The mRNA Binding Track in the *Escherichia coli* Ribosome for mRNAs of Different Sequences†

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ABSTRACT: Interactions between mRNA and rRNA on the 30S ribosomal subunit or 70S ribosome have been determined by photochemical cross-linking experiments using synthetic mRNA analogs substituted with 4-thiouridine. A set of RNA molecules containing different sequences has been used to determine the extent to which binding contacts are sequence dependent. The 16S rRNA and 23S rRNA nucleotides that form a part of the binding site have been identified by reverse transcription. The nucleotides are U1381, G1338, G1300, G1156, A845, U723, G693, A532, G497, U420, G413/A412, and G436 of 16S rRNA and U887 of 23S rRNA. Several additional nucleotides (U1065 of 23S rRNA and A1227, G818, G524, and G423 of 16S rRNA) are seen for some, but not all, of the mRNAs. Results obtained with two mRNAs containing the Shine–Dalgarno sequence were similar to those obtained with mRNAs lacking the Shine–Dalgarno sequence. Eight of these cross-linking sites were also seen when a mixture of RNA was used in which there are 12 random nucleotides preceding and seven random nucleotides succeeding an AUG codon. These results indicate that to a large extent placement of the mRNA in the ribosome does not depend upon its primary sequence.

The mRNA binding site on the *Escherichia coli* ribosome as defined by the nucleotides of rRNA and the ribosomal proteins is currently under investigation by many groups. Titration experiments indicate that mRNA binds to 30S and 70S ribosomes in 1:1 stoichiometry, providing strong evidence for a single specific binding site (Katunin et al., 1980). The locations of S4, S5, and S12 give a circumstantial location for the mRNA, since it is known that mutations in any of these alter the fidelity of translation (Brimacombe, 1986). Moreover, proteins S1, S3, S4, S7, S9, S12, S18, and S21 have been found to be in contact with the mRNA by affinity-labeling experiments (Cooperman, 1980; Liljas, 1982; Spirin & Vasiliev, 1989; Vladimirov et al., 1990). The locations of these proteins indicate that the track probably lies around the middle of the 30S subunit in the indentation between the head and body (Spirin & Vasiliev, 1989).

The identification of the rRNA nucleotides contacted by the mRNA in the ribosome will also be informative. 16S rRNA interacts with mRNA during initiation, elongation, and termination of translation (Dahlberg, 1989). During the initiation phase the Shine–Dalgarno sequence in the mRNA interacts with the 3′ terminus of the 16S rRNA through base-pairing interactions (Shine & Dalgarno, 1974). Interactions via base-pairing between mRNA and 16S rRNA during the elongation phase of translation have also been proposed to be a key component in the mechanism of programmed ribosomal frameshifting within the RF2 gene in *E. coli* (Weiss et al., 1987). In addition, in 1988 Murgola et al. reported the isolation of a mutant 16S rRNA that was found to be a codon-specific translational suppressor, suggesting the involvement of the rRNA in termination.

Wagner et al. (1976) reported that mRNA analogs would bind to two sites in the 16S rRNA at positions G462 and C474. Stiege et al. (1988) used poly(A) to locate mRNA in the translating ribosome and reported a UV-induced cross-link to the 16S rRNA in the region 1394–1399. Graifer et al. (1990) reported cross-links between a U_6 derivative and ribosomes from human placenta; the region in the 18S rRNA where cross-links occurred corresponds to nucleotides 703–784 in the *E. coli* 16S rRNA. Recently, Brimacombe and co-workers (Stade et al., 1989; Tate et al., 1990; Rinke-Appel et al., 1991) have used synthetic mRNAs containing single 4-thiouridine (s^4U)[1] nucleotides to investigate contacts between the mRNA and the ribosome. The site in the 1390–1400 region of the 16S rRNA and a site at A532 have so far been identified.

Recently we reported sites of contact of mRNA with 16S rRNA and 23S rRNA in the *E. coli* ribosome identified by cross-linking experiments using an mRNA randomly substituted with 4-thiouridine (Wollenzien et al., 1991). 4-Thiouridine is an analog of uridine that can be activated with 365-nm light to form covalent adducts with nucleic acids and proteins (Hajnsdorf et al., 1986). Twelve sites in the 16S rRNA (C1395, U1381, G1338, G1300, G1131/C1132, A845, U723, G693, A532, G424, U421, and G413) and two sites in the 23S rRNA (U1065 and U887) were detected. Since only one mRNA analog was used in that study, the extent to which the pattern would be dependent on the sequence of the mRNA was not clear. Because of the complex architecture of the ribosome and many defined functional sites on its components, the mRNA may follow a definite track irrespective of its sequence. To determine if the contact sites between the 16S rRNA and 23S rRNA and mRNA are independent of the primary sequence of the mRNA, we have undertaken a

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[1] Abbreviations: s^4U, 4-thiouridine; Tris, tris(hydroxymethyl)aminoethane; EDTA, ethylenediaminetetraacetic acid; EF-G, elongation factor G; EF-Tu, elongation factor Tu.

