± 0.03	30	70
2	I	0.75 ± 0.04	0.47 ± 0.03	40	60
3	I	0.14 ± 0.02	0.10 ± 0.01	50	50
-tRNA	II	0.02 ± 0.01	<0.01	n.d.	n.d.
1	II	0.53 ± 0.04	0.36 ± 0.03	30	70
2	II	0.52 ± 0.04	0.25 ± 0.02	45	55
3	II	0.11 ± 0.02	0.05 ± 0.01	70	30

^a1, 2, 3: types of complexes according to Figure 2.^bmRNA analogs: (azidoU)UUGUU, I; UUU(azidoG)UU, II.^cAccuracy of the determination was within 10%.^dn.d.: not determined.

of the crosslinked 18S rRNA in the presence of cDNA 1612–1639 resulted in the occurrence of the cross-linked mRNA analog only in the small fragment rather than in the large one (Fig. 5, lane 2). More precise determination of the crosslinking site was achieved with the use of cDNA 1812–1831. Cleavage of the cross-linked 18S rRNA in the presence of this cDNA resulted in disappearance of the majority of ³²P label from high-molecular-weight rRNA (compare lanes 1 and 3 in Fig. 5). This means that the crosslinking site was not 5' to position 1812. On the other hand, the 3'-terminal sequence with the crosslinked labeled derivative II that would be formed if the crosslinking site was 3' to position 1831 was not detected. Therefore, the crosslinking site was located within positions 1812–1831. The same experiments for complex 1 showed that the cross-

linked site was also located within the sequence 1812–1831 of 18S rRNA (data not shown).

The exact identification of the crosslinked 18S rRNA nucleotides was made using reverse transcriptase analysis (Fig. 6). Primer extension makes a stop or pause at the crosslinked rRNA nucleotide. The crosslinking site is generally assumed to be nucleotide 5' to the primer extension stopping site. Therefore, in the case of (azidoU)UUGUU, the crosslinking sites are G1207 for both complexes 1 and 2, and G961 for complex 3; in the case of UUU(azidoG)UU, the crosslinking sites are A1823 and A1824 for complexes 1 and 2. In complex 2, the stops are less pronounced, which is consistent with the lower crosslinking extent in complex 2 as compared to complex 1.

DISCUSSION

Both types of the UUUGUU derivatives used in this study were unable either to bind or to crosslink to human 80S ribosomes without the cognate tRNAs (see Fig. 3 and Table 1). This is typical for all short mRNA analogs that had been used earlier for investigation of the decoding center of human 80S ribosomes (e.g., see Malygin et al., 1994; Bulygin et al., 1997a, 1997b; Smolenskaya et al., 1998). Therefore, crosslinking of the UUUGUU derivatives actually occurred at the ribosomal decoding center and the contribution of any unspecific (tRNA-independent) crosslinking was almost negligible. The binding properties of the mRNA analogs depended mostly on the codon involved in the codon-anticodon interaction at the ribosomal P site; the modifying groups did not affect the binding significantly. tRNA^{Phe} (cognate to the UUU codon) stimulated binding of each of the UUUGUU derivatives to 80S ribosomes more effectively than tRNA^{Val} (cognate to the GUU codon). The same effect had been observed ear-

FIGURE 3. Analysis of crosslinking of [5'-³²P]p(azidoU)UUGUU (A) and [5'-³²P]pUUU(azidoG)UU (B) to rRNA and tRNA by electrophoresis in 4% polyacrylamide gels. The images are from autoradiograms of the gels. Numbers on lanes correspond to complex types (see Fig. 2). Lanes 4 are crosslinking without tRNAs. Positions of 18S rRNA and tRNA are marked as they were seen in the gels stained with "Stains All."

