Arrangement of Messenger RNA on *Escherichia coli* Ribosomes with Respect to 10 16S rRNA Cross-Linking Sites†

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ABSTRACT: The arrangement of the mRNA on the *Escherichia coli* ribosome with respect to ribosomal RNA sites has been investigated by photochemical cross-linking experiments. mRNA analogues 51–54 nucleotides in length contained a Shine–Dalgarno sequence, a single codon for $tRNA^{Gly}$, and 4-thiouridine (s^4U) in the 5′ third of the mRNA (−20 to −12), in the middle third of the mRNA (−3 to +6), or in the 3′ third of the mRNA (+20 to +26), where the position numbers are counted from the first nucleotide of the codon. Complexes were formed with these mRNAs and 70S ribosomes in the absence or presence of $tRNA^{Gly}$ and were irradiated. The extent of cross-linking and the identity of cross-linked rRNA sites were determined on agarose gels and by primer extension. 16S rRNA nucleotides A412, A532, G693 (weakly), U723, and U1381 (weakly) cross-linked with s^4U in the 3′ third; A532, G693, U723, A1167 (weakly), U1381, G818 (weakly), and A845 cross-linked with s^4U in the middle; A532, G693, U723, A1167, G818 (weakly), and A845 cross-linked with s^4U in the 5′ third. All of these cross-links occur with tRNA independence. Cross-links at C1395 and A1196 occur for all three mRNAs with tRNA dependence. The pattern of these sites provides information about the order of the rRNA sites along the mRNA track, and they also point out the apparent overlapping neighborhoods for the mRNA track. Models for the track of the mRNA on the 30S subunit are considered to explain this pattern of interactions.

The arrangement of the mRNA on the ribosome is a key question addressed in ribosome research because the answer should give insights into the orientations and geometry of the tRNAs in the P and A sites and in the mechanism of tRNA binding and translocation. Just as importantly, a description of the interaction sites between mRNA and rRNA and ribosomal proteins should give new information about the internal structure of the ribosome. In lieu of atomic structures, constraints like this will offer the opportunity to construct better predicted molecular models.

Numerous data on mRNA binding suggest its location and general features. Kang and Cantor (1985) determined that the size of poly(U)[1] protected from nuclease digestion on the 70S ribosome is about 49 nt; two accessible internal cleavage sites result in fragments of 20, 16, and 13 nt. The model they proposed is that the 20-nt fragment is the segment upstream of the decoding site, the 16-nt fragment is the segment just downstream of the decoding site and the 13-nt fragment is the 3′ fragment, which is not as tightly associated with the ribosome (Kang & Cantor, 1985). This model is generally consistent with the toeprinting experiments that show that reverse transcriptase is able to proceed on a ribosome-bound mRNA to a point 15 nt from the first nucleotide of the codon that is being used (Hartz et al., 1989).

The immunoelectron microscopy approach using dinitrophenyl hapten attached to the 5′ or 3′ end of poly(U) of different lengths indicated that the mRNA track is located in the groove (or neck) in the 30S subunit between its head and body (Evstafieva et al., 1983; Spirin & Vasiliev, 1989). This location for the mRNA track is consistent with the location of the 3′ end of the 16S rRNA (complementary to the Shine–Dalgarno sequence) as determined by electron microscopy (Olsen et al., 1988) and is consistent with the location of the tRNA anticodon as determined by electron microscopy (Gornicki et al., 1984). Affinity-labeling experiments have been used to determine the proteins associated with the mRNA track. The identity of the proteins and their location in the subunits also indicate that the track is in the middle part of the ribosome (Cooperman, 1980; Vladimirov, 1990).

Localization of the mRNA path through the ribosome using fluorescence spectroscopy has been recently reported (Bakin et al., 1991; Czworkowski et al., 1991). Both of these studies are consistent with a location in the middle of the 30S subunit with a significant turn or loop in the mRNA so as to bring the 5′ and 3′ ends of the mRNA closer together than they would be were the mRNA completely stretched out.

In photoaffinity experiments using an mRNA analogue with s^4U on the 3′ side of the decoding site at position +11 from the first nucleotide in the codon, Brimacombe and his collaborators have shown that the mRNA cross-linked to 16S rRNA in the interval 1390–1400, to position 532, and to ribosomal proteins S5, S3, and S1 (Rinke-Appel et al., 1991). With a series of mRNA analogues in which an s^4U was present at position −2, −5, or −8, only cross-links in the 3′-terminal region of the 16S rRNA were detected and cross-links to protein S7 occurred in the presence of cognate tRNA and to S21 in the absence of tRNA (Stade et al., 1989). An additional

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[1] Abbreviations: AMV, avian myeloblastosis virus; dsDNA, double-stranded DNA; EDTA, (ethylenedinitrilo)tetraacetic acid; mRNA binding buffer, 10 mM Tris·HCl, pH 7.4, 50 mM KCl, and 10 mM $MgCl_2$; nt, nucleotide(s); poly(U), poly(uridylic acid); s^4U, 4-thiouridine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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16S rRNA cross-link in the region of 1050 was also detected and was attributed to an s^4U at position +6 in the mRNA (Dontosova et al., 1991). This site was later shown to be at position U1052 and was shown to be tRNA dependent in 70S ribosomes (Dontsova et al., 1992).

In experiments using a 51-nt mRNA analogue randomly substituted with s^4U, 12 mRNA cross-linking sites in the 16S rRNA and two sites in the 23S rRNA were detected (Wollenzien et al., 1991). The mRNA sequence was found to a large extent not to make a difference in determining contact with most of the rRNA sites, ruling out that the cross-linking occurred by base pairing between mRNA and rRNA (Bhangu & Wollenzien, 1992). In both of these studies mRNAs containing 16 uridine positions were synthesized with two to four s^4U residues distributed throughout the mRNA; thus there was no direct indication of the sites in the mRNA that were responsible for the cross-linking in the rRNA.