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Table I: Sequences of the mRNAs Used for the Analysis of the Binding Site on the Ribosome

mRNA 0
5'GGGAAAGCUCCCGUGCCUCCUCU,GUC,UUC,UAC,UAGCCUCCUUGAUCGGAUC 3'
Val, Phe, Tyr

mRNA SD
5'GGGAAAGCUCCUACACGGAGGAACAGU,AUG,UUC,GAA,GUA,UAC,UAGCCUCCUUGGAUC 3'
S.D. fMet, Phe, Glu, Val, Tyr

mRNA 1B
5'GGGAAAGCUCAGGCGCCUCCUCU,AUG,GUC,UUC,UAC,UAGCCUCCUUGAUCCAGGGAUC 3'
fMet, Val, Phe, Tyr

mRNA 1B-SD
5' GGGAAAGCUCAGGAGGCUCCUCU,AUG,GUC,UUC,UAC,UAGCCUCCUUGAUCCAGGGAUC 3'
S.D. fMet, Val, Phe, Tyr

mRNA 7
5' GGGAAAGCUCAGUGGUCGUAGUCG,AUG,UGG,UAGUUGCCGUCGUUUGAUCCAGGAUC 3'
fMet, Trp

mRNA MIX
5' GGGAGCUCUGCCUC -N_{12}- AUG -N_7- UGCCUACCUAGCCC 3'
fMet

cross-linking study using five mRNAs of various sequences and one mRNA containing random nucleotide positions before and after an AUG codon. Data obtained with these mRNA samples complement and extend our earlier results. These nucleotides identify a part of the track the mRNA follows on the ribosome.

Materials and Methods

Materials. Nucleotides, deoxynucleotides, and dideoxynucleotides were purchased from Pharmacia. DNA oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and were gel-purified before use. Restriction nucleases were purchased from New England Biolabs. DNase I was from Promega. AMV reverse transcriptase (type MP) was purchased from Life Sciences. Sequenase reagents for DNA sequencing and T4 polynucleotide kinase were purchased from U.S. Biochemicals. $tRNA^{Phe}$, $tRNA^{fMet}$, and puromycin were purchased from Boehringer Mannheim, and [^{3}H]Phe was purchased from Amersham. tRNA was charged according to Traub et al. (1971). [^{32}P]Orthophosphate was purchased from ICN, and [γ-^{32}P]ATP was made from it by the method of Walseth and Johnson (1979). 70S ribosomes and 30S ribosomal subunits were prepared by the method described by Makhno et al. (1987). T7 RNA polymerase was purified by the procedure of Davanloo et al. (1984). 4-Thiouridine diphosphate was purchased from Sigma, and 4-thiouridine triphosphate was enzymatically synthesized by the method described by Tanner et al. (1988).

Construction of the Plasmid 19U-mRNA and in Vitro Transcription. The sequences of the synthetic mRNA used in this study are written in Table I. Plasmid DNA was used as templates for in vitro transcription of these RNAs. The plasmids were made by annealing phosphorylated complementary DNA strands starting with a *Hin*dIII site and ending in a *Bam*HI site and containing the desired internal DNA sequence and then ligating these into pTZ19U (USB) that had been digested with *Hin*dIII and *Bam*HI and dephosphorylated. *E. coli* HB101 cells were transformed with the ligation mix; double-stranded plasmid DNA was purified from individual colonies and was sequenced. Plasmid DNA from colonies containing the correct insert was purified on ethidium bromide/cesium chloride density gradients, digested with *Bam*HI, and then used for in vitro transcription. The transcription reactions contained 5 μg of digested 19U-mRNA plasmid in a total volume of 25 μL with 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 24 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol, 1.25 mM each ATP, CTP, and GTP, 0.3 mM UTP, 0.72 mM s^4UTP, 1 mM GMP, and 20 units of T7 RNA polymerase. Transcription was carried on for 1 h at 37 °C. The mixture was treated with 10 units of RNase-free DNase (Promega) and then was phenol extracted, ether extracted, and separated from the free nucleotides on a Sephadex G-50 column equilibrated with 50 mM triethylammonium carbonate, pH 7.5 (TEAC), and ethanol precipitated. The incorporation of s^4U, expressed as a ratio of moles of s^4U to moles of mRNA, was determined by the UV absorbance ratio at 330 and 257 nm. The RNA was redissolved in H_2O, and then the 5′ monophosphate was exchanged with [γ-^{32}P]ATP in 150 μL containing 50 mM sodium cacodylate (pH 6.6), 10 mM $MgCl_2$, 0.1 mM spermidine, 0.1 mM EDTA, 5 mM dithiothreitol, 0.3 mM ADP, 100 pmol of [γ-^{32}P]ATP (sp act. = 3000 Ci/mmol), and 30 units of T4 polynucleotide kinase. The exchange reaction was for 30 min at 37 °C. The reaction was stopped by adding EDTA to 20 mM. This mixture was again phenol extracted, and the RNA was ethanol precipitated and redissolved in H_2O.

For transcription of random mRNAs, a template of 67 nucleotides and a strand of 31 nucleotides that was complementary to the promoter region of the template were synthesized (Milligan et al., 1987; Tuerk & Gold, 1990). Five picomoles each of template and complementary strand were hybridized and then used for transcription as described above. Transcription and labeling were done as with the plasmids described above and then the reaction was electrophoresed on an 8% polyacrylamide gel; the band seen on the autoradiogram corresponding to the full-length transcript was cut out and eluted in RNA elution buffer (500 mM NH_4OAc, 0.2% SDS, 10 mM $MgCl_2$, and 1 mM EDTA) at room temperature overnight. The RNA was ethanol precipitated and used for complex formation.