FIGURE 4. Ribonuclease H digests of 18S rRNA bearing the cross-linked [5'-³²P]-p(azidoU)UUGUU analyzed on 4% polyacrylamide gels. The images are from autoradiograms of the gels. Crosslinked RNA was isolated from the irradiated complexes 1 (A) or 3 (B). Lanes 2–4 correspond to the digestion experiments in the presence of oligodeoxyribonucleotides complementary to 18S rRNA positions 1197–1216, 1081–1100, and 821–840, respectively. Lanes 1 are control rRNA treated with ribonuclease H without cDNAs. Positions of 18S rRNA and the excised fragments (the expected lengths are marked) are indicated as they were seen in the stained gels.

lier in a crosslinking study with derivatives of hexaribonucleotides that contained Phe and Val codons and a photoreactive group at the 5'-phosphate (Bulygin et al., 1997a). This implies that the P site of human 80S ribosome has higher affinity to tRNA^{Phe} with the cognate codon UUU than to tRNA^{Val} with its cognate codons. The binding data indicate that a modifying group at either the C5 atom of the 5'-terminal uracil or at the N7 atom of the guanine of UUUGUU actually did not interfere significantly with involvement of the respective modified codon in the codon–anticodon interaction. In accordance with all previous data on crosslinking of various mRNA analogs to human 80S ribosomes (Graifer et al., 1990; Karpova et al., 1992; Mundus et al., 1993; Malygin et al., 1994; Bulygin et al., 1997a, 1997b;

FIGURE 5. Analysis of ribonuclease H digests of 18S rRNA cross-linked to [5'-³²P]-pUUU(azidoG)UU in complex 2. The images are from autoradiograms of the gels. Lanes 2 and 3 correspond to the digestion experiments in the presence of oligodeoxyribonucleotides complementary to 18S rRNA positions 1621–1640 and 1812–1831, respectively. Lane 1 is control rRNA treated with ribonuclease H without cDNAs. Positions of 18S rRNA, tRNA, and the excised fragments (the expected lengths are marked) are indicated as they were seen in the stained gel.

Smolenskaya et al., 1998), the 40S subunit was the main target for crosslinking; only the ratio of the 18S rRNA/proteins modification depended on the reagent and complex types. It should be noted that for both mRNA analogs used in this study, yields of the crosslinking were very high (up to 65% of the bound mRNA analog). Similar yields of photocrosslinking have been observed for mRNA analogs with a *p*-azidotetrafluorobenzoic group attached to the 5'-phosphates (Bulygin et al., 1997a). The same photoactivable group attached to the 5'-phosphates of deoxyoligoribonucleotides indicated similarly high (up to 70%) yields of the crosslinking of these derivatives to the complementary DNA sequences (Levina et al., 1996). Thus, the high yield of photocrosslinking of nucleic acids derivatives that bear the *p*-azidotetrafluorobenzoic group is an advantage caused by the features of the crosslinking group itself.

In the majority of complexes, the mRNA analogs crosslinked not only to the 18S rRNA and proteins, but also to tRNA (Fig. 3). Moreover, in complex 3, derivative II crosslinked to tRNA much more effectively than

FIGURE 6. Identification of sites of cross-linking of 18S rRNA to (azidoU)UUGUU (**A, B**) and to UUU(azidoG)UU (**C**) by reverse transcription. Extension of [5'-³²P]-labeled primer complementary to 18S rRNA positions 1222–1241 (**A**), 1081–1100 (**B**), or 1830–1849 (**C**) on 18S rRNA isolated from irradiated complexes. Lane numbers correspond to complex types (see Fig. 2). Lanes 4 correspond to the experiments without tRNA. Lanes 5 are control 18S rRNA isolated from 80S ribosomes irradiated without azido-mRNAs and treated in the same way as the experimental ones. Lanes U, G, C, and A: sequencing of 18S rRNA. Positions of the reverse transcription stops caused by the crosslinking are indicated.

to 18S rRNA (Fig. 3B). This means that the photoactivated group at the N7 atom of the guanine of the P site-bound modified GUU codon was shielded by tRNA^{Val} from contacts with the 18S rRNA. In contrast, the reactive group at the C5 atom of the 5'-terminal uracil of the P site-bound modified UUU codon (derivative I, complex 1; see Fig. 2) crosslinked much more effectively to the 18S rRNA than to the tRNA^{Phe} molecule (Fig. 3B). In a complex where the modified nucleotide of the mRNA analog was far from the codon-anticodon interaction area (complex 3, derivative I), crosslinking to tRNA was negligible (see Fig. 3A). The photoreactive group at the guanine of the A site-bound modified GUU codon (complex 1, derivative II) was able to crosslink to tRNA^{Phe} bound at the neighboring P site; occupation of the A site (complex 2, derivative II) resulted in much more effective crosslinking to tRNA (evidently, to the A site-bound molecule) and less ef-

fective crosslinking to 18S rRNA (see Fig. 3B). Thus, the tRNA^{Val} molecule base paired with the modified GUU codon at the A site shields the reactive group at the N7 atom of the guanine from contacts with the 18S rRNA similar to its behavior when the modified GUU codon is at the P site (see above).

