Cross-Linking of mRNA Analogues Containing 4-Thiouridine Residues on the 3′- or 5′-Side of the Coding Triplet to the mRNA Binding Center of the Human Ribosome†

Dmitri M. Graifer,‡ Dalia I. Juzumiene,§,‖ Paul Wollenzien,*,§ and Galina G. Karpova‡

Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the Russian Academy of Sciences, 630090 Novosibirsk, Russia, and Department of Biochemistry, Box 7622, North Carolina State University, Raleigh, North Carolina 27695

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ABSTRACT: The interaction between mRNA and 18S rRNA within complexes of human placenta 80S ribosomes has been investigated by photochemical cross-linking experiments using mRNA analogues substituted with 4-thiouridine at specific locations. mRNA analogues 51 or 54 nucleotides long were prepared from synthetic DNA templates. These mRNA analogues contained either the sequence GGG–ACC (coding for glycine and threonine, respectively) or the single triplet GGG together with 2–4 4-thiouridine residues located at various positions with respect to the coding triplets. The products of cross-linking of the mRNA analogues to 18S rRNA within different model complexes without tRNA or in the presence of cognate tRNAs were analyzed by reverse transcription. Two cross-linking sites in the 18S rRNA were detected. The first site, U630, was cross-linked by mRNA 8′ (s^4U at +20, +22, +24, and +26), mRNA 9e′ (s^4U at −16, −18, and −20), and mRNA 10 (s^4U at +4, +6, −1, and −3) but, unexpectedly, not with either mRNA 10b (s^4U at +4 and +6) or mRNA 10c (s^4U at −1 and −3). The second site, U1111/A1112, was cross-linked by mRNA 10 and mRNA 10c but not by any of the other mRNA analogues. There is significant tRNA dependence on cross-linking only for mRNA analogue 9e′. Both of the sites detected correspond to sites of mRNA cross-linking in *Escherichia coli* 16S rRNA.

The interaction of mRNA with ribosomes is a topic of various investigator groups. For ribosomes from *Escherichia coli*, ribosomal proteins and sites in the rRNAs interacting with mRNA at different steps of initiation and elongation have been identified by affinity labeling techniques. A fruitful approach for this purpose has been the use of synthetic mRNA analogues with 4-thiouridine (s^4U)[1] as a photoaffinity probe placed at a single definite position (Stade et al., 1989; Rinke-Appel et al., 1991, 1993; Dontsova et al., 1991) or placed randomly (Wollenzien et al., 1991; Bhangu & Wollenzien, 1992). The former mRNA analogues allowed the identification of a limited number of mRNA cross-linking sites to 16S rRNA [principally cross-links to the region 1390–1400, the 3′-terminal region, and the nucleotide A532 (Rinke-Appel et al., 1991, 1993; Dontsova et al., 1992)]; the latter approach allowed the identification of a larger number of mRNA cross-linking sites, including two sites in the large subunit rRNA (Wollenzien et al., 1991; Bhangu & Wollenzien, 1992).

mRNA analogues with a random distribution of s^4U residues were used for the investigation of the mRNA track on human ribosomes (D. Graifer, D. Juzumiene, G. Karpova, and P. Wollenzien, unpublished results). A single nucleotide, U630, was the main target for two different mRNA analogues as detected by reverse transcription analysis. This nucleotide is located in a highly conserved part of the small subunit rRNA secondary structure and corresponds to U534 in the *E. coli* 16S rRNA (Neefs et al., 1990; Gutell, 1993). In addition, three other minor sites (U966, U1046, and A1060) were seen in human 18S rRNA. Thus, the labeling of human ribosomes was shown to proceed much more selectively compared to *E. coli* ribosomes. These data indicate some similarities, but also substantial differences in the organization of mRNA binding centers on prokaryotic and eukaryotic ribosomes, requiring study of the problem in more detail.

The goal of the present study is the investigation of cross-linking of human ribosomes with synthetic mRNA analogues containing s^4U residues at various definite positions with respect to the coding triplets. The pattern of reaction of two sites in 18S rRNA cross-linked by s^4U upstream and downstream of the codon in the mRNA analogue is described.

MATERIALS AND METHODS

Materials. Nucleoside triphosphates and dideoxynucleoside triphosphates were purchased from Pharmacia. DNA oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and were purified by electrophoresis on 12% polyacrylamide gels containing urea before use. AMV reverse transcriptase (type 007) was purchased from Life Sciences. T4 polynucleotide kinase was from U.S. Biochemicals. $tRNA^{Gly}$, $tRNA^{Thr}$, and glycogen were purchased from Boehringer Mannheim. [γ-^{32}P]ATP was from Amersham. Qiagen columns were purchased from Qiagen, Inc. (Chatsworth, CA). Ribosomal subunits (40S and 60S) containing intact ribosomal RNA were isolated from unfrozen human placenta by the method described by Matasova et al. (1991). The subunits were reactivated by incubation at 37 °C for 10 min in binding buffer (13 mM $MgCl_2$, 0.5 mM EDTA, 120

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* Author to whom correspondence should be addressed.
‡ Novosibirsk Institute of Bioorganic Chemistry.
§ North Carolina State University.
‖ Permanent address: Institute of Biochemistry, Lithuanian Academy of Sciences, Vilnius, Lithuania.