Ribosome–mRNA Complex Formation and Cross-Linking. Complex formation between activated 70S ribosomes or 30S subunits and mRNA was done in 10 mM Tris-HCl, pH 7.4, 50 mM KCl, and 10 mM $MgCl_2$ (mRNA binding buffer; Katunin et al., 1980). Twelve picomoles of 70S ribosomes or 30S subunits in a total volume of 100 μL was incubated 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 Phe-$tRNA^{Phe}$ or $tRNA^{fMet}$. The samples were cooled on ice. The samples were irradiated at 365 nm for 10 min in a thermostated high-intensity mercury light irradiator (Isaacs et al., 1977); the intensity of light in this device [after filtration with the $Co(NO_3)_2$ filter] is about 100 mW/cm^2. The cross-linked samples and control samples were digested with proteinase K, phenol extracted, ethanol, precipitated, and redissolved at a final concentration of 200 ng (16S rRNA)/μL.

tRNA Binding Assay. Binding of the [^{3}H]Phe-$tRNA^{Phe}$ to the ribosome was assayed by a filter-binding assay (Wower et al., 1988). The complex was formed as described earlier and the mixture was chilled on ice for subsequent treatments. The placement of the [^{3}H]Phe-$tRNA^{Phe}$ in the P site was determined by incubating the ribosomal complexes with 1 mM puromycin for 15 min at 25 °C followed by measuring the reduction of ^{3}H on the nitrocellulose filter.

Reverse Transcription Analysis. Primer extension analysis of the mRNA–rRNA cross-links using reverse transcriptase was done as previously described (Wollenzien, 1988). In the current experiments, 200 ng of 16S rRNA (or an equivalent amount of total RNA) of each sample, 1 pmol of ^{32}P-labeled oligonucleotide primer, and 3.0 units of AMV reverse transcriptase were used in each 20-μL reaction. For the 16S rRNA, 10 primers described previously were used (Ericson et al., 1989). These primers allow us to examine all of the 16S rRNA sequence except for the last 40 nucleotides. Two

primers were used to examine 23S rRNA; their sites of complementarity are 23S-One (1197–1219) and 23S-Six (940–959).

Results

Characteristics of the Synthetic mRNAs. The mRNAs for these experiments were synthesized by in vitro transcription of plasmids containing a T7 promoter and the desired DNA sequences. Each of these encodes an RNA that is from 50 to 57 nucleotides long. The mRNA sequences are written in Table I. Each mRNA has an average 15 U residues, which were partially substituted with s^4U during transcription to levels of 3–4 s^4U/mRNA to make photoreactive analogs of the mRNA. mRNA 0 was used earlier (Wollenzien et al., 1991) and the sequence does not contain the Shine–Dalgarno sequence because it was designed to imitate a mRNA during the elongation phase of translation. The sequence of mRNA 1B was based on mRNA 0 but a possible stem–loop structure in positions 1–12 was removed and an initiation codon was also included in addition to the three codons. mRNA 7 was designed with only two codons and different 5′ and 3′ regions than the previous four molecules. mRNA SD was designed with a conventional Shine–Dalgarno sequence for translation initiation. A second mRNA containing a Shine–Dalgarno sequence was made by making two base changes in mRNA 1B to yield mRNA 1B-SD. There are no uridine residues in the first eight nucleotides in any of the mRNAs, which are part of the optimum T7 RNA polymerase promoter and *Hin*dIII site, and in addition, mRNA SD has a stretch of 14 nucleotides (including the Shine–Dalgarno sequence) in which uridine residues are not present.

An additional sample was made to probe the ribosome with a large mixture of different RNA molecules. To do this, DNA containing 19 random positions surrounding an AUG codon was chemically synthesized and used as a template for in vitro transcription of the corresponding RNA. The resulting RNA contains 12 random nucleotides preceding the AUG and seven random nucleotides succeeding it with constant sequences in the 5′ and 3′ regions of the RNA (Tuerk & Gold, 1990; see Table I).

mRNA 1B and 1B-SD were tested for their ability to direct tRNA binding under the conditions used in the cross-linking experiment. The stoichiometry of Phe-$tRNA^{Phe}$ binding with a 5-fold molar excess of mRNA over ribosomes and a 5-fold molar excess of Phe-$tRNA^{Phe}$ over ribosomes for both of the mRNAs was 0.7 Phe-$tRNA^{Phe}$/ribosome. For both of the mRNAs, incubation of the mixtures containing the complexes with puromycin reduced the stoichiometry of Phe-$tRNA^{Phe}$ binding to 0.45 Phe-$tRNA^{Phe}$/ribosome. This indicates that at least part of the Phe-$tRNA^{Phe}$ binding is in the P site, but these results are qualitative since there is some unchanged $tRNA^{Phe}$ in the reactions, and since there is an excess of Phe-$tRNA^{Phe}$ over ribosomes, we cannot rule out rebinding of Phe-$tRNA^{Phe}$ after the puromycin reaction.

The complex between 70S ribosomes or 30S subunits, mRNA, and Phe-$tRNA^{Phe}$ was formed under the ionic conditions described by Kirillov et al. (1980) and Katunin et al. (1980) and was irradiated. As controls, complexes formed between 70S ribosomes or 30S subunits and mRNA containing only uridine were irradiated, or 70S ribosomes or 30S subunits were irradiated without mRNA.

Analysis of Cross-Linking by Electrophoresis on Agarose Gels. The level of ^{32}P-labeled mRNA cross-linked to rRNA was determined by agarose gel electrophoresis. After removal of proteins, the RNA samples were denatured by heating in formamide and then electrophoresed on 2% gels. A fraction

