Cross-Linking of the Anticodon of *Escherichia coli* and *Bacillus subtilis* Acetylvalyl-tRNA to the Ribosomal P Site. Characterization of a Unique Site in both *E. coli* 16S and Yeast 18S Ribosomal RNA†

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ABSTRACT: The nucleotide residues involved in the cross-link between P site bound acetylvalyl-tRNA (AcVal-tRNA) and 16–18S rRNA have been identified. This cross-link was formed by irradiation of *Escherichia coli* or *Bacillus subtilis* AcVal-tRNA bound to the P site of *E. coli* ribosomes or by irradiation of *E. coli* AcVal-tRNA bound to the P site of yeast ribosomes. The three cross-linked RNA heterodimers were obtained in 10–35% purity by disruption of the irradiated ribosome–tRNA complex with sodium dodecyl sulfate followed by sucrose gradient centrifugation. After total digestion with RNase T_1, and labeling at either the 5′- or the 3′-end, the cross-linked oligomers could be identified and isolated before and after photolytic splitting of the cross-link. One of the oligomers was shown to be UACACACCG, a unique rRNA nonamer present in an evolutionarily conserved region. This oligomer was found in all three heterodimers. The other oligomer of the dimer had the sequence expected for the RNase T_1 product encompassing the anticodon of the tRNA used. The precise site of cross-linking was determined by two novel methods. Bisulfite modification of the oligonucleotide dimer converted all C residues to U, except for any cross-linked C which would be resistant by being part of a cyclobutane dimer. Sequencing gel analysis of the UACACACCG oligomer showed that the C residue protected was the 3′-penultimate C residue, C_{1400} in *E. coli* rRNA or C_{1626} in yeast rRNA. Another method, statistical hydrolysis followed by diagonal gel electrophoresis [Ehresmann, C., & Ofengand, J. (1984) *Biochemistry* (following paper in this issue)], was used to confirm this assignment and to exclude the possibility of a small amount of cross-link at other positions. In particular, cross-linking of C_{1399} or C_{1625} was excluded. This method also directly proved that the site of cross-linking in tRNA was the wobble base, cmo^5U_{34} in *E. coli* or mo^5U_{34} in *B. subtilis*. Since both mo^5U_{34} and cmo^5U_{34} cross-link to the same C_{1400}, the COOH of cmo^5U_{34} cannot be involved in either cross-link formation or nucleotide selection. The cross-link site occurs in the middle of a sequence-conserved 17-mer in the 3′-region of the 16S rRNA which has been shown by others to be single stranded, accessible on the surface of the subunit, and at the interface between the subunits. The conservation of sequence, structure, location, and cross-linking site all imply the existence of a very specific three-dimensional structure involving this region of rRNA and the tRNA anticodon which is necessary for both prokaryotic and eukaryotic protein synthesis.

Although it has long been known that ribosomal RNA (rRNA)[1] and ribosomal proteins associate with each other in specific ways both during assembly and in the mature particle [reviewed by Nierhaus (1982) and Zimmermann (1980)], a role for rRNA in the various functions of the ribosome has only begun to be appreciated in recent years. The directive role of the sequence of 16S rRNA near the 3′-end in initiation of protein synthesis is now well-known [see Steitz (1980)], and the occurrence of a segment of 23S rRNA at the peptidyl transferase center is also well documented [reviewed in Ofengand (1980)]. In addition, there is evidence for a role in tRNA binding of an rRNA sequence complementary to the TΨCG found in most tRNAs (Ofengand, 1980; Ivanov et al., 1981) although the specific rRNA involved is still a matter of debate (Pace et al., 1982). Resistance to several antibiotics is also known to involve base changes in rRNA (Cundliffe, 1980; Ofengand et al., 1984). Small-subunit rRNA is also involved with the ribosomal decoding site. Affinity labeling studies with an mRNA analogue have implicated residues 462–474 of 16S rRNA (Wagner et al., 1976), and the anticodon of tRNA has been cross-linked to the 16–18S rRNA of ribosomes from *Escherichia coli*, spinach chloroplasts, yeast, and *Artemia salina* (Schwartz & Ofengand, 1978; Ofengand et al., 1979, 1982; Gornicki et al., 1983; Prince et al., 1979).

The properties of this latter cross-linking reaction have been extensively studied. Cross-linking by irradiation between 310 and 330 nm only occurs at the ribosomal P site of 70S or 80S ribosome–tRNA complexes (Ofengand et al., 1979, 1982; Gornicki et al., 1983), is codon specific (Ofengand & Liou, 1981), and is limited to tRNAs containing cmo^5U_{34} or mo^5U_{34} at the 5′-anticodon position (Ofengand et al., 1979). This reaction is of particular interest because the cross-link which is formed is a pyrimidine–pyrimidine cyclobutane dimer between the 5′-anticodon base of the tRNA and a residue of 16–18S rRNA (Ofengand & Liou, 1980; Ofengand et al., 1982). Formation of such a dimer requires that the cross-linkable residues be stacked on each other or be adjacent such that the π-orbitals of the C_5–C_6 double bonds of the two pyrimidines can interact. Thus, the ability to form such a cross-link demonstrates the existence of a hitherto unsuspected intimate (<4 Å) contact between the anticodon of P site bound tRNA and 16S rRNA.