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[1] Abbreviations: dsDNA, double-stranded DNA; EDTA, (ethylenedinitrilo)tetraacetic acid; nt, nucleotide(s); poly(U), poly(uridylic acid); s^4U, 4-thiouridine; TBE buffer, 89 mM Tris, 89 mM boric acid, and 10 mM EDTA; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol.

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Table 1: Sequences of the mRNA Analogues[a]

		+20.....+26
mRNA 8′	5′ GGCAGAGCGGCACAGGAGCGCAAC GGG ACCGCACAGCCGAGAGUCUGUCUA 3′	
	Gly Thr	
	-20.....-16	
mRNA 9e′	5′ GGCACACUAUCUGCACAGGAGCGCAAC GGG ACCGCACAGCCGAGAGCCAGACGA 3′	
	Gly Thr	
	-3...-1 +4...+6	
mRNA 10	5′ GGCAGAGCGGCACAGGAGCGCUAU GGG UGUGCACAGCCGAGAGCCAGACGA 3′	
	Gly	
	-3...-1	
mRNA 10c	5′ GGCAGAGCGGCACAGGAGCGCUAU GGG ACCGCACAGCCGAGAGCCAGACGA 3′	
	Gly Thr	
	+4...+6	
mRNA 10b	5′ GGCAGAGCGGCACAGGAGCGCCAC GGG UGUGCACAGCCGAGAGCCAGACGA 3′	
	Gly	

[a] The codon (GGG) for $tRNA^{Gly}$ is aligned in the middle of each mRNA. For mRNA analogues 8′, 9e′, and 10c, the codon ACC coding for $tRNA^{Thr}$ is also present. The beginning and end of each block of uridines that is substituted with s^4U in the test RNA molecules are numbered. +1 is the first nucleotide of the codon.

mM KCl, and 20 mM Tris-HCl, pH 7.5) before use. The 80S ribosomes were obtained by reassociation of the subunits in the binding buffer at room temperature. The activity of 80S ribosomes in poly(U)-dependent binding of [^{14}C]Phe-$tRNA^{Phe}$ was 1.6 mol of the tRNA per mole of ribosomes (80% of maximum).

mRNA Analogues. mRNA analogues were prepared by transcription with T7 RNA polymerase from synthetic DNA templates (Bhangu et al., 1994). Their sequences are written in Table 1. For photoaffinity experiments, uridine positions in the mRNA were completely substituted with s^4U by the use of 0.25 mM s^4UTP, 3 mM each of ATP, GTP, and CTP, and 1 mM GMP in the transcription reactions. RNA for control experiments was made with 3 mM UTP in place of 0.25 mM s^4UTP. After transcription, RNA was purified on Qiagen-tip 5 columns according to directions from the manufacturer. Before use, the mRNA analogues were labeled at their 5′-termini by exchange with [γ-^{32}P]ATP and T4 polynucleotide kinase (Bhangu & Wollenzien, 1992). All mRNA analogues were checked for complete transcription by gel electrophoresis before use.

Complex Formation and Cross-Linking. Formation of complexes of 80S ribosomes with mRNA analogues and tRNAs was done with 4-fold excess of mRNA and tRNA over ribosomes in binding buffer for 1 h at room temperature. Typically, 7 pmol of 80S ribosomes were reacted in 32 μL total volume. Cross-linking was done at 25 °C for 10 min in the irradiation device described by Isaacs et al. (1977), which produces near-UV light in the range 320–365 nm. After cross-linking, the samples were phenol-extracted in the presence of 1% SDS and 20 mM EDTA and concentrated by precipitation with 10 vol of 2% $LiClO_2$ in acetone. The RNA was washed with 70% ethanol and vacuum-dried before being redissolved at a final concentration of 6 pmol of 18S rRNA/μL in H_2O.