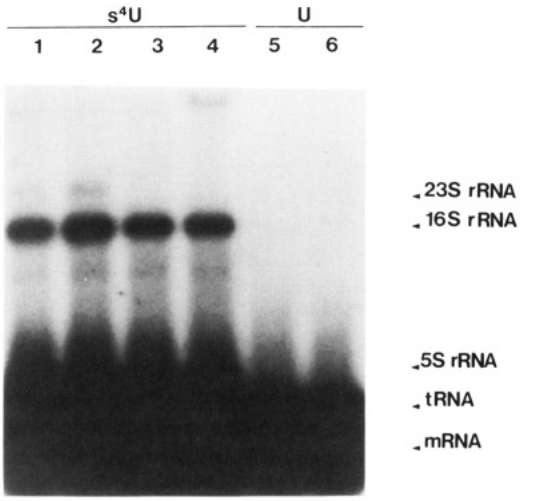

FIGURE 1: Gel electrophoresis of rRNA after cross-linking to mRNA 1B-SD. 70S ribosomes with or without Phe-$tRNA^{Phe}$ (lanes 1 and 2) or 30S subunits with or without Phe-$tRNA^{Phe}$ (lanes 3 and 4) were complexed with ^{32}P-labeled mRNA 1B-SD containing s^4U. As controls, 70S ribosomes with or without Phe-$tRNA^{Phe}$ (lanes 5 and 6) were complexed with ^{32}P-labeled mRNA 1B-SD containing only U. These complexes were then irradiated, digested with proteinase K, deproteinated, and denatured in formamide before gel electrophoresis. The gel was dried and an autoradiogram, shown here, was made. The positions of the various RNAs were determined by ethidium bromide straining of the agarose gels. The samples are marked according to the use of s^4U or U.

of the radioactive mRNA migrates with 16S and 23S RNA only when thiolated mRNA is used, as shown in Figure 1. In all cases mRNA not containing the s^4U did not comigrate with the 16S or 23S RNA. The pattern of cross-linking seen on gels run for a longer time than that shown in Figure 1 with RNA from 70S ribosomes does not give evidence for new low molecular weight species that would result if cross-linking occurred between mRNA and 5S RNA. Similarly, in experiments done with tRNA, the patterns do not indicate low molecular species that would result from cross-links between tRNA and mRNA (results not shown). The amount of rRNA cross-linked to mRNA was found to be dependent on the incorporation of s^4U in the mRNA. With mRNAs containing 3–4 s^4U/mRNA, the level of cross-linking to 16S rRNA was 11% with mRNA 1B, 14% with mRNA 0, 18% with mRNA 7, 27% with mRNA SD, and greater than 30% with mRNA 1B-SD. The level of cross-linking to 23S rRNA was 4% with mRNA 1B, 6% with mRNA 0, 4% with mRNA 7, 6% with mRNA SD, and 7% with mRNA 1B-SD. No difference in the frequency of cross-linking to 16S rRNA was observed with 30S subunits and 70S ribosomes. The inclusion of Phe-$tRNA^{Phe}$ in the complex did not alter the frequency of cross-linking (Figure 1 and results not shown).

Localization of rRNA–mRNA Binding Sites. The sites of cross-linking in rRNA were analyzed by reverse transcription elongation patterns obtained using various oligodeoxynucleotide primers. The basis of this assay is that the reverse transcriptase does not transcribe through modified nucleotides and hence generates a stronger than normal stop at the site of a cross-link compared to control samples. The location of these modified nucleotides can then be identified by gel electrophoresis of the reverse transcripts in parallel with lanes containing sequencing and control reactions. The controls are reverse transcripts made from RNA from complexes formed with nonthiolated mRNA and irradiated or made from RNA from ribosomes that were irradiated without added mRNA.

The detection of the cross-linking depends upon the visual inspection of the autoradiograms. At some cross-linking sites