The sequences of the small-subunit rRNAs are now known, and secondary structures have been proposed (Noller & Woese, 1981; Stiegler et al., 1981a; Zwieb et al., 1981). In order to use this knowledge to assess the significance of the close contact between 16–18S rRNA and the anticodon loop of tRNA revealed by the above-mentioned cross-linking re-

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[1] Abbreviations: rRNA, ribosomal RNA, Mes, 2-(*N*-morpholino)-ethanesulfonic acid; AcVal-tRNA, acetylvalyl-tRNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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action, the residue(s) of rRNA which is (are) cross-linked must be determined. This has been done in the present work. We have determined the site of cross-linking in *E. coli* 16S RNA for *E. coli* $tRNa_1^{Val}$ which has the cmo^5U_{34} residue (Murao et al., 1970) and have also used *Bacillus subtilis* $tRNA^{Val}$ which has mo^5U at position 34 (Murao et al., 1976) in order to see if the cross-linked residue in rRNA remains the same when the carboxyl group is absent from the tRNA anticodon. In addition, the site of cross-linking in yeast 18S rRNA has been determined in order to see if the same cross-linking site is involved in both prokaryotes and eukaryotes. Preliminary accounts of some of this work have appeared (Ofengand et al., 1982; Gornicki et al., 1982).

Experimental Procedures

Materials. The following materials were obtained or prepared as described previously: *E. coli* AcVal-$tRNA_1^{Val}$, *B. subtilis* AcVal-$tRNA^{Val}$ (*B. subtilis* $tRNA^{Val}$ was the generous gift of H. Ishikura), *E. coli* 70S tight-couple ribosomes, and poly(U_2,G) (Ofengand et al., 1979); pGUU (Ofengand & Liou, 1981); yeast 80S ribosomes (Ofengand et al., 1982; Dasmahapatra & Chakraburtty, 1981). T_4 polynucleotide kinase was purchased from New England Nuclear; T_4 RNA ligase, ribonuclease from *Bacillus cereus*, and ribonucleases Phy M and P_1 were purchased from P-L Biochemicals; ribonucleases T_1 and U_2 were purchased from Sankyo; chicken liver ribonuclease CL_3 was from BRL; alkaline phosphatase was from Boehringer. [γ-^{32}P]ATP and 5′-[^{32}P]pCp were from Amersham. Cellulose thin-layer plates (catalog no. G1440) (20 × 20 cm) were obtained from Schleicher & Schuell.

General Information. Electrophoresis was carried out on slab gels (40 × 30 × 0.04 cm) containing either 20% or 25% polyacrylamide, 100 mM Tris–borate (pH 8.3), 0.25 mM Na_2EDTA, and 8 M urea. The weight ratio of acrylamide to bis(acrylamide) was 20:1. Electrophoresis was conducted at room temperature, and the gels were preelectrophoresed for 2 h at 1800 V. The RNA bands were visualized by autoradiography. The selected RNA products were excised and eluted according to the procedure described by Maxam & Gilbert (1977). The cross-link was photolyzed (Ofengand & Liou, 1980) by irradiation under a 30-W Sylvania germicidal lamp at a distance of 7 cm for 7 min. Either cross-linked RNA could be directly irradiated in the polyacrylamide gel or, as an alternative, the RNA samples were resuspended in a small volume of water or appropriate buffer, spotted on Parafilm, and irradiated.

Formation, Assay, and Isolation of Covalent tRNA–rRNA Complexes. (*i*) *E. coli* $tRNA_1^{Val}$–*E. coli Ribosomes.* P-site complexes were prepared, cross-linked by irradiation, and assayed as previously described (Ofengand & Liou, 1981) with the following variations: [AcVal-tRNA], 100–150 nM; [Mg^{2+}], 9–15 mM; [poly(U_2,G)], 20 μg/mL; [pGUU], 25–40 μM. Irradiation was for 3–3.5 h at >310 nm with the Mylar filter (Ofengand et al., 1979) and for 54 min at ca. >290 nm in its absence. In the latter case, 1 cm of 1.7 M $NiSO_4$ was used to filter out (<10% transmission) light >350 nm. Ribosomes and tRNA were precipitated with 2% KOAc, pH 5, and 72% EtOH and resuspended in 20 mM Mes, pH 6.0, 100 mM NH_4Cl, 0.5 mM Mg^{2+}, and 3 mM EDTA (buffer A) containing 2% SDS; 25–100 μL/mL of reaction mixture was used. After incubation for 1 min at 37 °C, the sample was diluted with buffer A as needed and applied to 5–27% isokinetic sucrose gradients in buffer A plus 0.1% SDS. The equivalent of 2.5–3.0 mL of reaction mixture was applied to each SW40 tube, which was then centrifuged at 20 °C. After location of the tRNA–rRNA complex peak by radioactivity determination, the pooled fractions were precipitated 2 times or more with 2% KOAc, pH 5, and 67% EtOH. Depending on the batch of ribosomes used and the exact incubation conditions, the isolated complexes were 15–35% pure, assuming 67 pmol/A_{260} unit for 16S rRNA.

(*ii*) *B. subtilis* $tRNA^{Val}$–*E. coli Ribosomes.* The same procedure described above was followed except that when pGUU was used as the codon, 20 mM Mg^{2+} was used instead of 15 mM and the AcVal-tRNA was used at 60–85 nM. Irradiation was performed with pGUU as well as with poly(U_2,G) both with and without the Mylar filter. The purity of the isolated complexes ranged from 14 to 31%.