The aim of the work described here was to find the rRNA neighborhoods surrounding different sections of the mRNA track. The approach was to use mRNA analogues with clusters of s^4U in the 3′ third, the middle, or the 5′ third of the molecule. This strategy allows investigation of the mRNA track from position −20 to +26 with respect to the codon with levels of cross-linking sufficient for analysis by reverse transcription. Several models for the arrangement of the mRNA on the ribosome are considered to account for the pattern of reaction.

MATERIALS AND METHODS

Materials. Nucleotide triphosphates, deoxynucleotide triphosphates, and dideoxynucleotide triphosphates were purchased from Pharmacia. DNA oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and were gel purified before use. AMV reverse transcriptase was purchased from Life Sciences. $tRNA^{Gly}$-1 (anticodon CCC) was purchased from Subriden RNA. 70S ribosomes and 50S and 30S ribosomal subunits were prepared as described by Makhno et al. (1988) except that the subunits, after separation on sucrose gradients, were concentrated by centrifugation. 4-Thiouridine diphosphate was purchased from Sigma and enzymatically phosphorylated by the method described by Tanner et al. (1988).

Preparation of DNA Templates and mRNA. DNA templates of 68 or 71 nt and primers of 20 nt for the leftward and rightward directions of DNA polymerization were synthesized for each of the mRNA molecules (Milligan et al., 1987; Tuerk & Gold, 1990). DNA amplification was done with 10 pmol of template and 100 pmol of each primer in 100 μL under standard conditions (Tuerk & Gold, 1990). dsDNA was purified on Qiagen columns and redissolved in 10 mM Tris·HCl, pH 7.5, and 1 mM EDTA. The usual yield was about 100 pmol of dsDNA. *In vitro* transcription reactions for uridine-containing mRNA were done in 300 μL with 40 mM Tris·HCl (pH 7.5), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol, 1.25 mM each ATP, CTP, GTP, and UTP, 1 mM GMP, 300 units of RNasin (Promega), 200 units of T7 RNA polymerase (Promega), and 50 pmol of dsDNA template. For reactions to incorporate s^4U, UTP was replaced by 0.125 mM s^4UTP. Transcription proceeded for 2 h at 37 °C. mRNA was purified on Qiagen columns, 2-propanol precipitated, and redissolved in H_2O. The incorporation of s^4U was measured according to Favre et al. (1986). The RNA 5′-monophosphate was exchanged with [γ-^{32}P]-ATP (Bhangu & Wollenzien, 1991), and the RNA was checked for complete transcription on 8% polyacrylamide/urea gels.

Ribosome–mRNA Complex Formation and Cross-Linking. Complex formation between activated 70S ribosomes and mRNA was done in mRNA binding buffer (Katunin et al., 1980). Ten picomoles of 70S were incubated in a total volume of 100 μL for 15 min at 37 °C with a 5-fold molar excess of mRNA and, in some experiments, with a 5-fold molar excess of $tRNA^{Gly}$. The samples were cooled on ice. They were then irradiated at 320–365 nm for 10 min in a thermostated mercury light irradiator (Isaacs et al., 1977); the intensity of light in this device is about 100 mW/cm^2. The cross-linked samples were digested with proteinase K, phenol extracted, ethanol precipitated, and redissolved at a final concentration of 200 ng (16S rRNA)/μL.

Binding Assays. The stoichiometry of binding mRNA analogues was determined by the filter-binding assay. For each mRNA analogue, mixtures contained 10 pmol of 70S ribosomes, 50 pmol of ^{32}P-labeled mRNA analogue, and 50 pmol of unlabeled $tRNA^{Gly}$ in a total volume of 100 μL of mRNA binding buffer. After incubation for 30 min on ice, the mixture was filtered through nitrocellulose filters (Schleicher and Schuell, 0.45 μm). The filters were washed with 5 mL of mRNA binding buffer before drying and scintilation counting.

The activity of the ribosomes in binding [^{14}C]Phe-$tRNA^{Phe}$ directed by poly(U) was tested by the filter-binding assay. Mixtures contained 10 pmol of 30S subunits, 9 nmol of poly(U), and 50 pmol of [^{14}C]Phe-$tRNA^{Phe}$ in mRNA binding buffer. After incubation for 30 min on ice, this was filtered through the nitrocellulose filters (Schleicher and Schuell, 0.45 μm); 0.95 pmol of the [^{14}C]Phe-$tRNA^{Phe}$ was bound to 1 pmol of the 30S. For determining the stoichiometry of [^{3}H]-Gly-$tRNA^{Gly}$ binding in the presence of mRNA analogues **8**, **9**, and **10** (see Table 1), 5-fold excesses of mRNA analogue and [^{3}H]Gly-$tRNA^{Gly}$ over 70S ribosomes were used. The placement of the [^{3}H]Gly-$tRNA^{Gly}$ in the P site was determined by puromycin reaction. Ribosomal complexes formed by using a 5-fold molar excess both of the mRNA analogue and of [^{3}H]Gly-$tRNA^{Gly}$ under the standard conditions were incubated with 1 mM puromycin for 15 min at 25 °C before filtration.