Cross-Link Analysis. Levels of cross-linking of mRNA analogues to ribosomal RNA were determined by agarose gel electrophoresis. RNA from cross-linking experiments was mixed with 2 vol of formamide and heated at 45 °C for 5 min before electrophoresis on 1% agarose gel made in TBE buffer. Levels of cross-linking were determined by laser densitometry of the autoradiograms of the dried gels. Identification of the cross-linked nucleotides in 18S rRNA was performed by primer extension using AMV reverse transcriptase as previously described (Wollenzien, 1988). Eight synthetic DNA oligomers (20-mers) complementary to the 18S rRNA were used as primers. For each reverse transcription reaction, samples usually contained 1 pmol of 18S rRNA and 2 pmol of ^{32}P-labeled primer. The terminal 57 nucleotides in the 3′-end of 18S rRNA were not examined. The sites of the primers' complementarity are as follows: 18S-1A, 1812–1831; 18S-2, 1222–1241; 18S-3, 655–674; 18S-10, 1398–1417; 18S-12, 1142–1161; 18S-20, 950–969; 18S-23, 372–391; and 18S-27, 1548–1567. The primers allowed the examination of most of the 18S rRNA sequence at single-nucleotide resolution, except for about 150 nucleotides at the 5′-end. After reverse transcription, samples were phenol-extracted, treated with 0.16 M NaOH for 1 h at 45 °C to hydrolyze RNA, and ethanol-precipitated. They were washed with 70% ethanol, dried, and redissolved in 3 μL of H_2O. Samples were then analyzed by electrophoresis on 8% polyacrylamide/8 M urea gels.

RESULTS

Characteristics of the Synthetic mRNA Analogues. The mRNA analogues for these experiments were synthesized by *in vitro* transcription from synthetic DNA templates. These RNAs are 51–54 nucleotides long and contain either the sequence GGG–ACC (coding for glycine and threonine, respectively) or the single triplet GGG approximately in the middle of each RNA. The mRNA analogues contain two or four s^4U residues located at various positions with respect to the coding triplets (Table 1). All of the RNA molecules

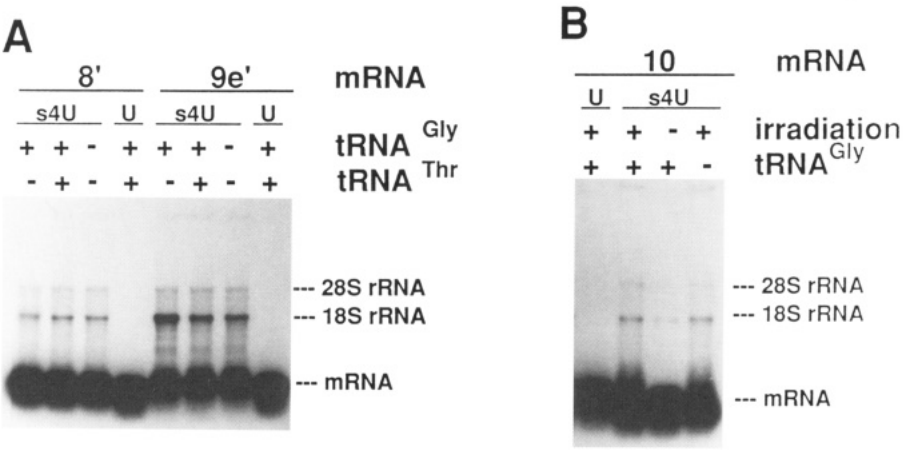

FIGURE 1: Agarose gel electrophoresis of RNA after cross-linking reactions with mRNA analogues 8′, 9e′, and 10. The mRNAs analogues were synthesized with uridine (U) or 4-thiouridine (s^4U) for use in control or cross-linking experiments and were ^{32}P-labeled. The figure shows autoradiograms of dried agarose gels. $tRNA^{Gly}$ and $tRNA^{Thr}$ were included in the complexes as indicated. The locations of 28S, 18S, and mRNA bands seen by ethidium staining are indicated. All samples shown in panel A were irradiated; all samples in panel B were irradiated except the control samples in lane 3, as indicated in the figure.

contain the Shine–Dalgarno sequence, AGGAG, upstream of the triplet GGG because they were used initially in experiments with eubacterial ribosomes; in eukaryotic cells, these sequences should not influence the binding of the mRNA analogues to the ribosome since it is known that the eukaryotic ribosome does not recognize the Shine–Dalgarno sequence. The mRNA analogues were tested for their ability to bind to human 80S ribosomes under the conditions used in the cross-linking experiments. The stoichiometry of binding of the mRNA analogues at 4-fold excess of mRNA over ribosomes was about 0.8–0.9 mol of mRNA/mol of ribosomes, as determined by the nitrocellulose filter binding assay.

The complexes between 80S ribosomes, mRNA, and a cognate $tRNA^{Gly}$ at the P site, or $tRNA^{Gly}$ at the P site and $tRNA^{Thr}$ at the A site, were obtained under the ionic conditions described by Graifer et al. (1990) and then were irradiated with saturating amounts of light in the wavelength range 320–365 nm. As controls, complexes of 80S ribosomes, tRNA, and mRNA containing uridine instead of s^4U were irradiated. In some experiments, complexes were formed with 80S ribosomes, tRNA, and s^4U-containing mRNA analogues, but they were not irradiated as controls to determine whether cross-linking depended on irradiation.