(*iii*) *E. coli* $tRNA_1^{Val}$–*Yeast Ribosomes.* P-site binding, cross-linking by irradiation for 45 min, and assays were as described previously (Ofengand et al., 1982) using 40–50 μM pGUU as the codon and 150 nM AcVal-tRNA. Approximately 90% cross-linking was obtained. After the addition of 0.1 volume of 20% KOAc, pH 5, the reaction mixtures were deproteinized by Vortex mixing for 3 min at 23 °C with an equal volume of phenol equilibrated with 2% KOAc, pH 5, and 10 mM mercaptoethanol. This extraction was repeated, and the two phenol phases were back-extracted with 2% KOAc, pH 5. The two aqueous extracts were pooled and precipitated with 2 volumes of EtOH at −20 °C for 30 min. The precipitate was dissolved in buffer A containing 0.1% SDS (0.25–0.30 mL/mL of reaction mixture) and incubated at 37 °C for 1 min. Sucrose gradients were run as described above but with 10–30% sucrose at 15 °C. The purity of the isolated product ranged from 10 to 20% in different preparations, assuming 54 pmol/A_{260} unit for 18S rRNA.

Isolation of the Covalent tRNA–rRNA T_1 Oligonucleotide Duplex. The covalent tRNA–rRNA complexes were resuspended in 10 mM Tris-HCl (pH 8.0) at a concentration of 1 mg/mL. An aliquot was photolyzed to provide a reference system. Ribonuclease T_1 was added to both irradiated and nonirradiated samples at an enzyme to substrate ratio of 20 units/60 μg of RNA. Incubation was at 37 °C for 60 min. The digestion mixtures were adjusted to 10 mM $Mg(OAc)_2$ and 6 mM 2-mercaptoethanol and incubated for 45 min at 37 °C in the presence of 4 units of T_4 polynucleotide kinase and 50 μM [γ-^{32}P]ATP. The 5′-labeled oligonucleotides were recovered by precipitation with ethanol, dried under vacuum, and resuspended in 8 M urea, 0.025% xylene cyanol, and 0.025% bromophenol blue. They were fractionated by electrophoresis on 20% polyacrylamide slab gels until the xylene cyanol marker migrated 8–11 cm into the gel, as indicated in the figure legends. After autoradiography, the selected bands were excised and eluted.

In several experiments, the T_1 digestion products were labeled at their 3′-ends. The 3′-terminal phosphate was first removed by alkaline phosphatase, in the presence of 0.1% SDS, as described by Shinagawa & Padmanabhan (1979). After destruction of the phosphatase activity by heating in the presence of 5 mM nitrilotriacetic acid (Silberklang et al., 1977), the dephosphorylated oligonucleotides were labeled with ^{32}P at their 3′-terminus with 5′-[^{32}P]pCp in the presence of RNA ligase (England & Uhlenbeck, 1978).

Two-Dimensional Gel Electrophoresis of Total T_1 Digestion Products. Preirradiated and nonirradiated tRNA–rRNA complexes were subjected to total T_1 digestion and 5′-terminal labeling, as described above. The first dimension was run on a 20% polyacrylamide slab gel. After direct irradiation of the gel, the strip containing the fractionated oligonucleotides was cut out and included at the top of a second 20% polyacrylamide slab gel for the second dimension. Electrophoresis at 800 V

was terminated when the bromophenol blue marker had traveled 12 cm.

Bisulfite Modification. Sodium metabisulfite, $Na_2S_2O_5$, was used as the source of bisulfite ions. Preirradiated and nonirradiated oligonucleotide duplexes were incubated in 100 μL of freshly prepared 2 M $NaHSO_3$–1 M Na_2SO_3 (pH 5.9) containing 100 μg of carrier tRNA for 24 h at room temperature (Singhal, 1971). At the end of the incubation, the reaction mixture was diluted 4-fold, desalted on Sephadex G25, and precipitated with 2 volumes of ethanol. The pellet was dissolved in 50 mM H_3BO_3–$Na_2B_4O_7$ buffer (pH 8.5) and incubated overnight at 37 °C. After being desalted again on Sephadex G25, the RNA was recovered by ethanol precipitation. The modified duplex was purified by polyacrylamide gel electrophoresis when subsequent postlabeling was required.

Sequencing Procedure. The 5′-labeled oligonucleotides were analyzed by enzymatic cleavage methods. The reaction mixtures were 5 μL and contained 1 μg of unlabeled tRNA. Partial digestion with RNase T_1 (G specific) or U_2 (A specific) was carried out under the conditions described by Donis-Keller et al. (1977), the enzyme to substrate ratio being 0.025 and 0.25 unit/μg of RNA, respectively. Digestion with RNase Phy M (A + U specific) was also carried out in the same buffer conditions, according to Donis-Keller (1980), with 1–2 units/μg of RNA. Digestion with RNase from *B. cereus* (C + U specific) was carried out in the absence of 7 M urea, as described by Lokard et al. (1978) with 1 unit/μg of RNA for 15 min at 55 °C. Digestion with chicken liver RNase CL_3 (C specific) was carried out in 10 mM phosphate buffer (pH 6.5) as described by Boguski et al. (1980) for 30 min at 37 °C with 1–2 units/μg of RNA. The ladder was obtained by limited alkaline hydrolysis, as described by Donis-Keller et al. (1977). Electrophoresis was at 1800 V in 25% polyacrylamide gel and was terminated when the bromophenol blue marker had traveled 11 cm.

The 3′-labeled oligonucleotides were analyzed by the chemical cleavage methodology described by Peattie (1979).

Identification of 5′-Terminal Nucleotides. Total digestion of 5′-labeled oligonucleotides with ribonuclease P_1 was carried out in 10 μL of 50 mM NH_4OAc (pH 5.3) for 1 h at 37 °C with 0.2 unit of enzyme and 10 μg of carrier tRNA. Analysis was by one-dimensional thin-layer chromatography with concentrated HCl–2-propanol–H_2O (17.6:68.0:14.4) as solvent. The 5′-labeled nucleotides were visualized by autoradiography and marker 5′-nucleotides by inspection under UV light.