Reverse Transcription Analysis. Primer extension analysis of the mRNA–rRNA cross-links using reverse transcriptase was done as previously described (Wollenzien, 1988). For the 16S rRNA, 10 primers described previously were used (Ericson et al., 1989). One microliter of sample (0.2 μg of 16S rRNA) and 1 pmol of ^{32}P-labeled primer were dried together and then redissolved in 1 μL of 1× R-loop mix, which is 80% formamide, 40 mM Pipes, pH 6.8, 1 mM EDTA, and 400 mM NaCl. The mixture was annealed for 10 min at 45 °C and then cooled on ice. Reverse transcription was done by adding 20 μL of 50 mM Tris·HCl, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, 0.5 mM dNTPs (each), and 10 mM DTT containing 2 units of reverse transcriptase. Reaction mixtures for determining the sequence contained, in addition, 0.05 mM ddNTP. The extension reaction proceeded for 10 min at 42 °C. The samples were phenol extracted, and when radioactive mRNA was used, it was hydrolyzed in the presence of 0.16 M NaOH for 1 h at 45 °C. The samples were then ethanol precipitated overnight, using 2 μg of glycogen as a carrier. Electrophoresis was usually performed on 8% denaturing polyacrylamide gels.

RESULTS

mRNA Structure and Complex Formation with Ribosomes. Each of the mRNA analogues made for this study was 51 nt

Table 1: Sequences of mRNAs Used for Analysis of the Arrangement of mRNA on the Ribosome[a]

mRNA **8**	5′ GGCAGAGCGGCAC<u>AGGAG</u>CGCAAC	GGG AGCGCACAGCCGAGAGUCUGUCUA 3′ (+20 ..... +26)
mRNA **9**	5′ GGCAGAUCUGUAU<u>AGGAG</u>CGCAAC	GGG AGCGCACAGCCGAGAGCCAGACGA 3′ (−18 ..... −12)
mRNA **9e**	5′ GGCACACUAUCUGCAC<u>AGGAG</u>CGCAAC	GGG AGCGCACAGCCGAGAGCCAGACGA 3′ (−20 ... −16)
mRNA **10**	5′ GGCAGAGCGGCAC<u>AGGAG</u>CGCUAU	GGG UGUGCACAGCCGAGAGCCAGACGA 3′ (−3 ... −1; +4 ... +6)
mRNA **10a**	5′ GGCAGAGCGGCAC<u>AGGAG</u>CGCUAU	GGG CGCGCACAGCCGAGAGCCAGACGA 3′ (−3 ... −1)
mRNA **10b**	5′ GGCAGAGCGGCAC<u>AGGAG</u>CGCCAC	GGG UGUGCACAGCCGAGAGCCAGACGA 3′ (+4 ... +6)

[a] The Shine–Dalgarno sequence is underlined and the codon (GGG) for $tRNA^{Gly}$ is aligned in the middle of each mRNA; +1 is the first nucleotide of the codon. The beginning and end of each block of uridines that are substituted with s^4U are numbered.

long (except for mRNA **9e**, which was 54 nt) and had a Shine–Dalgarno sequence with a spacing of 6 nt before the triplet GGG, the codon for $tRNA^{Gly}$-1. The mRNA sequences are different from each other with respect to the position of the uridines, as shown in Table 1. None of the molecules should have any significant secondary structure on the basis of inspection and folding of their sequences compared to that of random sequences of the same base composition. The mRNAs **8**, **9**, and **10** in the first series of experiments have four uridine residues which were completely substituted with s^4U during *in vitro* transcription.

Complexes between 70S ribosomes, mRNA, and $tRNA^{Gly}$ were formed with a 5-fold molar excess of mRNA and $tRNA^{Gly}$ over ribosomes. Under these conditions the amount of mRNA binding for uridine-containing RNA is 0.4, 0.7, and 0.6 of mol mRNA/mol of 70S ribosome for mRNA analogues **8**, **9**, and **10**, respectively, and for the same RNA analogues, the amount of $tRNA^{Gly}$ binding is 0.4, 0.6, and 0.3 mol of [^{3}H]-Gly-$tRNA^{Gly}$/ mol of 70S ribosome. About 80% of the bound $tRNA^{Gly}$ is puromycin reactive. Uridine-containing mRNA competes s^4U-containing mRNA analogue in binding and cross-linking; with a 10-fold excess of cold competitor mRNA over ^{32}P-labeled s^4U-containing mRNA analogue, in each case both binding and cross-linking were decreased to about 20% of the uncompeted value (results not shown).

Cross-Linking and Analysis by Agarose Gel Electrophoresis. The 70S–mRNA complexes were irradiated with a high-intensity device that produces wavelengths in the range 320–365 nm (Isaacs et al., 1977). As controls, complexes formed between 70S ribosomes, mRNA containing normal uridine, and tRNA were irradiated. As an additional control, complexes were formed with 70S ribosomes and mRNA containing s^4U, but were not irradiated. The level of ^{32}P-labeled mRNA cross-linking to 16S and 23S rRNA was determined by agarose gel eletrophoresis. As seen in Figure 1, for mRNAs **8**, **9**, and **10**, a fraction of the radioactive mRNA was found to comigrate with rRNA only in the irradiated samples with s^4U-containing mRNA. The stoichiometry of cross-linking indicates an extensive reaction with 16S rRNA and only a low level of reaction with 23S rRNA (Table 2). The amount of cross-linking increases in the presence of $tRNA^{Gly}$ for mRNAs **9** and **10**.