18S rRNA nucleotides crosslinked to mRNA analogs

Results obtained in this study demonstrate the 18S rRNA environment of the first nucleotide of mRNA codon at the A, P, and E sites; the data are summarized in Table 2. Crosslinks of mRNA analogs that bear a photoreactive group at the 5'-phosphate in complexes analogous to complex 1 (the reactive group at position +1) to G1207 of human 18S rRNA were detected earlier (Bulygin et al., 1997a). Thus, crosslinking to G1207 from mRNA position +1 does not depend on the point of attachment of the reactive group in the modified nucleotide, and G1207 is a key element of the ribosomal environment of the first nucleotide of the P site-bound codon. It should be noted here that a derivative of pUUUUUU that carried an alkylating group at the 5'-terminus was found to crosslink to 18S rRNA nucleotide C1057 in complexes with human 80S ribosomes and tRNA^{Phe} molecules at both the A and P sites (Malygin et al., 1994). In this case, the alkylating group was at mRNA position +1, but crosslinking occurred at C1057 rather than at G1207, most probably because of the different chemical and/or stereochemical selectivity of the reactive intermediates formed by the aryl azide and the alkylating group (discussed in Bulygin et al., 1997a).

Crosslinking to 18S rRNA nucleotides A1823 and A1824 was not detected earlier. The mRNA analog UUUC with an alkylating group at the 3'-terminus was found earlier to crosslink to 18S rRNA nucleotides G1702 and G1763/G1764 in a complex with human 80S ribosomes and Phe-tRNA^{Phe} at the P site (Malygin et al., 1994). In the latter case, the position of the reactive group on the 80S ribosome was +4 (if codon UUU was base paired with Phe-tRNA^{Phe}) or +3 (codon UUC base paired with Phe-tRNA^{Phe}). Data on the crosslinking of a reactive group at mRNA analog position +4 to 18S rRNA nucleotides A1823 and A1824 (results of this study) and G1702 (Malygin et al., 1994) are in a good agreement, because both 18S rRNA sites are located close to each other in a region of 18S rRNA secondary structure (see Fig. 7), which corresponds to the "16S rRNA decoding site" (Van Loock et al., 1999; Yoshizawa et al., 1999). Nucleotide G1207, which crosslinks to a modified mRNA nucleotide at position +1, is also located in the vicinity of the region mentioned (Fig. 7).

Crosslinking of mRNA analogs to 18S rRNA nucleotide G961 from position -3 has also been detected for the first time. In our previous work, synthetic mRNAs

TABLE 2. Summary of the data on crosslinking of mRNA analogs to human 18S rRNA.

mRNA position	Cross-linked rRNA nucleotide	Reference	Corresponding nucleotide in <i>E. coli</i> 16S rRNA		
			Position	Extent of conservation ^a	Relation to the 16S rRNA decoding site
+4	A1823 A1824	This study	G1491 A1492	Conserved Invariant	Suggested to form hydrogen bond with mRNA position +5 (Yoshizawa et al., 1999)
+3/+4	G1702	Malygin et al., 1994	G1401	Invariant	Keystone nucleotide at the decoding site (Cunningham et al., 1992); G1402 crosslinks to mRNA position +4 (Rinke-Appel et al., 1994)
	G1763, G1764	Malygin et al., 1994	1450, 1451	Variable	Not found
+1	G1207	This study and Bulygin et al., 1997a	G926	Invariant	Crosslinks with mRNA Position +2 (Sergiev et al., 1997)
+1	G1057	Malygin et al., 1994	U789	Conserved	Is located in about 25 Å from G926 and G693 (Mundus & Wollenzien, 1998)
−3	G961	This study	G693	Invariant	Crosslinks with mRNA positions −1/−3 (Bhangu et al., 1994)

^aAccording to Neefs et al., 1990.

that contained 4-thiouridines at positions −1 and −3 crosslinked to human 18S rRNA nucleotides U1111/A1112 (Graifer et al., 1994b). However, the crosslinking was tRNA independent, and this may be the reason for the discrepancy with the results of the present study.