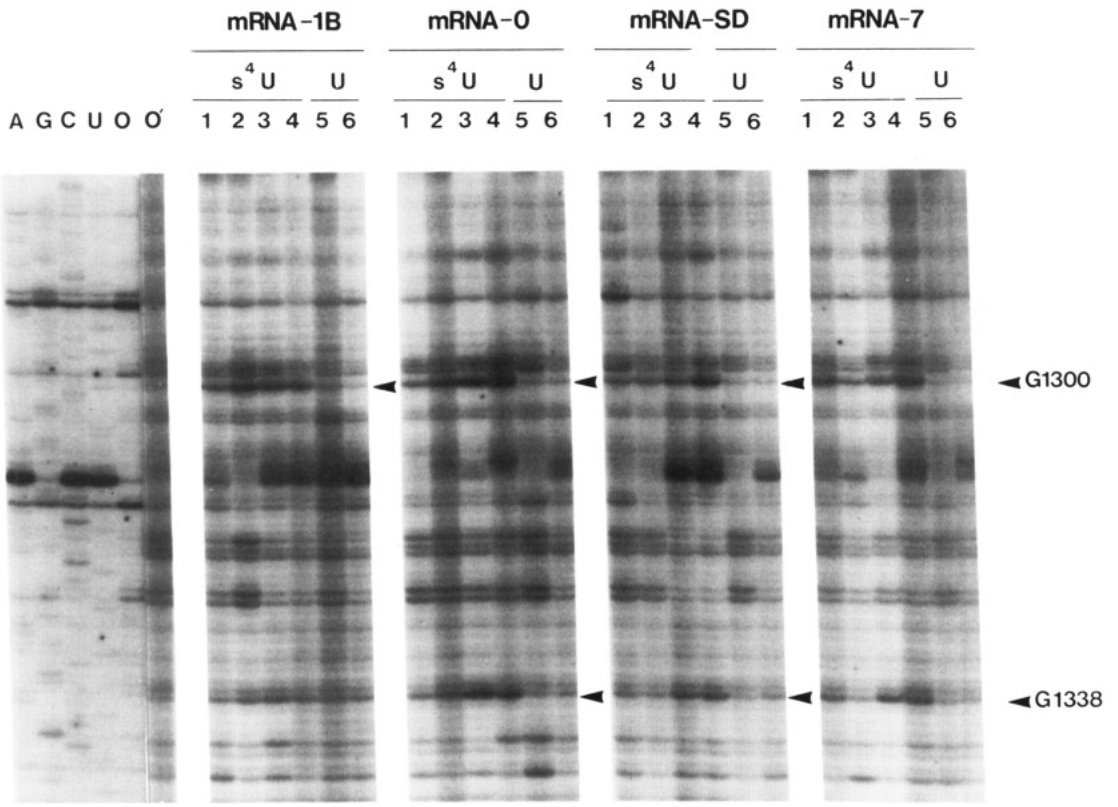

FIGURE 2: Reverse transcription analysis of 16S rRNA cross-linking sites at G1338 and G1330. A ^{32}P-labeled oligonucleotide primer complementary to nucleotides 1453–1466 in the 16S rRNA sequence was hybridized to unirradiated 16S rRNA for sequencing lanes or to the RNA purified from the cross-linking experiments; AMV reverse transcriptase was used for primer extension. The mRNAs that were used for complex formation are indicated. Sequencing and control reactions are marked A, G, C, U and O. The control lane marked O′ was done with RNA from irradiated 70S ribosomes. Lanes numbered 1 and 2 are from irradiated complexes containing 70S ribosomes and s^{4}U-containing mRNa (with and without Phe-tRNA^{Phe}, respectively). Lanes numbered 3 and 4 are from irradited complexes containing 30S subunits and s^{4}U-containing mRNA (with and without Phe-tRNA^{Phe}). Lanes numbered 5 and 6 are from complexes formed with 70S ribosomes or with 30S subunits with U-containing mRNA and with Phe-tRNA^{Phe}. Positions indicated with arrows on the right of each panel are the nucleotides on which cross-linking occurs.

there are only modest differences in the intensity of the band in the experimental lanes compared to control lanes, due presumably to the low extent of cross-linking at these sites. In the current experiments all cross-linking sites were observed in at least two of three independent experiments. There may be additional sites at which cross-linking occurs at lower efficiency, but these would not be reported if they cannot be reproducibly seen. In addition, most nucleotide positions show some degree of nonspecific reverse transcription stopping. This must be due to natural stops of the reverse transcriptase, UV-induced changes in the RNA from the irradiation step, and/or breaks in the rRNA that occur during purification or from radiation damage during storage of the samples. At some positions this nonspecific stopping is so great that a cross-linking site would be overlooked.

The actual cross-linking point on the rRNA must be the nucleotide on the 5′ side of the reverse transcription stopping site. The sites reported are the actual cross-linking sites. Sometimes a two-band stopping site is observed; in this case, one cross-linking point must be the 5′ nucleotide of the two nucleotides, and in addition, a cross-link could also be present on the next nucleotide on the 5′ side of the two bands. Due to this uncertainty one site has been listed with a question mark in the tabulation of the cross-linking sites in Table II.

Autoradiograms showing the pattern of the reverse transcription are shown in Figures 2, 3, and 4. These show examples of gels in which cross-links were detected at G1338 and G1330 (Figure 2), A532 and A524 (Figure 3), and U1381 (Figure 4) with the indicated mRNA samples. A complete list of sites seen in the 16S and 23S RNA which were identified in at least two out of three experiments is given in Table II. No attempt has been made to determine the frequency of cross-linking at individual sites since there are several factors that affect the strength of the reverse transcription signal from one primer extension experiment to another.

Discussion

Strategy of Designing the Various mRNAs and Their Properties. About 50 nucleotides of mRNA are shielded on the ribosome as determined by nuclease trimming experiments (Kang & Cantor, 1985). The synthetic mRNAs designed for these experiments were between 50 nucleotides (mRNA MIX) and 57 nucleotides (mRNAs SD, 1B, and 1B-SD), and since they were chosen not to have extensive secondary structure they should be able to span the ribosome. The A + G base compositions were 35% (mRNA 0) to 54% (mRNA SD), and the G + C base compositions were 49% (mRNA SD) to 59% (mRNA MIX). All of the mRNAs studied in these experiments associate with the ribosome and cross-link to it without the presence of tRNA, and for all six of these mRNA samples, the pattern of cross-linking does not have any apparent dependence on the presence of tRNA. This indicates that these mRNAs are associating efficiently with the ribosome even without tRNA. This is similar to the behavior of poly(U) of approximately this same length (Kirillov et al. 1980; Katunin et al., 1980; Bakin et al., 1991).

The results presented in this study indicate that the sequences of these RNAs do not have a major effect on the track they follow on the ribosome. Four of the mRNAs contained different 5′ regions and different arrangements of sequences

Table II: Sites of mRNA–16S rRNA Cross-Linking Determined by Using mRNA of Various Sequences

	mRNA 1B		mRNA 0		mRNA 7		mRNA SD		mRNA 1B-SD		mRNA MIX	
cross-link site	70S	30S	70S	30S	70S	30S	70S	30S	70S	30S	70S	30S
					16S rRNA							
U1381	+[a]	+	+	+	+	+	+	+	+	+	+	ND[b]
G1338	+	+	+	+	+	+	+	+	+	+	–	ND
G1300	+	+	+	+	+	+	+	+	+	+	+	ND
A1227	–	–	+	+	–	–	+	+	+	+	–	ND
G1156	+	+	+	+	+	+	+	+	+	+	–	ND
A845	+	+	+	+	+	+	+	+	+	+	+	+
G818	–	–	–	–	–	–	–	–	+	+	–	ND
U723	+	+	+	+	+	+	+	+	+	+	+	+
G693	+	+	+	+	+	+	+	+	+	+	–	ND
A532	+	+	+	+	–	–	+	+	+	+	–	ND
G524	–	–	+	+	–	–	–	–	+	+	–	ND
G497	–	–	+	+	+	+	+	+	+	+	–	ND
G423	–	–	+	+	–	–	–	–	–	–	–	ND
U420	+	+	+	+	+	+	+	+	–	–	–	ND
G413/A412?	+	+	+	+	+	+	+	+	+	+	+	ND
G346	+	+	+	+	+	+	+	+	+	+	+	ND
					23S rRNA							
U887	+		+		+		+		+		+	
U1065	–		+		–		–		+		+	

[a] + indicates cross-links seen by reverse transcriptase analysis; – indicates cross-links not seen. [b] ND, not determined.