Determination of Contact Sites in the rRNA by Primer Extension. Primer extension by reverse transcriptase was used to analyze cross-linking sites on 16S rRNA. Reverse transcriptase is unable to transcribe through certain chemically or photochemically modified bases, and these sites can be identified by gel electrophoresis. The principal controls in each set of experiments are RNA samples that come from irradiated complexes of 70S ribosomes, tRNA, and mRNA containing uridine. Thus all discernible differences in the reverse transcription pattern are attributable to the presence of s^4U in the mRNA. All experiments have been repeated at least four times with different mRNA preparations. We have

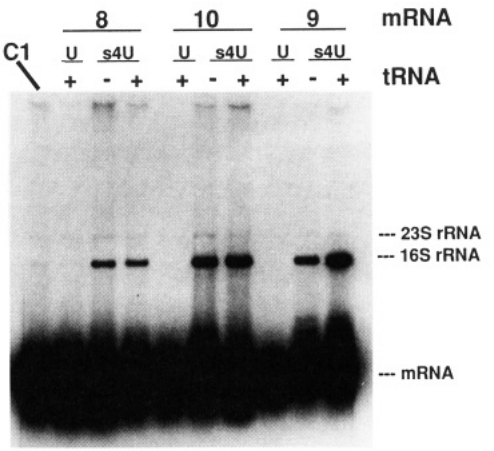

FIGURE 1: Agarose gel electrophoresis of rRNA after cross-linking to mRNAs **8**, **10**, and **9**. Samples from experiments using mRNAs **8**, **10**, and **9** are shown. These mRNAs were synthesized with uridine (U) or 4-thiouridine (s^4U) for use in control or cross-linking experiments and were ^{32}P-labeled. The figure shows an autoradiogram of the agarose gel. $tRNA^{Gly}$ was included in the complexes as indicated. The locations of 23S, 16S, and mRNA bands seen by ethidium staining are indicated. The control lane labeled C1 is a sample made with 70S ribosomes, mRNA **10** containing s^4U, and $tRNA^{Gly}$ but not irradiated. All other samples were irradiated.

Table 2: Frequency of Cross-Linking mRNA Analogues **8**, **9**, and **10**[a]

mRNA	tRNA	16S rRNA	23S rRNA
8	−	12	1.0
8	+	12	0.5
9	−	29	<0.2
9	+	58	0.5
10	−	49	1.0
10	+	56	1.0

[a] Frequency is expressed as a percentage of the 16S or 23S rRNA that is modified.

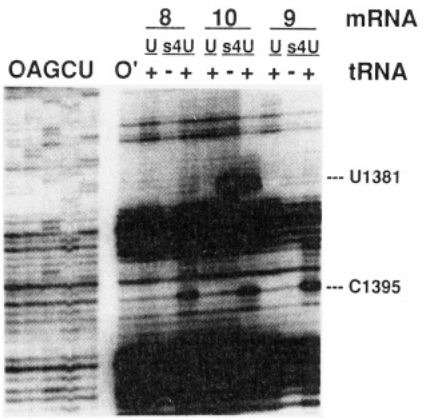

FIGURE 2: Reverse transcription analysis of mRNA cross-linking to 16S rRNA in the region 1408–1364. Samples from cross-linking experiments prepared with mRNA **8**, **10**, and **9** containing uridine (U) or thiouridine (s^4U) are indicated. The complexes included $tRNA^{Gly}$ when indicated. The control lane marked O′ was prepared from a sample of 70S ribosomes without mRNA or tRNA. All samples on the right part of the gel were irradiated. 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 indicated sequence could be read.

seen no reproducible differences with ribosomes that have been ethanol precipitated for their final concentration step after separation on sucrose gradients. A set of 10 primers complementary to various parts of the 16S rRNA are used; these allow the analysis of all but the terminal 30 nt of the 16S rRNA. Four primers complementary to the first 1200 nt of the 23S rRNA were used because cross-links in this part of the 23S rRNA were detected previously (Wollenzien et al., 1991; Bhangu & Wollenzien, 1992). However, no cross-links were detected, and additional primer extension experiments have not been done in the present studies.

Analysis of two mRNA cross-linking sites in the 16S rRNA region 1430–1360 is shown in Figure 2. The site U1381 is cross-linked only by s^4U in the middle of the mRNA and occurs with tRNA independence. On the other hand, C1395 in the 16S rRNA is cross-linked by all three of the mRNA analogues in a tRNA-dependent manner. The intensity of cross-linking is nearly equal for all three, in spite of the fact that the s^4U placements in mRNAs **8**, **9**, and **10** are separated by large intervals.

Several regions of the 16S rRNA contain additional mRNA cross-linking sites. Figure 3 contains examples of experiments in which the remaining cross-links for mRNA **8**, **9**, and **10** are shown. For several of these rRNA sites there again is specific or restricted localization to mRNA regions. A412 is cross-linked to a much greater extent to the 3′ region than to other regions. The sites at G1166/A1167, G818, and A845 are cross-linked to the middle and 5′ regions of the mRNA. The sites A532, G693, and U723 are reactive with all three regions of the mRNA analogues. One other site, A1196, also cross-links to all mRNA analogues but with tRNA dependence. Both of the tRNA-dependent cross-linking sites (C1395 and A1196) require cognate tRNA since $tRNA^{Phe}$ did not substitute for $tRNA^{Gly}$ in these reactions (results not shown).

Because the s^4U-containing mRNA analogues show a slightly larger affinity to ribosomes than uridine-containing mRNA analogues of the same sequence, we tested for nonspecific cross-linking. Cross-linking experiments were done with each ^{32}P-labeled s^4U-containing mRNA analogue with 10-fold excess unlabeled uridine-containing mRNA of the same sequence as competitor. In each case cross-linking was decreased by about 5-fold (results not shown). To determine whether all the cross-linking sites were competed equally, reverse transcription analysis was done to determine levels of cross-linking at each site. Comparisons were made between two samples. The first was a sample that contained one part of RNA from ribosomes that had been cross-linked with s^4U-containing mRNA mixed with four parts of non-cross-linked 16S rRNA; reverse transcriptions performed with this mixture yielded patterns that had intensities of bands at cross-linking sites depressed by about 5-fold (results not shown). The first sample was the mock competition sample. The second sample was RNA from ribosomes that had been cross-linked with a mixture of s^4U-containing mRNA analogue with a 10-fold excess of uridine-containing mRNA analogue. Figure 4 shows the results of the reverse transcription analysis at three cross-linking sites. Cross-linking at U1381 and C1395 is the same in the mock competition samples (lanes labeled "1") as in the real competition experiments (lanes labeled "2"). This indicates that the decrease in cross-linking at these sites by the competitor mRNA analogue is to the same extent as the overall decrease in the amount of cross-linking. This same result is seen for all eight other sites we report. At one other 16S rRNA site, G1300, for all three mRNA analogues, cross-linking was not competed out by competitor RNA (Figure 4B).