The level of the mRNA cross-linking to ribosomal RNA was evaluated by agarose gel electrophoresis of denatured samples isolated from the irradiated reaction mixtures (Bhangu et al., 1994). Cross-linking was dependent on the presence of s^4U in the mRNA analogues and on irradiation, and both 18S and 28S rRNA were labeled, but with labeling preferentially to 18S rRNA in all cases (see Figure 1). The percentages of 18S and 28S rRNA cross-linked to mRNA were about 8% and 2% for mRNA analogue 8′, 14% and 2% for mRNA analogue 10, and 20–40% and 5% for mRNA analogue 9e′, respectively. The variation for 18S rRNA cross-linking by 9e′ depended on the presence of $tRNA^{Gly}$, with the lower amount without and the higher amount with the tRNA. The levels of cross-linking with the mRNA analogues 10c and 10b were similar to those with mRNA analogue 10.

Localization of Cross-Linking Sites in 18S rRNA. Sites of cross-linking of the mRNA analogues to 18S rRNA were determined by reverse transcription analysis using a set of oligonucleotide primers complementary to different sites. The locations of the cross-linked nucleotides in 18S rRNA were identified by polyacrylamide gel electrophoresis of the reverse transcripts in parallel with lanes containing sequencing and control reactions. The principal controls for this analysis were reverse transcripts made from RNA from irradiated complexes formed with nonthiolated mRNA analogues. The cross-linking sites reported here were observed in at least two, and in some cases as many as four, independent irradiation and reverse transcription experiments. Reverse transcriptase stops were assigned as cross-linking sites if they were visibly stronger in the autoradiograms in the experimental lanes as compared to the stops in control ones. There is always some degree of banding at each position due to some natural feature of the RNA or partial damage of the RNA upon isolation or during analysis. Irradiation of ribosomes by itself did not induce any extra intensity of stops on the autoradiograms (data not shown). The cross-linking sites reported here correspond to the nucleotide on the 5′-side of the reverse transcription stopping site.

Autoradiograms showing the pattern of reverse transcription in the 650–610 region are shown in Figure 2. All three of the mRNA analogues 8′, 9e′, and 10 produce a strong cross-link at the same position, U630, in this region (Figure 2A). There is no significant tRNA dependence on the intensity of the reverse transcription stop for mRNA analogues 8 and 10, but there is a discernible increase in cross-linking by mRNA analogue 9e′ when $tRNA^{Gly}$ is present. For mRNA analogues 8′ and 9e′, which contain a threonine codon in addition to a glycine codon, there is no significant change in efficiency in the presence of $tRNA^{Thr}$.

Since the distribution of s^4U in mRNA 10 was on both the 5′- and 3′-sides of the codon (positions –3, –1, +4, and +6), an experiment was performed to determine whether a subset of the s^4U's could be assigned as the site from which the cross-link to U630 was made. However, in the experiments using mRNA 10c (s^4U at positions –3 and –1) or mRNA 10b (s^4U at positions +4 and +6), the cross-link to U630 was not made by either mRNA analogue (Figure 2B). Similar anomalous behavior of mRNAs 10a (s^4U positions –3 and –1) and 10b was previously seen in *E. coli* ribosomes at the corresponding site in the 16S rRNA (Bhangu et al., 1994).

The other position recognized as a cross-linking position in 18S rRNA in these experiments is the site U1111/A1112. Figure 3 shows autoradiograms of a reverse transcription experiment in the region that contains this site. mRNA 10