Functional conservation at the small subunit rRNA decoding site

The majority of nucleotides of 18S rRNA found to be crosslinked to the mRNA analogs in this study and in previous reports are located in the most highly conserved regions of the small subunit rRNA secondary structure (see Fig. 7 and Table 2). Four of them correspond to 16S rRNA nucleotides A1492, G1401, G926, and G693, which had been found earlier at the 16S rRNA decoding site (see Table 2). 18S rRNA nucleotide C1057, which crosslinks to mRNA position +1 (Malygin et al., 1994), corresponds to 16S rRNA nucleotide U789, which has never before been crosslinked to a mRNA. On the other hand, U789 is located not far from the 16S rRNA decoding site (see Table 2). In general, it is seen that at each ribosomal site, A, P, or E, there are strongly conserved small subunit rRNA nucleotides that surround the mRNA codons in both *E. coli* and human ribosomes.

Because the structures of mammalian ribosomes are not available at a resolution that permits the location of specific helices of the rRNA, our data may be interpreted in terms of the partial atomic structures of the bacterial 30S ribosomal subunit. Thus, the approximate positions of 18S rRNA nucleotides G961 (G693, the tip of helix 23), C1057 (U789, helix 24), G1207

(G926, helix 27) and G1702 (G1401), G1763–G1764 (~1450–1451), and A1823–A1824 (G1491–A1492) in helix 44 can all be discerned on the crystallographic maps of the *Thermus thermophilus* 30S subunit at a 5.5 Å resolution (Clemons et al., 1999). Nucleotides G1207 (G926), G1702 (G1401), and A1823–A1824 (G1491–A1492), which are labeled from the +1 to +4 mRNA positions, are nicely clustered on the segment of 30S subunit known as the decoding site (see Clemons et al., 1999); nucleotide C1057 (U789) is located in the vicinity of this segment. Two other crosslinking sites, G961 (G693) and G1763–G1764 (1450–1451) are separated from this cluster and from each other. Positions 1450–1451 lie at the tip of the long penultimate helix 44 very far from the decoding site (Cate et al., 1999; Clemons et al., 1999). Helix 44 is a very well conserved feature of small subunit rRNAs and it is quite unlikely that it differs substantially in eukaryotes and bacteria. Thus, crosslinking of mRNA positions +3/+4 to G1763/G1764 should be considered as an artifact. The source of the error might be the prolonged incubations at room temperature (required for the labeling of 80S ribosomes with the 3'-alkylating mRNA analogs; see Malygin et al., 1994) which could lead to a partial degradation of the ribosomes and, subsequently, to artifactual crosslinking in the fraction of the degraded ribosomes. As for nucleotide G961 (G693), which crosslinks to the E site-bound codon, we do not see any reasons to doubt the validity of the result; moreover, the analogous crosslink has been detected for *E. coli* ribosome (see Table 2). One of the possible explanations for the separate position of this nucleotide from the decoding site on the structure of the free 30S

FIGURE 7. Secondary structure of human 18S rRNA according to Gutell (1993). mRNA positions (in boxes) and the crosslinked nucleotides of the 18S rRNA are marked. Solid arrows indicate cross-links found in this study. Dotted arrows indicate crosslinking sites determined earlier, namely, C1057, G1702, and G1763/1764 (Malygin et al., 1994) and U1111/A1112 (Graifer et al., 1994b). Numbers of the corresponding nucleotides of *E. coli* 16S rRNA are given in parentheses.

subunit (Clemons et al., 1999) may be that binding of mRNA and tRNA to the subunit alters its structure and brings this rRNA position closer to the decoding site.

MATERIALS AND METHODS

tRNA and DNA

Val-tRNA^{Val} (1,800 pmol/A₂₆₀ unit) and Phe-tRNA^{Phe} (1,300 pmol/A₂₆₀ unit) were kindly provided by Dr. T. Shapkina (B.P. Konstantinov's St. Petersburg Institute of Nuclear Physics, Gatchina, Russia). DNA 20-mers complementary to human 18S rRNA sequences 821–840, 1081–1100, 1197–1216, 1621–1640, and 1830–1849 were synthesized in the Novosibirsk Institute of Bioorganic Chemistry; oligomers complementary to the sequences 1222–1241 and 1812–1831 were kindly donated by Prof. P. Wollenzien (North Carolina State University). 4-[(N-2-chloroethyl-N-methylamino)]benzylamine, CIRCH₂NH₂, and *p*-azidotetrafluorobenzoic acid *N*-oxysuccinimide ester were provided by Dr. T.M. Ivanova (Novosibirsk Institute of Bioorganic Chemistry).