The sites of cross-linking are summarized in Table 3. The entries in this table are a qualitative description of the patterns since there is variation in the intensities of the reverse transcription patterns from one experiment to another. The results in Table 3 are based on at least four independent cross-linking and reverse transcription analyses for each site; in several cases these do not correspond exactly to the appearance of the gels in Figures 2 and 3. In several experiments there was a low degree of cross-linking of mRNA analogue **8** to position U1381. In spite of the appearance of Figure 3, A532 in many experiments was cross-linked to nearly the same levels for mRNA analogues **8**, **9**, and **10**. The site at A412 is cross-linked nearly exclusively to mRNA analogue **8** as seen in the original of the autoradiogram and in other experiments. The levels of cross-linking at G693 and U723 are nearly equal for mRNA analogues **8**, **9**, and **10** in other experiments, and for G693, the band intensity for the control sample O′ was much lower in other experiments. Cross-linking at G845 is not seen at all for mRNA analogue **8** in other experiments.

Several RNA molecules were designed to narrow the identification of cross-linking sites. mRNA 10a contains s^4U positions at –1 and –3, and mRNA **10b** contains s^4U positions at +4 and +6. Figure 5A shows that the cross-link to position U1381 occurs with mRNA **10a** and mRNA **10** but not with mRNA **10b**. Thus positions –1 to –3 are responsible for the interaction with the U1381 site. The behavior of one additional mRNA related to mRNA **9** has also been investigated to see if s^4U positions further upstream of the Shine–Dalgarno sequence were also reactive. mRNA **9e** is 54 nt and has s^4U positions at –16 to –20 from the codon. Figure 5B shows the cross-linking pattern in the 540–520 region for mRNAs **8**, **10a**, **10b**, and **9e**. mRNA **8** is cross-linked to A532 in a tRNA-independent manner just as seen before. Unexpectedly, neither mRNA **10b** nor mRNA **10a** is cross-linked to A532 even though together they contain all the uridine positions contained in the parent mRNA **10**. The reactivity of mRNA **9e** is somewhat different from that of mRNA **9**. mRNA **9e** cross-links to A532 but also to A533, U531, and G530, and all of its reaction is tRNA dependent (see Figure 5B). The reactivities at all of the rRNA sites are summarized in Table 3.