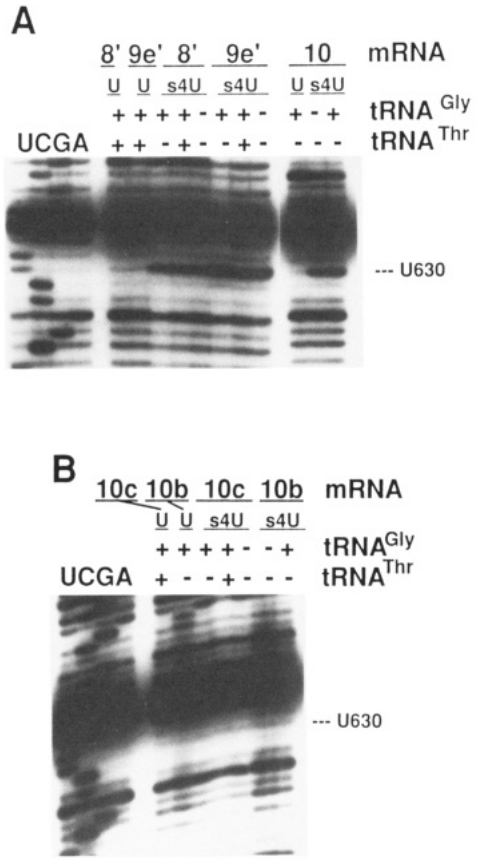

FIGURE 2: Reverse transcription analysis of mRNA cross-linking to 18S rRNA in the region of U630. In panel A, samples from cross-linking experiments were prepared with mRNA analogues 8′, 9e′, and 10 containing uridine (U) or s^4U as indicated. In panel B, samples of RNA from experiments with mRNA 10b and 10c are shown; this panel shows the *absence* of cross-linking at U630 for the mRNA analogues 10b and 10c. The complexes included $tRNA^{Gly}$ and $tRNA^{Thr}$ when indicated by a +. All samples on the right part of the gel were irradiated before purification of the RNA. The control and sequencing lanes on the left part of the gel were made with RNA that had not been irradiated; reverse transcription reactions contained dideoxynucleotides so that the sequence in the template could be read.

was the only mRNA in the first series of three mRNAs (8′, 9e′, and 10) that formed a cross-link at this site. In a further comparison, mRNA 10c (s^4U at positions –1 and –3) still made the cross-link, but mRNA 10b (s^4U at positions +4 and +6) did not (Figure 3B). Thus, the rRNA site U1111/A1112 is associated with the mRNA neighborhood just upstream of the codon. The presence of one (or two) cognate tRNAs filling the P site (and A site) did not alter the cross-linking efficiency. The results for the cross-links at U630 and U1111/A1112 are summarized in Table 2.

Table 2: Summary of Cross-Linking Sites in the 18S rRNA[a]

		18S rRNA sites	
mRNA	s^4U position(s)	U630	U1111/A1112
8′	+26, +24, +22, +20	+/+	
10b	+6, +4		
10	+6, +4, –1, –3	+/+	+/+
10c	–1, –3		+/+
9e′	–16, –18, –20	+/++	

[a] The two entries in the boxes for the cross-linking sites indicate the response without and with one cognate tRNA ($tRNA^{Gly}$). One tRNA effect was seen in mRNA analogue 9e′. Empty boxes indicate no detected cross-linking without or with cognate tRNA.

The remainder of the 18S rRNA, except for the 3′-terminal 60 nt, was analyzed for additional cross-linking sites, particularly in the regions that correspond to cross-linking sites made by the same mRNA analogues in 16S rRNA (Bhangu et al., 1994) or by alkylating agents in 18S rRNA (Malygin et al., 1993; Matasova et al., 1993; Mundus et al., 1993). No additional cross-links were seen.

DISCUSSION

Two sites—U630 and U1111/A1112—have been detected in human 18S rRNA as cross-linking sites in this study (Figure 4). The site U630 has also been seen in studies using two mRNA analogues with s^4U distributed throughout the mRNA sequence (Graifer et al., unpublished results). There is good, but not exact, correspondence between this mRNA cross-linking site and the mRNA cross-linking site A532 in *E. coli* 16S rRNA (Rinke-Appel et al., 1991, 1993; Wollenzien et al., 1991; Dontsova et al., 1992; Bhangu & Wollenzien, 1992). U630 in human 18S rRNA corresponds to U534 in *E. coli* 16S rRNA, given the primary sequence and secondary structure alignments. Even though the sites are two nucleotides apart, they probably represent the same mRNA functional interaction in the two types of ribosomes. U1111/A1112 correspond approximately to positions G838/C839 in *E. coli*

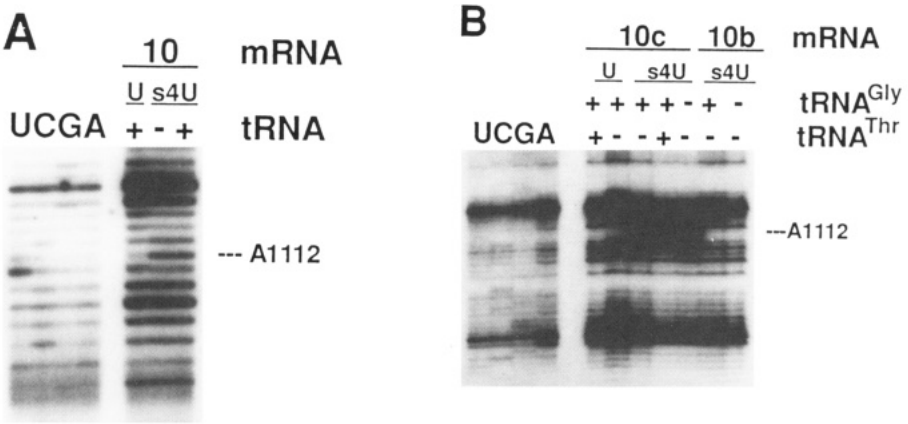

FIGURE 3: Reverse transcription analysis of mRNA cross-linking to 18S rRNA in the region surrounding U1111/A1112. In panel A the region 1120–1105 is shown (as detected with primer 18S-12). In panel B the region 1136–1085 is shown (as detected by primer 18S-2). mRNA analogues 8′ and 9e′ do not produce any cross-links in this area and are not shown. In both panels, the position corresponding to cross-linking at A1112 is indicated; because two bands are more intense in the pattern, the preceding nucleotide U1111 is probably a cross-linking point. In panel A, $tRNA^{Gly}$ was used as the tRNA where indicated, and in panel B, the complexes included $tRNA^{Gly}$ and $tRNA^{Thr}$ as indicated. All experimental samples in both panels were irradiated.