Results

Isolation of Covalent tRNA–rRNA Complexes. *E. coli* Ac[^{3}H]Val-tRNA$_1^{Val}$ or *B. subtilis* Ac[^{3}H]Val-tRNA^{Val} was bound to *E. coli* 70S ribosomes in the presence of pGUU or poly(U_2,G) as codon. Cross-linking was performed by irradiation for varying lengths of time at 0 °C with 300-nm lamps in a Rayonet photoreactor, either with or without a Mylar plastic filter. When the filter was used, only wavelengths >310 nm reached the sample. In the absence of a filter, the short-wavelength transmission is limited by the Pyrex glass cooling chamber to ca. >290 nm (Ofengand et al., 1979). Typical cross-linking kinetics under these various conditions are illustrated for *E. coli* AcVal-tRNA in Figure 1. Clearly, the two different irradiations conditions only affect the rate of reaction (4-fold difference) but not the yield. The difference between the two codons is also clearly illustrated. While there was 88% cross-linking with pGUU, there was only 51% with poly(U_2,G). Note also that the amount of tRNA bound to the ribosome was not affected by these irradiation conditions. Cross-linked complexes were prepared by using both pGUU

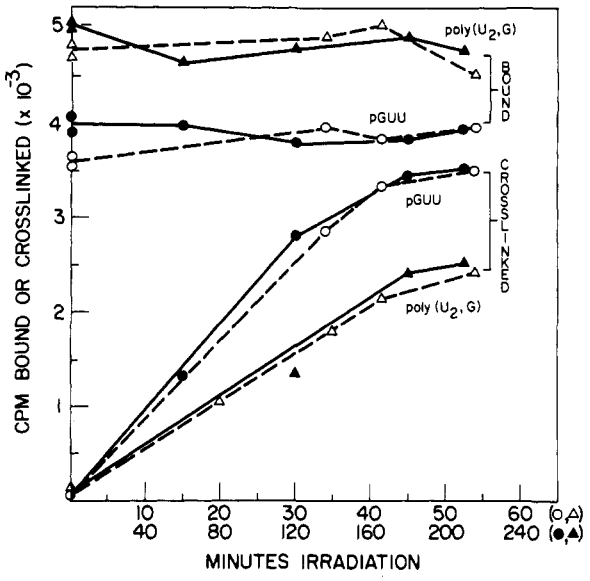

FIGURE 1: Kinetics of cross-linking of *E. coli* AcVal-tRNA to *E. coli* ribosomes using pGUU or poly(U_2,G) as codons and with different irradiation conditions. P-site complexes were prepared, irradiated, and assayed as described under Experimental Procedures: with pGUU (○, ●); with poly(U_2,G) (△, ▲); irradiation without the Mylar filter (○, △); irradiation with the filter (●, ▲). The lower two sets of curves are covalent binding assays (0.1 mM Mg^{2+}); the upper two are total binding assays (20 mM Mg^{2+}).

and poly(U_2,G) with each of the two irradiation conditions in order to see if variation in either of these parameters would affect the cross-linking site in rRNA. This possibility was considered since there was strong indirect evidence that the tRNA residue which cross-links is the 5′-anticodon residue (Ofengand et al., 1979). For the preparation of *E. coli* AcVal-tRNA cross-linked to yeast ribosomes, only pGUU and irradiation without the Mylar filter were used because cross-linking with poly(U_2,G) was only 20–25% efficient (Ofengand et al., 1982), and the yeast noncovalent complexes were not sufficiently stable to the long incubation times required in the presence of the Mylar filter.

In all cases, the cross-linked tRNA–rRNA was isolated by SDS–sucrose-gradient centrifugation as described under Experimental Procedures. All cross-linking was to 16S RNA, and the complex was readily separated from non-cross-linked tRNA. Thus, in one step cross-linked complexes could be obtained whose only contaminant was un-cross-linked rRNA. This un-cross-linked rRNA could, however, be readily removed in subsequent steps of the fractionation (see below). After SDS–sucrose-gradient centrifugation, the tRNA–rRNA peak was recovered by ethanol precipitation, dissolved in water, and assayed. The purity ranged from 10 to 35% for the various samples. The samples were then precipitated with ethanol twice, washed with ethanol, dried, and resuspended in RNase T_1 hydrolysis buffer.

Isolation of Cross-Linked tRNA–rRNA Oligonucleotides. In each case, both covalent tRNA–rRNA complex and a photolyzed (Ofengand & Liou, 1980) complex were subjected to total T_1 RNase hydrolysis followed by 5′- or 3′-labeling. Besides the normal T_1 oligonucleotides from both tRNA and rRNA molecules, one (or more) pair(s) of linked oligonucleotides was (were) expected. These duplex structures should be specifically observed only in the digest of the nonphotolyzed complexes. The digestion products of both photolyzed and nonphotolyzed samples were fractionated on a polyacrylamide slab gel. Comparison of the two fractionation patterns showed the presence of such covalent duplexes as an additional band, present only in the nonirradiated complex.