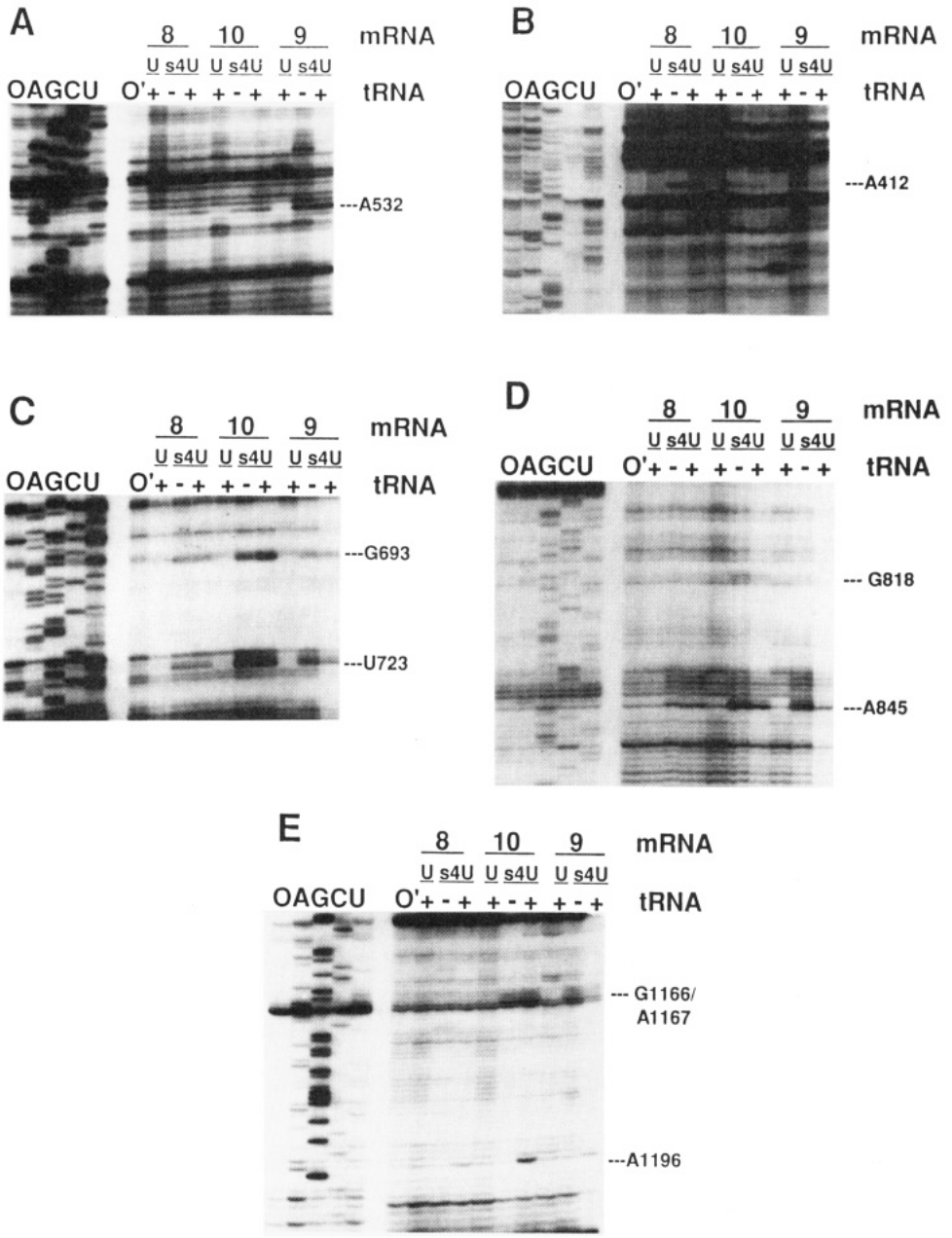

FIGURE 3: Reverse transcription analysis of mRNA cross-linking in six regions of the 16S rRNA. In panel A, reverse transcription in the 16S from 540 to 514 is shown. In panel B, reverse transcription in the 16S from 450 to 380 is shown. In panel C, reverse transcription in the 16S from 747 to 670 is shown. In panel D, reverse transcription in the 16S from 863 to 795 is shown. In panel E, reverse transcription in the 16S from 1204 to 1150 is shown. The lanes in each panel are identified as described in the caption to Figure 2.

DISCUSSION

Order of the rRNA Sites along the mRNA Track. These experiments have been done with mRNA analogues containing s^4U positions that span altogether –20 to +26 with respect to the first nucleotide of the codon. A total of 10 sites in the 16S rRNA have been detected as being cross-linking sites, and many of these can be assigned to specific or at least restricted neighborhoods in the mRNA track. A412 is cross-linked by s^4U in the 3′ region of the mRNA; U1381 is cross-linked by s^4U within the interval –1 to –3 in the mRNA, and several sites (G1166/A1167, G818, A845) are cross-linked by s^4U in the middle or the 5′ region of the mRNA. The mRNA analogues are also functional in that they direct cognate tRNA binding to the P site as evidenced by puromycin reactivity of the majority of the bound aminoacyl-tRNA. Also for those three sites (C1395, A1196, and, for mRNA 9e, A532) that are strictly tRNA dependent, the tRNA must be cognate tRNA. These observations suppport the idea that the mRNA analogues form specific complexes with the ribosome mediated by the strong Shine–Dalgarno sequence even without the presence of cognate tRNA. However, at the same time, a number of rRNA sites (A532, C1395, G693, U723, and A1196) are cross-linked by very widely spaced regions of the mRNA. The most surprising result is the reactivity of s^4U at positions upstream of the Shine–Dalgarno sequence with numerous rRNA sites that must be located in the body of the 30S subunit.

Based on the patterns of cross-linking for all the mRNA analogues reported here, an approximate order of the sites along the track can be proposed; this is indicated in the arrangement of Table 3. If the track across the interface side of the 30S subunit is viewed from the 50S subunit, A412 must occur at the left (3′) end of the track. A number of rRNA sites (A532, C1395, A1196, G693 and U723) are grouped in

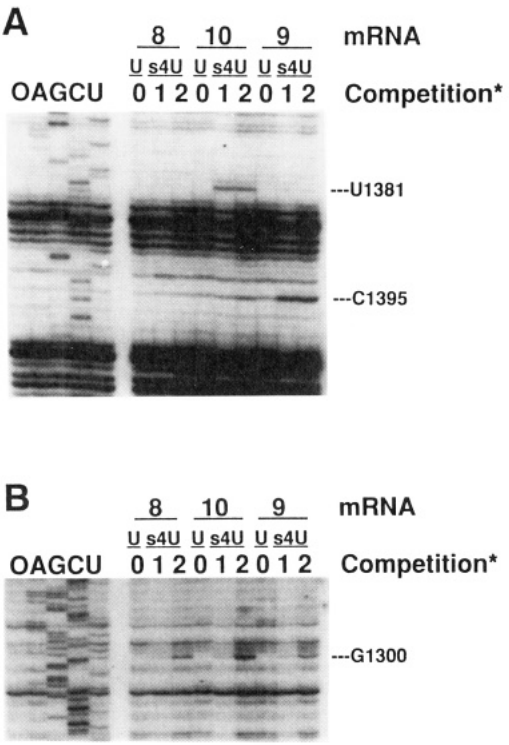

FIGURE 4: Reverse transcription analysis of rRNA samples from competition cross-linking experiments. Experiments were done with mRNA analogues **8**, **9**, and **10** as indicated to determine whether competition by uridine-containing mRNA analogues decreases the amount of cross-linking at each rRNA cross-linking site. Cross-linking was done with uridine-containing mRNA analogue (U) or with s^4U-containing mRNA analogue (s^4U) under different conditions. Competition conditions were as follows: for lanes 0, irradiation was done with uridine-containing mRNA as control; for lanes 1, cross-linking was done with s^4U-containing mRNA and the sample was then mixed with four parts of unirradiated 16S rRNA to give a mock competition experiment; for lanes 2, cross-linking was done with a mixture of s^4U-containing mRNA and uridine-containing mRNA that was known to decrease the overall cross-linking 5-fold. In panel A, reverse transcription in the interval 1468–1371 is shown. In panel B, reverse transcription in the region 1331–1270 is shown.

the middle of the track because of their reactivity with s^4U in all of the mRNA analogues. The order of the sites within this group is arbitrary. The rRNA site at U1381 is made by s^4U only on the 5′ side of the codon, so it is placed just to the right of the middle. Lastly, a series of sites (G818, A845, and G1166/A1167) are placed on the right of the track because they are all reactive with s^4U in placement sites in both the middle and the 5′ third of the mRNA. Within this last group the order of sites is again arbitrary at the present time.