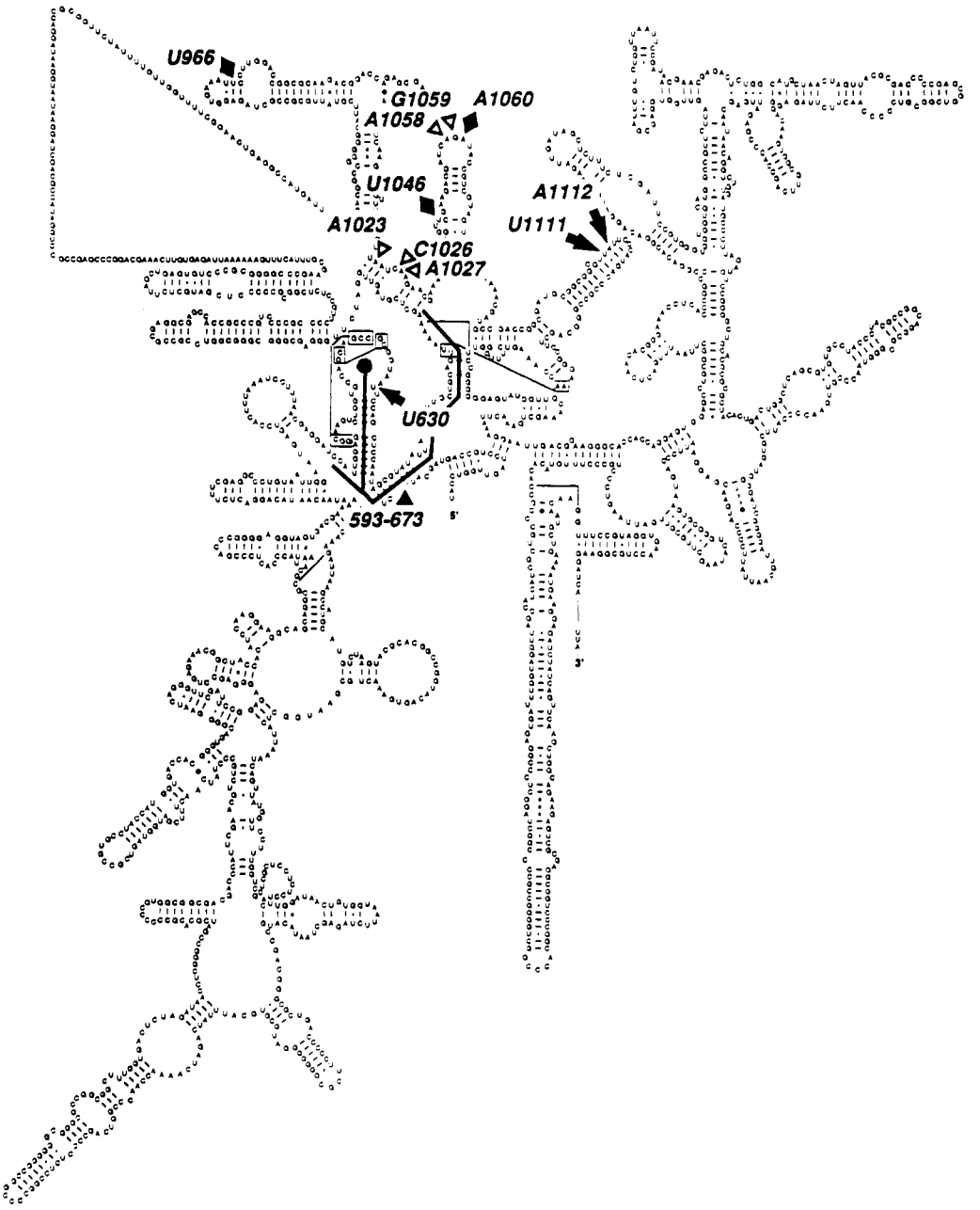

FIGURE 4: Summary of the sites of cross-linking in 18S rRNA. Cross-linking sites in 18S rRNA determined in this study and in previous studies are shown on the secondary structure proposed by Gutell (1993). Arrows indicate the sites U630 and U1111/A1112 determined in this study. Diamonds indicate three sites previously detected using mRNA analogues randomly substituted with s^4U (Graifer et al., unpublished results); U630 was also detected as the major cross-linking site in that study. Positions indicated by open triangles are sites cross-linked by an alkylating agent attached to the 5′-terminus of oligouridylate (Malygin et al., 1993). The interval 593–673, indicated by the filled triangle, contains the cross-linking site(s) made by alkylating agents at position −6 or +7 on mRNA analogues with respect to the codon being used (Matasova et al., 1993; Mundus et al., 1993).