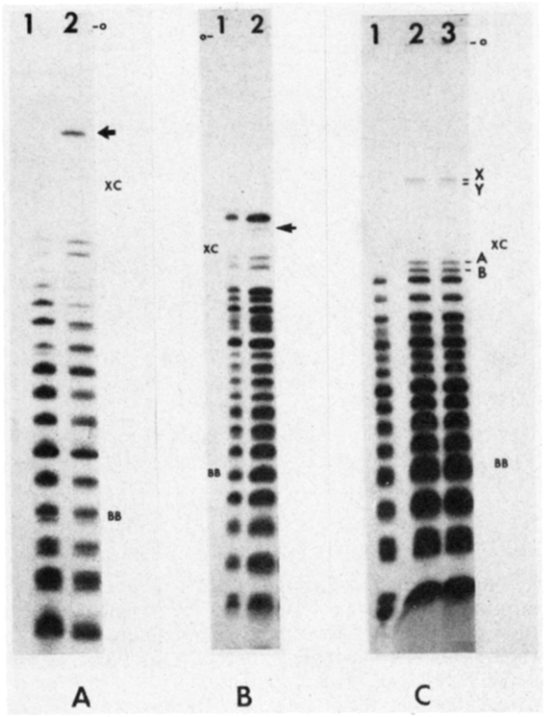

FIGURE 2: Gel electrophoresis fractionation of oligonucleotides resulting from total T_1 RNase digestion of cross-linked complexes. (A) *E. coli* $tRNA_1^{Val}$–*E. coli* 16S RNA complex; (B) *E. coli* $tRNA_1^{Val}$–yeast 18S RNA complex; (C) *B. subtilis* $tRNA^{Val}$–*E. coli* 16S RNA complex; (lanes 1) photolyzed controls; (lanes 2 and 3) covalent complexes. Fractionation was carried out on 20% polyacrylamide gels. The positions of xylene cyanol (XC) and bromophenol blue (BB) markers are indicated. The positions of the covalent T_1 oligonucleotide duplexes are indicated by an arrow or designated by A, B, X, and Y. Cross-linking of *B. subtilis* $tRNA^{Val}$ to *E. coli* ribosomes was made in the presence of pGUU (lanes 1 and 2) or poly(U_2,G) (lane 3).

In order to confirm that this band was the cross-linked dimer, two-dimensional gels were used. After the first dimension, the gel was irradiated to cleave the cross-link (Ofengand et al., 1979; Ofengand & Liou, 1980), and the strip containing the fractionated products was included at the top of a second slab gel of an identical composition. After electrophoresis in the second dimension, oligonucleotides with unchanged mobility migrate along a diagonal while those oligonucleotides which are released by cleavage of the cross-link have an increased mobility and migrate as off-diagonal products.

(*i*) *E. coli* $tRNA_1^{Val}$–*E. coli 16S RNA Complex.* A typical fractionation pattern of 5′-labeled T_1 digestion products from both photolyzed and nonphotolyzed samples is shown in Figure 2A. One band, denoted by the arrow, is clearly observed in the nonirradiated sample but not in the photolyzed complex. After photolysis and electrophoresis in the second dimension, a unique pair of off-diagonal subfragments was revealed (see Figure 3, products A and B). When 3′-terminal labeling was used instead of 5′-labeling, only one single off-diagonal product was observed, corresponding to the slower moving product A (data not shown).

(*ii*) *E. coli* $tRNA_1^{Val}$–*Yeast 18S RNA Complex.* Figure 2B shows the fractionation of 5′-terminal-labeled T_1 digestion products from irradiated and unirradiated samples, after the first gel electrophoresis. Again, one band is observed (denoted by an arrow) in the unirradiated sample, which is absent in the photolyzed control. In a second covalent tRNA–rRNA preparation, a minor band which migrated slightly faster than the major product was also observed. One pair of two major off-diagonal products was observed after irradiation and electrophoresis in the second dimension (Figure 4B, products

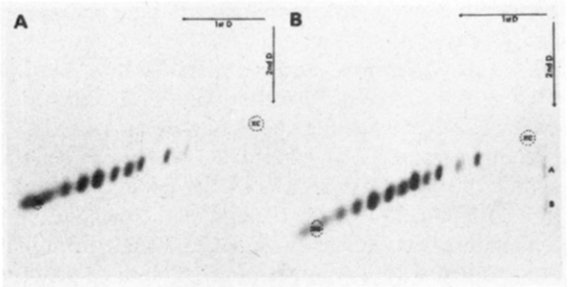

FIGURE 3: Two-dimensional gel electrophoresis of T_1 digestion products from the *E. coli* $tRNA_1^{Val}$–*E. coli* 16S RNA complex: (A) photolyzed; (B) nonphotolyzed. Oligonucleotides were ^{32}P labeled at their 5′-end. The first dimension was carried out as described in Figure 2. The second dimension, following photoreversal of the cross-link at 254 nm, was carried out the same way. Marker positions are indicated. The unique pair of off-diagonal products is denoted A and B.

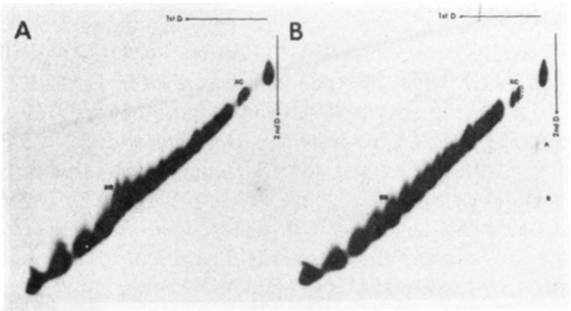

FIGURE 4: Two-dimensional gel electrophoresis of T_1 digestion products from the *E. coli* $tRNA_1^{Val}$–yeast 18S RNA complex: (A) photolyzed; (B) nonphotolyzed. Conditions as in Figure 3.

A, B). A background of minor off-diagonal products is present but is also observed in the control sample (see Figure 4A). In this case again, only the slow-moving off-diagonal product A can be detected after 3′-terminal labeling (data not shown).