Several rRNA cross-linking assignments have been made previously with s^4U placed at single positions. s^4U at –2, –5, or –8 cross-linked to the 3′-terminal region of 16S rRNA (Stade et al., 1989); s^4U at +11 cross-linked to A532 and to the region 1390–1400 (Rinke-Appel et al., 1991); s^4U at +6 cross-linked to U1052 (Dontsova et al., 1991, 1992). In an article by Dontsova et al. (1992) seven mRNA analogues were used in 70S complexes with $tRNA^{fMet}$ and sometimes with additional cognate tRNAs. Three distinct correspondences were reported: s^4U at +6 cross-linked to U1052, s^4U at +7 cross-linked to C1395, and s^4U at +11 cross-linked to A532. The present experiments with mRNA analogues **8**, **9**, and **10** do not suggest such a specific set of interactions. A532 occurs as a tRNA-independent cross-linking site with mRNAs **8**, **9**, and **10** and as a tRNA-dependent site with mRNA **9e**. However, A532 did not occur with mRNAs **10a** and **10b** (s^4U placement sites at –3 and –1 or at +4 and +6). Thus there must be some anomolous absence of cross-linking with some mRNA analogues that have s^4U placement close to the codon, but otherwise there is cross-linking to A532 even in the extreme 3′ end (+20 to +26), in the extreme 5′ end (–12 to –20), and for some molecules with s^4U in the middle. C1395 occurs as a cross-link in a tRNA-dependent manner for mRNAs **8**, **9**, **9e**, and **10**. However, cross-links to C1395 do not occur with as great an efficiency for s^4U in placement sites in the interval –1 to –6 (results not shown), so again there seems to be an anomolous region in the mRNA for cross-linking to C1395. We have no evidence for cross-linking at U1052. However, the site we detect at A1196 is on the complementary strand of the base-paired region involving U1052 and is about one-half turn away, so these two sites may be very close in space, and it is likely that they represent the same interaction site. In terms of reactivity, A1196 is cross-linked by mRNA analogues **8**, **10**, **9**, and **9e** in a tRNA-dependent manner but not by analogue **10a** or **10b** (Table 3).

It must be emphasized that all of the molecules in the current experiments are designed from a common parent with uridine substitutions at selected locations. All of the molecules have about a 65% purine nucleoside composition, and this is probably very relevant to their behavior in the photoaffinity experiment. In earlier experiments with mRNA analogues that were on average 50% in purine content, the level of covalent cross-linking was about the same as in the current experiments, but the number of sites in the 16S rRNA that were cross-linked was larger —as many as 15 sites in the 16S rRNA for some mRNA analogues (Bhangu & Wollenzien, 1992). On the other hand, the mRNA analogues used by the Berlin group and their collaborators are from 74% to 90% purine in composition, show much stronger tRNA dependence for cross-linking, and show a smaller number of cross-linking sites but at the same time with a greater specificity than in our series of mRNA analogues [see above and Stade et al. (1989), Tate et al. (1990), Rinke-Appel et al. (1991), and Dontsova et al. (1991, 1992)].

A total of 10 16S rRNA cross-linking sites are detected in the present experiments. This large number may be partly due to both the composition of the mRNA analogues and the sensitivity of the direct reverse transcription analysis. Since a number of factors, such as the distance between the s^4U and the nucleoside target, the geometry of the cross-linking sites, and the flexibility of the sites, probably determine cross-linking efficiency [see Bergstrom and Leonard (1974) and Lemaigre-Dubreuil et al. (1990)], a low cross-linking efficiency at an rRNA site does not mean that the site is necessarily further away from the mRNA track than an rRNA site that is cross-linked more efficiently. Therefore all of these sites should be relevent in the description of the mRNA track through the subunit.

Models To Explain the Pattern of mRNA Cross-Links with rRNA. A number of models for the arrangement of the 16S rRNA in the 30S subunit have been proposed (Expert-Bezancon & Wollenzien, 1985; Nagano & Harel, 1988; Brimacombe et al., 1988; Stern et al., 1988; Oakes et al., 1990; Hubbard & Hearst, 1991). All of these are tempered by the fact that some of the rRNA sites to which mRNA is cross-linked are not suitably located in the middle part of the 30S subunit. Given the number of rRNA sites, their locations in the 16S rRNA secondary structure, and other constraints that have to be met in the three-dimensional model, it is likely that it will be impossible to accommodate all of the mRNA–rRNA cross-linking sites simultaneously. This suggests some heterogeneity in the exact arrangement of the mRNA on the

Table 3: Summary of the rRNA Cross-Linking Sites[a]

	16S rRNA Sites									
mRNA	A412	A532	C1395	A1196	G693	U723	U1381	G818	A845	A1167
8	+/+	+/+	(+)/+	−/(+)	(+)/(+)	+/+	(+)/(+)			
10b			−/(+)		+/+	+/+			+/+	
10		+/+	−/+	−/+	+/+	+/+	+/+	(+)/(+)	+/+	(+)/(+)
10a			−/(+)		+/+	+/+	+/+		+/+	
9		+/+	−/+	−/+	+/+	+/+		(+)/(+)	+/+	+/+
9e		−/+	−/+	−/+	(+)/(+)	+/+			+/+	+/+

[a] The two entries for each box indicate the amount of cross-linking without or with cognate tRNA: + indicates strong cross-linking; (+) indicates weak cross-linking; − indicates no cross-linking without cognate tRNA; open boxes indicate no cross-linking without or with cognate tRNA.