Preparation of ³²P-labeled mRNA analogs

Synthesis of a derivative of hexaribonucleotide UUUGUU that carries the 2-(4-azido-2,3,5,6-tetrafluorobenzoyl)-aminoethyl group at the C5 atom of the 5'-terminal uracil, (azidoU)UUUGUU (Fig. 1) and confirmation of its structure was carried out as described by Repkova et al. (1999). The synthesized (azidoU)UUUGUU was dissolved in water and stored in liquid nitrogen. Labeling of the derivative by [γ -³²P]ATP and polynucleotide kinase and subsequent purification of the [5'-³²P]p(azidoU)UUUGUU was carried out according to Smolenskaya et al. (1998).

Synthesis of unmodified hexaribonucleotide UUUGUU was carried out using the solid-phase H-phosphonate method as described earlier (Ven'yaminova et al., 1990). Perfluoroaryl azide group was introduced at the guanine in two steps. First, the hexaribonucleotide was alkylated with CIRCH₂NH₂ by incubating the hexaribonucleotide (0.5 A₂₆₀ units) with 500 nmol of the reagent in 0.16 mL of 8 mM Tris-HCl, pH 7.5, for 5 h at 37°C. The product of alkylation was isolated by a reversed-phase chromatography on a microcolumn packed with a C18-containing sorbent (Smolenskaya et al., 1998); it was eluted at a higher concentration of methanol than the initial hexamer. The extent of incorporation of —RCH₂NH₂ group into UUUGUU was about 90%. The fraction harboring the alkylated UUUGUU was evaporated to dryness in vacuum. Then, the photoactivatable moiety was coupled to the aliphatic amino group by treatment with *p*-azidotetrafluorobenzoic acid *N*-oxysuccinimide ester in the same way as at the final step of (azidoU)UUUGUU synthesis (see above). The product was precipitated from the reaction mixture by 2% LiClO₄ in acetone in the presence of 1 μ L of 0.1 M ATP as a carrier, then reprecipitated, dissolved in water, and purified by reversed-phase chromatography (see above). The obtained derivative, UUU(azidoG)UU, due to the presence of the aryl azide group, eluted at a much higher methanol concentration than the alkylated UUUGUU. The yield was about 50% per initial UUUGUU. The peak of the purified UUU(azidoG)UU

was evaporated in vacuum to dryness, dissolved in water, and stored in liquid nitrogen. Before use, it was 5'-labeled as in the case of (azidoU)UUUGUU.

Preparation of ribosomal complexes and analysis of crosslinking

Isolation of 40S and 60S ribosomal from unfrozen human placenta and their association into the 80S ribosomes were performed as described earlier (Matasova et al., 1991).

Complexes of 80S ribosomes (taken in a final concentration of 8×10^{-7} M) with the 5'-³²P-labeled derivatives of UUUGUU (4×10^{-6} M) and the cognate tRNAs (4×10^{-6} M for Phe-tRNA^{Phe} and 7×10^{-6} M for Val-tRNA^{Val}) were obtained and examined as described in Bulygin et al. (1997a). Irradiation of the complexes with mild UV light (wavelength > 280 nm), analysis of the crosslinking to the ribosomal subunits, and distribution of the crosslinks between the ribosomal RNA and the proteins within the subunits were carried out according to Bulygin et al. (1997a). Total RNA was isolated from the crosslinked ribosomes using a method described by Graifer et al. (1994b). Regions of 18S rRNA that contained the crosslinked mRNA analog were examined using hydrolysis of the crosslinked rRNA with RNase H in the presence of deoxy 20-mers complementary to various rRNA sequences as described by Graifer et al. (1997). The exact sites of crosslinking to the 18S rRNA were established by AMV reverse transcriptase analysis according to Wollenzien (1988). Each reaction mixture contained about 1 pmol of the crosslinked or control 18S rRNA and the same amount of the 5'-³²P-labeled primer. After reverse transcription reaction, the samples were treated as described earlier (Graifer et al., 1994b).

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