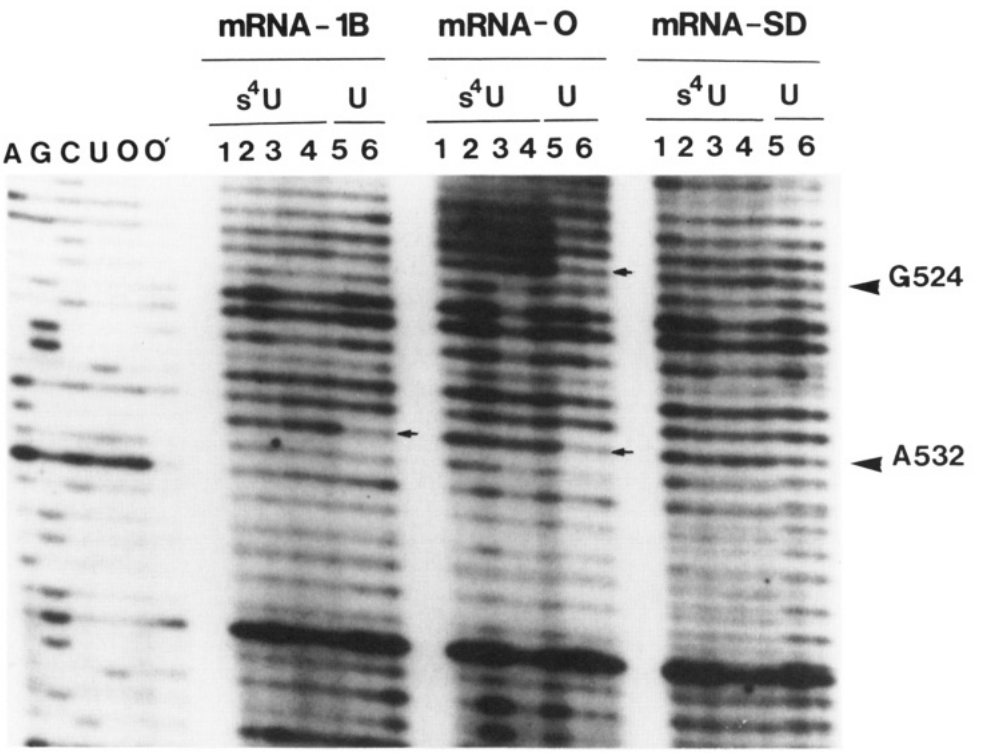

FIGURE 3: Reverse transcription analysis of 16S rRNA cross-linking sites at A532 and G524. A ^{32}P-labeled primer complementary to positions 559–575 was used for the primer extension. Sequencing and control lanes are marked A, G, C, U, and O and a control lane done with RNA from irradiated 70S ribosomes is marked O′. The experimental lanes done with 70S ribosomes and 30S subunits with s^{4}U-containing mRNA with and without Phe-tRNA^{Phe} are numbered 1–4 as described in the legend to Figure 2. The control lanes done with RNA from irradiated complexes containing 70S ribosomes and 30S subunits, U-containing mRNA, and Phe-tRNA^{Phe} are numbered 5 and 6, respectively.

containing triplets for the codons fMet, Val, Phe, Tyr, and Glu in the middle part of their sequence but had similar sequences in their 3′ third. Therefore, if the pattern of contacts were strictly sequence dependent, there would be differences and some similarities in the pattern of cross-linking in the first four samples. However, a fifth mRNA (mRNA 7) contained a different set of codons and different 5′ and 3′ sequences; using this mRNA, 12 of the same cross-linking sites seen with the first four mRNAs were observed. The sixth mRNA sample contained 19 random sequence positions surrounding a single AUG codon. With the sample amounts that were used in the experiments (25 pmol of mRNA incubated with 5 pmol of ribosomes), a very large number of different sequences were represented. In addition, the constant sequences at the 5′ and 3′ ends of mRNA MIX are different than any of the previous five mRNA sequences. With these differences the mRNA MIX still gave a cross-linking pattern very similar to those of the previous five samples; eight of the same sites were detected and no extra sites not seen in the previous experiments were detected.

Overall, for the 18 rRNA sites that have been detected, more than half of the sites (13 of 18) are cross-linked by at

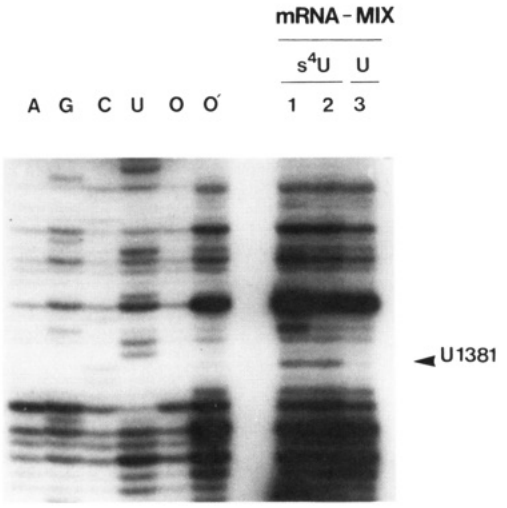

FIGURE 4: Reverse transcription analysis of 16S rRNA cross-linking sites at U1381 using mRNA MIX. Lanes marked A, G, C. U, and O are sequencing lanes; these and a control lane were all done with unirradiated RNA, and the lane marked O′ was done with RNA from irradiated 70S ribosomes. The lanes numbered 1 and 2 were done with RNA from irradiated complexes containing 70S ribosomes and s^4U-containing mRNA MIX, with or without Phe-$tRNA^{Phe}$. The lane numbered 3 was done with RNA from irradiated complexes containing 70S ribosomes, U-containing mRNA MIX, and Phe-$tRNA^{Phe}$.