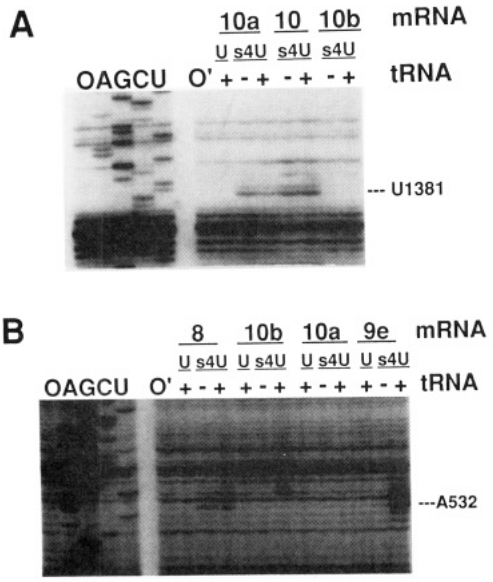

FIGURE 5: Reverse transcription analysis of mRNA cross-linking to 16S rRNA for the mRNAs **8**, **9e**, **10a**, **10b**, and **10**. In panel A, the pattern of reverse transcription at the U1381 site is shown for the indicated mRNAs. In panel B, the pattern of reverse transcription around A532 is shown for the indicated mRNAs. The lanes are identified as described in Figure 2.

ribosome and/or heterogeneity in the structure of the 30S subunit itself.

On the basis of protein footprinting data (Stern et al., 1986), the locations of the ribosomal proteins S4 and S7 (Capel et al., 1988), and electron microscopy localization (Trempe et al., 1982), it is thought that the 530 loop is in the indentation on the surface of the 30S subunit away from the cleft region [see Moazed and Noller (1990) and Noller (1991). This placement results in a significant distance between the 530 region and the 1400 region of the 16S rRNA. However, on the basis of the reactivity of s^4U with rRNA sites A532, C1395, and U1052, Stade et al. (1991) and Dontsova et al. (1992) have proposed that these three sites are closer than originally thought. In our studies we again see A532, C1395, and A1196 (equivalent to the site at U1052) reactive in the same way. However, G693 and U723 are also reactive with each given mRNA analogue, and all five of these sites are reactive with s^4U at widely spaced locations in the mRNA. Therefore the problem of determining reasonable locations for all the rRNA sites is potentially more severe.

One model that would be consistent with the observed mRNA cross-linking pattern is that the 16S rRNA sites A532, C1395, A1196, G693, and U723 are in a tight arrangement clustered in a central location in the 30S subunit and the mRNA track makes a large loop around this cluster. This would bring parts of the 3′ third, the middle, and the 5′ third of the mRNA in contact with these sites. However, since s^4U is a zero-length cross-linker and probably requires specific geometries for reaction, it is likely there would still have to be significant flexibility in the mRNA–rRNA interactions for the different cross-links to be made. The reactivity of s^4U in the 5′ third of the mRNA is particularly difficult to explain. s^4U placed at −2 to −8 between the codon and the Shine–Dalgarno sequence has been shown to be reactive with the 3′-terminal region of 16S rRNA and with protein S7, and this has been interpreted to indicate that the mRNA track is leaving the 30S subunit in an upward, rightward direction as viewed from the 50S subunit (Stade et al., 1990). An electron microscopy study supports this location and orientation for the 3′-terminal region of the 16S rRNA (Olson et al., 1988). On this basis, we would expect that the part of the mRNA analogue containing sequences 5′ to the Shine–Dalgarno sequence would not be located on the ribosome. However, the cross-links made by mRNA **9** (s^4U positions −12 to −18) and mRNA **9e** (s^4U positions −16 to −20) suggest that the mRNA returns to the middle part of the body or neck of the 30S subunit. Another explanation for the reactivity of s^4U at these positions on the 5′ side of the Shine–Dalgarno sequence is that the mRNA track goes all the way around the middle part of the 30S subunit so that some of the rRNA cross-linking sites contact the middle part of the mRNA track in one passage and the same rRNA site contacts the 5′ end in its second passage past that site. We cannot rule out this possibility on the basis of the data from the mRNA analogues used so far and current three-dimensional models.

Another way to explain how a large number of rRNA sites are cross-linked by s^4U at widely separated positions in the mRNA is to consider the possibility of significant structural flexibility in the 30S subunit itself. The rRNA site U1381 is cross-linked only to s^4U positions just upstream from the codon, so it must be associated with the mRNA in some specific way. However, a large number of the other rRNA sites to the left and the right of this site are able to be associated with different regions of the mRNA. One model to accommodate this pattern would be that the upper one-third of the 30S subunit (the head) including the domain III rRNA (nucleotides 920–1397) has rotational flexibility with respect to the lower two-thirds (the body and platform). This flexibility could occur around helix 28 [the base-paired interaction (921–933)·(1384–1396)] and around ribosomal protein S5 (Ramakrishnan & White, 1992). This rotation would result in different orientations of the head with respect to the body. If the mRNA at position −1 to −3 is strongly associated with rRNA at U1381 and this can be thought of as an anchoring point for the mRNA, this rotational motion would bring different sections of the mRNA past reactive rRNA sites in the body. For instance, in an intermediate rotation, the middle part of the mRNA would be brought close to the rRNA site C1395; in a counterclockwise rotation (looking down onto the head of the 30S subunit), regions in the 3′ side of the mRNA would be brought close to the C1395 site; and in a clockwise rotation,

regions on the 5′ side of the mRNA would be brought close to the C1395 site. Binding of cognate tRNA to its codon in the functionally defined P site seemingly would not interfere with this postulated rotational flexibility. In such a model it is not necessary to propose that all parts of the mRNA track are touching these sites simultaneously or that all of the rRNA sites are very close to one another in space. Even though this rotational flexibility model has some unexpected implications concerning the structure of the 30S subunit, it would account for the pattern of reaction of the mRNA analogues. In addition it suggests that certain distances within the 30S subunit should have great variation that should depend upon the rotational conformation of the head of the 30S subunit. This will be testable by additional cross-linking experiments or by other distance-measuring experiments such as fluorescence energy transfer.

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