16S rRNA (the primary sequence and secondary structure of human 18S rRNA are larger than that of *E. coli* 16S rRNA in this region). An mRNA cross-link to position A845 in *E. coli* 16S rRNA has been seen previously in several studies (Wollenzien et al., 1991; Bhangu & Wollenzien, 1992; Bhangu et al., 1994). Thus, as for the first site, the correspondence between the cross-link in the human 18S rRNA and *E. coli* 16S rRNA is not exact, but nevertheless indicates a close similarity in the arrangement of the rRNA region with respect to the mRNA track.

Three other minor cross-linking sites (U966, U1046, and A1060) seen using mRNA analogues randomly substituted with s^4U (Graifer et al., unpublished data) have not been detected in this study. This is not unexpected given that we have previously seen a reduced number of cross-linking sites in *E. coli* ribosomes when the mRNA analogues 8, 9e (similar to 8′ and 9e′ of this study), and 10 were used compared with the randomly substituted mRNA analogues (Wollenzien et al., 1991; Bhangu & Wollenzien, 1992; Bhangu et al., 1994).

The cross-linking of the mRNA analogues to U630 in human 18S rRNA has many parallels to the cross-linking of the same analogues to A532 in *E. coli* 16S rRNA (Bhangu et al., 1994). In human ribosomes, mRNA analogues 8′, 9e′, and 10, but not 10b or 10c, cross-linked to U630, and in *E. coli*, mRNA analogues 8, 9e, and 10, but not 10a (equivalent to 10c) or 10b, cross-linked to A532. Thus, U630 is cross-linked by the mRNA analogues 8′, 10, and 9e′, even though they contain s^4U placement sites separated by large distances (from position

–20 to position +26 with respect to the first nucleotide of the codon; see Table 1 for the sequences) along the mRNA molecule. With mRNA 9e′, the cross-link to U630 was to some extent more effective in the presence of $tRNA^{Gly}$ at the P site (see Figure 2A); the same effect occurred in *E. coli* ribosomes (Bhangu et al., 1994) with the mRNA analogue 9e, which contains s^4U in the same position as in mRNA analogue 9e′. The cross-link to A532 in *E. coli* has also been detected with a single s^4U residue placed at position +11 in an mRNA analogue (Rinke-Appel et al., 1991; Dontsova et al., 1992; D. I. Juzumiene and P. Wollenzien, unpublished results). Thus, the arrangement of the 3′- and 5′-sides of the mRNA track located relatively far from the coding triplet in the P site on both eukaryotic and bacterial ribosomes seems to be similar.

Cross-linking of mRNA analogues 10 (s^4U at positions –3, –1, +4, and +6) and 10c (s^4U at positions –3 and –1) with 18S rRNA at the site U1111/A1112 indicates that this site is located at the 5′-side of the area of the P site codon–anticodon interaction. This cross-link is observed at the same frequency without or with cognate tRNA. Since this site clearly is not reactive with s^4U in mRNA analogues 8′, 10b, and 9e′, this assignment between the rRNA site and the location in the mRNA track is quite specific. The behavior of the cross-linking site U1111/A1112 in humans is somewhat different from the behavior of the corresponding *E. coli* cross-linking site, A845. In *E. coli* 16S rRNA, A845 was cross-linked by s^4U placement sites in the middle and 5′-regions of the mRNA as far as positions –16 to –20, so that the assignment of it to a small interval of the mRNA track was much less specific.

The results on the cross-linking of mRNAs 10 and 10b are unexpected in some aspects. mRNA 10 (s^4U at positions –3, –1, +4, and +6) was cross-linked to U630, as well as to the site U1111/A1112. As described above, the U1111/A1112 site is closely associated with s^4U at mRNA positions –3 and –1. This would leave s^4U at positions +4 and +6 as the candidates for cross-linking to the U630 site. However, mRNA analogue 10b did not produce any detectable cross-links at U630. Two possibilities could explain this difference. First, the cross-link at U630 might depend upon the prior formation of the cross-link at U1111/A1112 for mRNA 10. In this case, the first cross-link could somehow alter the environment of the mRNA in the area of the codon (whether or not tRNA is in the P site). Alternatively, the conformation of the complex of mRNA analogue 10 containing s^4U at –3 and –1 with the ribosome may be slightly different from that with mRNA analogue 10b, in which positions –3 and –1 are both cytosines; this could allow the cross-linking of U630. s^4U should be chemically very similar to uridine (Hajnsdorf et al., 1986), and since there is good evidence that the interactions between the mRNA and the rRNA do not involve base-pairing interactions (Wollenzien et al., 1991; Bhangu & Wollenzien, 1992), we would not expect the substitution of uridine or cytidine for s^4U to matter. However, in competition experiments, s^4U-containing mRNAs have somewhat higher affinity binding constants than uridine-containing mRNAs of the same sequence (D. I. Juzumiene and P. Wollenzien, unpublished results), so that it is possible that there are also differences in the behavior of RNA that has s^4U or C at certain positions.