(*iii*) *B. subtilis tRNA*^{Val}–*E. coli 16S RNA.* A fractionation pattern of 5′-labeled T_1 digestion products from both irradiated and unirradiated samples is shown in Figure 2C. Two major bands, denoted A and B, are reproducibly present in the covalent complex and not in the photolyzed sample. In the experiment shown here, two additional slower moving products, denoted X and Y, were also detected. After irradiation and second-dimension electrophoresis, each band A and B yielded a single radioactive off-diagonal subproduct, with an electrophoretic mobility corresponding to a nonanucleotide (data not shown).

Sequence Analysis of the Cross-Linked Oligonucleotides. Oligonucleotides arising from photolysis of the T_1 oligonucleotide duplexes were isolated directly from two-dimensional (2D) gels (see Figures 3 and 4). As an alternative procedure, the covalent T_1 olgionucleotide duplexes were isolated by one-dimensional gel electrophoresis (Figure 2), eluted, and irradiated, and the resulting subproducts were fractionated by gel electrophoresis. The 5′- and 3′-labeled oligonucleotides were analyzed by enzymatic or chemical cleavage methodology, respectively.

(*i*) *E. coli* $tRNA_1^{Val}$–*E. coli 16S RNA.* A unique covalent T_1 oligonucleotide duplex was characterized (Figure 2A) which upon photolysis dissociated into oligonucleotides A and B (Figure 3). The sequence of oligonucleotide A, the slower moving product, was deduced to be pC-A-C-C-U-C-C-C-U-cmo^{5}U-A-C-m^{6}A-A-G (Figure 5A). This corresponds to the expected T_1 pentadecamer from *E. coli* $tRNA_1^{Val}$ which contains cmo$^5U_{34}$. This modified uridine, located at the 5′-anticodon position, has been argued to mediate the cross-linking reaction (Ofengand et al., 1979; Ofengand & Liou, 1981).

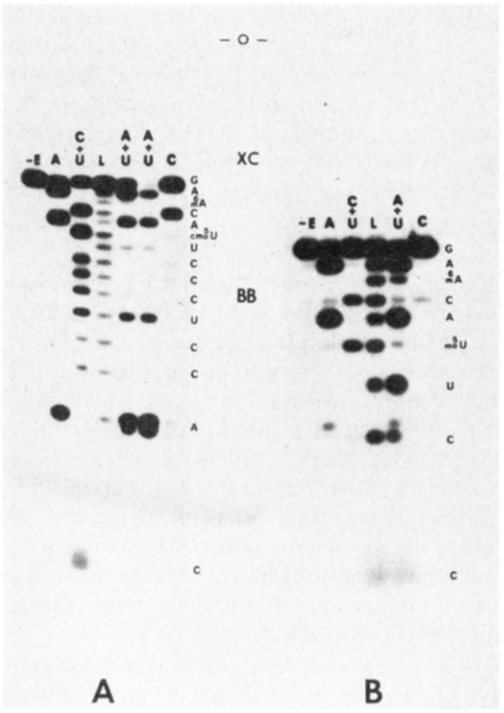

FIGURE 5: Sequence analysis of T_1 oligonucleotides on polyacrylamide gels. (A) Oligonucleotide A from the gel of Figure 3B; (B) oligonucleotide A from the gel of Figure 2C was photolyzed and electrophoresed on 25% gels; the radioactive product, faster moving than the starting oligomer A, was eluted and used for sequencing. Incubation mixtures (5 μL) contained 1 μg of unlabeled tRNA and (–E) no enzyme added, (A) 0.25 unit of RNase U_2, (C + U) 1 unit of RNase from *B. cereus*, (A + U) 0.25 unit of RNase Phy M, or (C) 1 unit of chicken liver RNase CL_3. The ladder (L) was obtained by limited alkaline digestion. Electrophoresis was carried out on 25% polyacrylamide slab gels until the bromophenol blue dye (BB) reached 11 cm from the origin. The modified residue m^6A is not cleaved by RNase U_2, and (c)mo^5U is nearly not cleaved by RNase CL_3.

Oligonucleotide B, the faster moving product, was unambiguously identified as pU-A-C-A-C-A-C-C-G, a unique nonanucleotide in the sequence of *E. coli* 16S RNA, located at positions 1393–1401 (Brosius et al., 1978; Carbon et al., 1978). The sequencing gel electrophoresis pattern is shown in Figure 6A. This result is in agreement with the findings of Taylor et al. (1981) obtained by a different approach.

When 3′-labeling was used, only one labeled oligonucleotide was released after irradiation as an off-diagonal product with a mobility corresponding to oligonucleotide A. Sequence analysis confirmed that this oligonucleotide was the tRNA pentadecamer (data not shown), thus indicating that RNA ligase is unable to add a pCp to the 3′-extremity of the 16S RNA nonamer when it is cross-linked to the tRNA fragment.

(*ii*) *E. coli* $tRNA_1^{Val}$–*Yeast 18S RNA*. The major covalent T_1 oligonucleotide duplex was dissociated into oligonucleotides A and B (see Figure 4). Sequence analysis showed that oligonucleotide A was the expected tRNA pentadecamer pC-A-C-C-U-C-C-C-U-cmo^5U-A-C-m^6A-A-G (data not shown), and oligonucleotide B was identical with the *E. coli* 16S RNA nonamer pU-A-C-A-C-A-C-C-G, a unique sequence (residues 1619–1627) in the yeast 18S rRNA sequence (Rubstov et al., 1980). An example of part of the analysis is shown in Figure 7A.

A minor oligonucleotide complex, which was isolated as a faster moving product after RNase T_1 hydrolysis in one preparation (see above), yielded three different products after UV irradiation. These products were identified as the tRNA pentadecamer, the 18S RNA nonamer pU-A-C-A-C-A-C-C-G, and the incomplete 18S RNA oligonucleotide pA-C-A-C-A-C-C-G (data not shown).