least four of the six mRNAs. However, there are some exceptional sites. Sites at G818 and G423 are seen only with mRNA 1B-SD and mRNA 0, respectively, and the site at G524 is seen with mRNA 0 and mRNA 1B-SD. The significance of seeing sites only with some mRNA samples is not clear since there are no obvious differences in the distribution of uridine residues within the mRNAs and the mRNAs otherwise give typical patterns of cross-linking. As additional mRNA sequences are used with the s^4U reagent, some more cross-linking sites may be revealed.

The 16S rRNA sites identified are from all three major domains of the 30S subunit but only two sites in 23S rRNA (both in Domain II) were detected. This indicates that, while the mRNA interaction with the 30S is extensive, it is limited in the 50S subunit. The presence of strong Shine–Dalgarno sequences (mRNA SD and 1B-SD) apparently does not alter where the mRNA binds on the main mRNA portion of the ribosome, although it has an effect on the energy of interaction between the mRNA and the ribosome as reported by Calogero et al. (1988) and is reflected in overall efficiency of cross-linking in our experiments.

The use of s^4U as the photoaffinity reagent has several desirable features. The reagent can be incorporated during transcription in the mRNA and the level of incorporation adjusted according to the experimental needs. Illumination of thiolated complex at 366 nm selectively photoactivates the incorporated s^4U. The photoactive triplet state has a lifetime of a few microseconds in the native tRNA structure (Shalitin et al., 1976; Salet et al., 1988). Since the triplet energy of this nucleoside (240 kJ) is lower than those of other nucleic acid residues (Daniels, 1976), all the cross-links generated should occur directly at the photoexcited s^4U. The ability of the photoactivated s^4U to cross-link to a group would depend on proximity, orientation, and the nature of the environment around the group. Lemaigre-Dubreuil et al. (1991) recently determined the pattern of cross-linking by s^4U in a section of 16S rRNA and concluded that cross-links were formed between nucleotides that were constrained to be close in the secondary structure but were not themselves base-paired and were at sites of significant flexibility. Therefore, the majority of interactions between the mRNA and rRNA detected by this reagent are more likely to be by close approximation or by stacking interactions rather than through Watson–Crick base-pairing.

Comparison to Other Results Using s^4U-Substituted mRNA. Stade et al. (1989), Tate et al. (1990), and Rinke-Appel et al. (1991) reported cross-linking with several mRNA analogs containing single s^4U residues. Depending upon the location of the s^4U in the mRNA and the tRNA that was used, cross-linking occurred to the 16S rRNA in the region between nucleotides 1390 and 1400, to position A532, and to the 3′-terminal region of the 16S rRNA. In their experiments the efficiency of cross-linking mRNA to 16S rRNA was as high as 50% and was largely dependent upon cognate tRNA. These results are different from ours, in which for each mRNA that was used 12–16 sites in the 16S rRNA were cross-linked, the overall efficiency of cross-linking was from 11% to 30%, and the cross-linking was tRNA independent. These differences in the behavior of the mRNA and the cross-linking pattern are likely to be due to differences in the length and base composition of the mRNAs. The mRNAs used by Stade et al. (1989), Tate et al. (1990), and Rinke-Appel et al. (1991) are somewhat shorter (38–43 nucleotides) than the mRNAs we have used, and in addition, they contained mostly adenosine and guanosine nucleotides (up to 90% A + G) so that a hybrid selection procedure with poly(dT) could be used to enrich for rRNA that was cross-linked to mRNA. This base composition might result in unusual properties since it is known that the homopolypurines have distinct and unusual configurations compared to random polynucleotides (see Saenger, 1983).

Previously, using one mRNA, we reported cross-linking to two 23S rRNA sites and 12 16S rRNA sites (Wollenzien et al., 1991). In the current experiments we see cross-linking to the same two 23S rRNA sites and cross-linking to 16 sites in the 16S rRNA. Ten of these 16 sites were previously seen, six are new sites, and there were two sites in the 16S rRNA that were reported previously that have not been seen in the present experiments. There are several reasons for these differences. The first is that, due to partial reactivity in the cross-linking step and the appearance of the transverse transcription patterns, the number of cross-links that are reported in each experiment is a minimum number. Therefore, depending upon a number of variables, a larger or smaller number of sites may be reported. Second, there are reproducible differences seen with different ribosome preparations. Previously, we noted two regions in the 16S rRNA that were cross-linked differently in different ribosomes samples. These cross-links were A1130/G1131 in one sample vs G1131/C1132 in another sample and A412/G413 and U420/U421 in one sample vs G423/G424 in another sample (Wollenzien et al., 1991). In the present experiments, we did not see any cross-link in the 1130–1132 region, but instead we saw a cross-link at G1156 and cross-links in all three regions at A412/G413, U420, and G423 were seen. In addition, cross-links at A1227, G524, G497, and G346 were seen that were not seen before.