It must be noted that the distribution of cross-links in the 18S rRNA determined by reverse transcription is mainly descriptive of the behavior of s^4U at different positions in the mRNA analogues. In general, there is poor quantitative correlation between the frequency of cross-linking determined from the autoradiogram of the agarose gel that separates cross-linked from un-cross-linked mRNA analogues (Figure 1) and the number and intensity of bands determined by reverse transcription. For instance, there is an approximately 8-fold difference in the amount of cross-linking of mRNA analogues 8′ and 9e′ with tRNA present, but the only 18S rRNA position detected for each of these is U630 and there is a much less than 8-fold difference in the intensity of this band. Another example is RNA analogue 10b, for which the frequency of cross-linking is not significantly different from that for mRNA analogue 10, yet for mRNA analogue 10b no cross-linking is seen either at U630 or at A1112. There are several possible reasons for these discrepancies that include both the nature of the reverse transcription patterns and the possibility of cross-linking in the terminal 57 nucleotides of the 18S rRNA; these have not yet been sorted out for s^4U-induced cross-linking.

In earlier affinity experiments, nucleotides A1023, C1026, A1027, A1058, and C1059 of 18S rRNA were found to be modified with mRNA analogues that were derivatives of tri-, tetra-, and hexauridylates bearing alkylating groups at their 5′-ends (Malygin et al., 1993). All of these nucleotides are located within the central domain (domain II) of 18S rRNA (see Figure 4). The differences in the exact location of the cross-linking sites for this reagent compared to the results for mRNA analogues 10 and 10c may be caused by the length of the spacer group (about 12 Å long) separating the 5′-terminal phosphate from the alkylating moiety in the case of the alkylating reagents (Malygin et al., 1993). In the same domain is the sequence 1173–1181, which has been proposed to be involved in the interaction with mRNA during translation initiation (Sarge & Maxwell, 1991). This sequence is complementary to the Kozak consensus sequence surrounding the mRNA translation start site (Kozak, 1987).

Labeling of human 18S rRNA in the region 593–673 (see Figure 4) was observed earlier within a post-translocational complex, in which only the 3′-terminal UUU triplet of the nonanucleotide AUGUUUUUU interacted with peptidyl-tRNA at the P site and the AUGUUUUUU bore an alkylating group at its 5′-end (Matasova et al., 1993). AUGUUU or AUG bearing the same alkylating group in similar complexes was not able to cross-link to the mentioned region of 18S rRNA (Matasova et al., 1993). Therefore, the contact to this region of the 18S rRNA was realized when the modifying group on the 5′-phosphate of the mRNA analogue was located at position –6 with respect to the first nucleotide of the codon being used. Labeling of human 18S rRNA within the interval 593–673 was also observed for the 40S initiation complexes containing mRNA analogue AUGUUUC bearing an alkylating group at its 3′-end (position +7). In the same type of complex with the AUGC derivative, this cross-link was not detected (Mundus et al., 1993). From this data, it is reasonable to suggest that mRNA is arranged on the ribosome in such a way that the 3′- and 5′-sides of the mRNA located far enough from the area of the codon–anticodon interaction are in close vicinity to each other and to the 18S rRNA in the interval 593–673. It has not yet been determined whether in all of these cases the cross-link occurred at position U630.

One interpretation of the data from both s^4U-induced cross-linking and chemical-induced cross-linking in the human ribosome is that the mRNA track forms a large loop across the ribosome surface. In such a model, the rRNA site at U630 is located in a central location in the ribosome subunit, and the mRNA track makes several close interactions with this site to bring different parts of the mRNA close to the site. As discussed above, the behavior of the mRNA at position

U630 in human and A532 in *E. coli* ribosomes is very similar; the proposal of looping of the mRNA track also has been an explanation for the pattern of cross-linking in *E. coli* ribosomes (Bhangu et al., 1994). By inference in the *E. coli* case, the main part of this track has to be on the side of the small ribosomal subunit facing the large ribosomal subunit, and because of the underlying similarities, the same should be true for the mRNA track on the human ribosome.

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