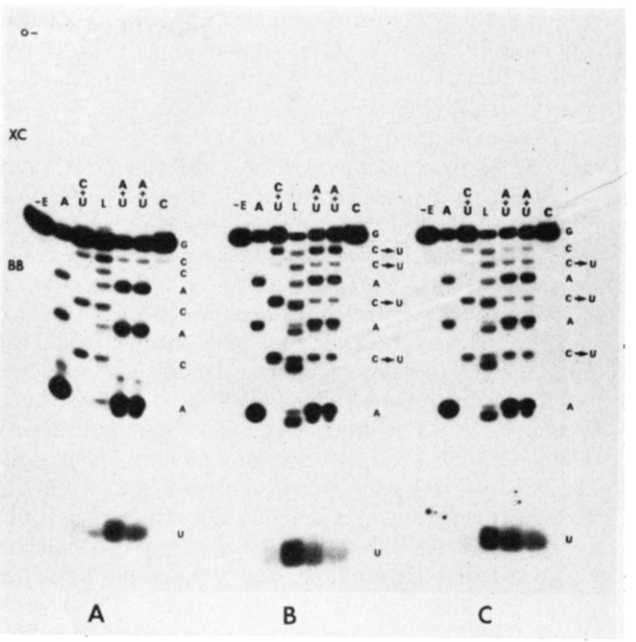

FIGURE 6: Sequence analysis on polyacrylamide gels of the ribosomal RNA oligomer derived from the *E. coli* tRNA–*E. coli* rRNA complex. (A) Unmodified; (B) modified by bisulfite treatment of the prephotolyzed oligonucleotide duplex; (C) modified by bisulfite treatment of the nonphotolyzed covalent oligonucleotide duplex. Hydrolysis conditions were as described in Figure 5 except that RNase Phy M digestion was carried out with 1.4 and 2 units. Fractionation was on 25% polyacrylamide gels until the bromophenol blue dye (BB) had traveled 11 cm.

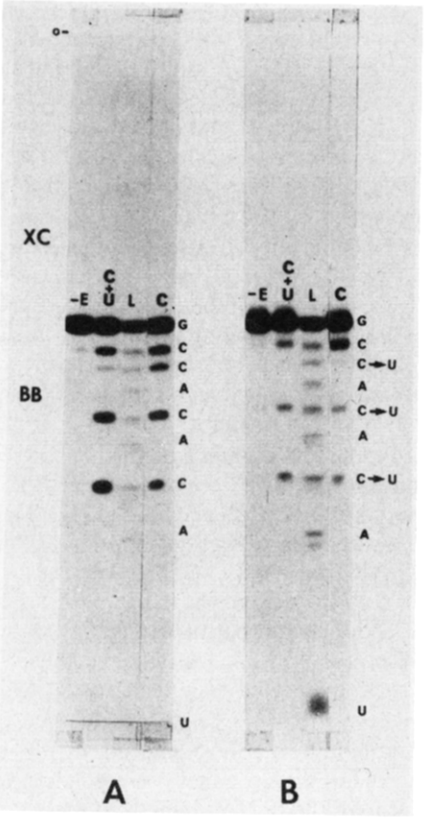

FIGURE 7: Sequence analysis of a polyacrylamide gel of the yeast 18S RNA nonamer containing the cross-linking site: (A) unmodified; (B) modified by bisulfite treatment within the covalent oligonucleotide duplex. Digestion and electrophoresis conditions are identical with those described in Figure 5.

After 3′-labeling of the major duplex, only one off-diagonal product was found, again corresponding to the tRNA oligonucleotide (data not shown).

(*iii*) *B. subtilis tRNA*^{Val}–*E. coli 16S RNA*. The 5′-labeled product released after photolysis of each of the two major covalent T_1 oligonucleotide duplexes A and B (Figure 2C) gave sequence ladders consistent with a single sequence rather than a mixture of two labeled oligonucleotides of the same length. The oligonucleotide released from duplex A by photolysis was found to be pC-C-U-mo^{5}U-A-C-m^{6}A-A-G (Figure 5B). It corresponds to the expected T_1 nonamer from *B. subtilis* tRNA^{Val} containing mo^{5}U, the modified uridine thought to mediate the cross-linking reaction. The oligonucleotide released from duplex B was unambiguously identified as pU-A-C-A-C-A-C-C-G by sequence analysis (data not shown), the unique *E. coli* 16S RNA nonamer already found cross-linked to *E. coli* tRNA$_1^{Val}$. Surprisingly, we obtained in this case two distinct covalent oligonucleotide duplexes in which only one of the two linked oligonucleotides was 5′-labeled. Each labeled oligonucleotide so obtained was >99% pure, as shown both by sequencing and by 5′-terminal nucleotide analysis. It was not possible to detect any trace (<1%) of the associated oligomer in labeled form.

In order to unambiguously identify the unlabeled associated oligonucleotide, each covalent oligomer duplex A and B was isolated by one-dimensional gel electrophoresis, irradiated, and again 5′-labeled. This time, both 5′-ends were labeled. The two 5′-labeled nonamers were then fractionated by extensive gel electrophoresis. By sequence analysis (data not shown), the slower moving band was identified as the tRNA nonamer pC-C-U-mo^{5}U-A-C-m^{6}A-A-G, and the faster moving band was shown to be the 16S RNA nonamer pU-A-C-A-C-A-C-C-G. Therefore, duplexes A and B are identical in terms of nucleotide length and sequence except for the location of the 5′-terminal [^{32}P]phosphate, but nevertheless exhibit different electrophoretic mobility. The same results were obtained whether tRNA cross-linking occurred in the presence of pGUU or poly(U_2,G) as codon.