Location of the mRNA Contact Points in the 23S rRNA Secondary Structure. Two 23S rRNA sites, U887 and U1065, were found to be part of the mRNA binding center. The U887 site was consistently observed with all mRNA sequences examined. The stem–loop structure containing position U887 is present in the large subunit rRNA from many species from the three kingdoms but is not present in mitochondrial rRNA, and the nucleotide sequence containing U887 is not conserved. This site at present has no known function. U1065 was seen

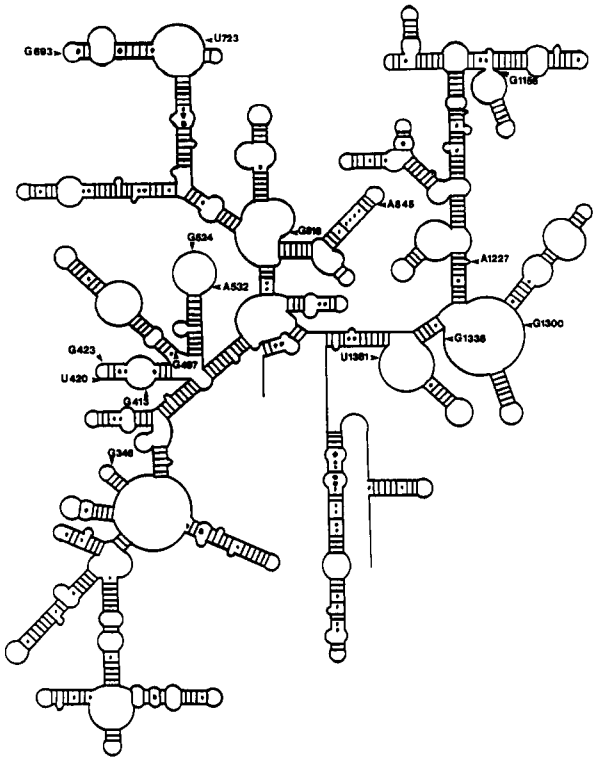

FIGURE 5: Locations of the mRNA cross-linking sites in the 16S rRNA secondary structure. All of the cross-linking sites seen with at least one mRNA are indicated. The secondary structure shown is from Gutell et al. (1985).

with mRNA 0, mRNA 1B-SD, and mRNA MIX. This site is in a stretch of 23S rRNA domain that is evolutionarily highly conserved (Moazed et al., 1988) and has been associated with the GTPase activity of EF-G (see Egebjerg et al., 1990).

Arrangement of the mRNA Contact Points on the 16S rRNA Secondary Structure. The 16S rRNA sites cross-linked to each particular mRNA are listed in Table II and the sites are marked in Figure 5, which shows the 16S rRNA secondary structure. Seven cross-linking sites (G346, G413, U420, G423, G497, G524, and A532) occur in Domain I. Of these, only G524 and A532 are in a region of the 16S rRNA that is highly conserved. Both of these nucleotides are in the loop containing m^7G527, which has been located on the 70S ribosome close to where elongation factors EF-Tu and EF-G have been mapped (Girshovich et al., 1981, 1986; Langer & Lake, 1986). EF-Tu is involved in A-site binding, and parts of the 518–533 loop are also protected by A-site-bound tRNA (Moazed & Noller, 1986, 1989). The involvement of G524 and A532 in this loop with mRNA is not unexpected. Four cross-linking sites (G693, U723, G818, and A845) occur in Domain II. The first three of these sites occur in highly conserved sections of the 16S rRNA, but the last one occurs in a region that shows variation in sequence and length even within known eubacteria sequences. Five cross-linking sites (G1156, A1227, G1300, G1338, and U1381) occur in Domain III. G1156 is in a nonconserved region and the rest are in highly conserved regions. Two additional cross-links that were previously reported, G1131/C1132 and C1395, are in Domain III. The cross-link at C1395 was seen was seen by us in 70S ribosomes but not in 30S subunits (Wollenzien et al., 1991). In addition, Stiege et al. (1988), using poly(A), and Stade et al. (1989), Tate et al. (1990), and Rinke-Appel et al. (1991), using synthetic RNA of defined sequences, have consistently reported cross-links to the region 1390–1400 using 70S ribosomes and, in one case using reverse transcription analysis, also determined that the site was C1395 (Tate et al., 1990).

Implications for the Three-Dimensional Arrangement of the 16S RNA Nucleotides around the mRNA Binding Center. Three-dimensional modeling of the 16S rRNA has been done by several research groups; partial and complete models have been proposed by Expert-Bezancon and Wollenzien (1985), Nagano and Harel (1988), Stern et al. (1988), Brimacombe et al. (1988), Oakes et al. (1990), and Hubbard and Hearst (1991). Some of the rRNA sites are in the middle part of the models (e.g., C1395 and A845), but in general, many of the rRNA sites are not situated in such a way to be consistent with the central location of the mRNA. In this report we have been able to document two sites in the 23S rRNA and 16 sites in 10 different regions of the 16S rRNA. Since these sites have been seen now with several different mRNAs, they must be representative of the track where the mRNA contacts the ribosome.

A key question is the identity of the mRNA sites (relative to the codon at the P site) to which the 16S rRNA is cross-linked, since this will have direct relevance for the arrangement of the rRNA around the mRNA and will also have consequences for the internal arrangement of the 16S rRNA. Rinke-Appel et al. (1991) presented data that the site at A532 was cross-linked from a s^4U placed 11 nucleotides in the 3′ direction from the beginning of the P site in the mRNA. This is consistent with the A and P tRNA sites located on the left and right, respectively, when the interior of the 30S subunit is viewed (see Noller, 1991). However, the same photoreactive nucleotide at +11 also reacted with the 16S rRNA between positions 1390 and 1400; therefore, the distance between A532 and the 1400 region is not yet clearly defined. Previously the 518–532 loop was determined to be on the side of the 30S subunit opposite from the decoding site in the cleft region. As noted by Rinke-Appel et al. (1991), if this site is much closer to the decoding site than previously thought, this will necessitate changes in the three-dimensional models. Similarly, once distances from the decoding site to the other sites of contact between mRNA and rRNA are known, these will add additional constraints for the rRNA folding.

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