Using 3′-labeling, only the tRNA oligomer was labeled in each of the two duplexes A and B of Figure 2C (data not shown). Thus, even though the 5′-end of the tRNA oligomer was unavailable for 5′-labeling in duplex B, it was available for 3′-labeling. The lack of labeling of the rRNA oligomer in both duplexes is consistent with the similar failure described immediately above in sections i and ii.

Small additional off-diagonal products observed in several experiments (not shown) were sequenced also. They were identified as pA-C-A-C-C-G and pA-C-C-G. These products most likely arose from partial degradation of the 16S RNA sequence U-A-C-A-C-A-C-C-G. The smaller fragment, pA-C-C-G, was found associated with a complete 5′-labeled tRNA nonamer. The fact that both fragments were derived from the 3′-part of the 16S rRNA oligomer suggests that the cross-link must be located near the 3′-end.

The two large duplexes, X and Y of Figure 2C, were also analyzed by photolysis and sequencing of the derived subfragments. A large subproduct corresponding to the entire anticodon loop of the tRNA, namely, pC-A-U-C-U-G-C-C-U-mo^{5}U-A-C-m^{6}A-A-G-C-A-G-A-G-(G), was identified as well as the 16S RNA nonamer pU-A-C-A-C-A-C-C-G (data not shown). It appears that these large duplexes arise from incomplete T_1 digestion of the tRNA.

Identification of the Cross-Linked Nucleotides. The structure of the cross-link was inferred to be a pyrimidine–pyrimidine cyclobutane dimer between the (c)mo$^5U_{34}$ residue of the tRNA and a pyrimidine in the ribosomal RNA (Ofengand et al., 1979; Ofengand & Liou, 1980). The facts that the rRNA oligomers could not be labeled at their 3′-ends and that A-C-C-G was found covalently linked to the *B. subtilis* tRNA oligomer suggested that the cross-link must be near the 3′-terminus of the U-A-C-A-C-A-C-C-G sequence. The most probable candidates are therefore the two last C residues. We have used two different approaches to unambiguously localize the cross-linking site.

(*i*) *Bisulfite Modification*. Bisulfite is known to add to the C_5–C_6 double bond of cytosine (Shapiro et al., 1970b; Hayatsu et al., 1970a,b). Cytidine is converted quickly by sodium bisulfite at pH 6 to an addition product which is slowly deaminated to the corresponding uridine adduct. This derivative readily loses its sulfite radical in a slightly alkaline solution. Bisulfite modification has been successfully used to convert cytidine into uridine in RNAs (Shapiro et al., 1970a; Singhal, 1971; Seno, 1975). Since the pyrimidine–pyrimidine cyclobutane dimer involves saturation of the C_5–C_6 double bond, it was anticipated that if a cytidine residue of the rRNA oligomer participated in the cross-link, it would be protected against C → U conversion.

The various covalent T_1 oligonucleotide duplexes of Figure 2 were isolated by one-dimensional gel electrophoresis. The isolated 5′-labeled duplexes were then divided into three equal parts. One third was photolyzed and the released 16S RNA oligomer fractionated by gel electrophoresis. This unmodified oligomer served as a control (Figure 6A). The second part was photolyzed and subjected to bisulfite modification for use as a control for total C → U conversion (Figure 6B). The remaining covalent duplex was treated with sodium bisulfite and subsequently photolyzed. The released 16S RNA oligomers were then fractionated and analyzed for sequence (Figure 6C).

This methodology was used for both *E. coli* tRNA$_1^{Val}$–*E. coli* 16S RNA and *E. coli* tRNA$_1^{Val}$–yeast 18S RNA oligonucleotide duplexes and for the *B. subtilis* tRNA^{Val}–*E. coli* 16S RNA duplex B (the faster moving duplex, with unlabeled tRNA oligomer). Duplex A (the slower moving duplex with unlabeled 16S RNA nonamer) was treated with bisulfite and purified by gel electrophoresis. After photoreversal of the cross-link, the mixture of the two released modified oligonucleotides was subjected to a second 5′-labeling step. The newly labeled 16S RNA oligomer could then be fractionated by extensive gel electrophoresis and used for sequence analysis.

A typical set of sequencing gels (control without bisulfite modification, control with total bisulfite modification on photolyzed duplex, and bisulfite modification on cross-linked duplex) is shown in Figure 6. Strictly superimposable hydrolysis patterns were obtained for each of the four duplexes studied (data not shown). Bisulfite modification of the free rRNA nonamer U-A-C-A-C-A-C-C-G resulted in the conversion of C residues at each position into U residues as shown by additional bands in the Phy M RNase lane (A + U specific) (see Figure 6B). Although it was not possible to evaluate the extent of C to U conversion, the yield appeared to be reasonably good since Phy M RNase has a higher affinity for A residues than for U residues. When the covalently linked rRNA nonanucleotide was modified by bisulfite treatment, the susceptibility to RNase Phy M of the C residue adjacent to the terminal G was not increased as in Figure 6B but was unchanged (Figure 6C) compared to the unmodified control (Figure 6A). None of the other C residues showed this behavior [compare (B) and (C) of Figure 6]. The unexpected weak but reproducible reactivity to Phy M RNase seen in all

the samples at the 3′-penultimate C residue is evidence for a partial C to U conversion there. This probably arises as a result of saturation of the C_5–C_6 double bond in the cyclobutane dimer, a condition known to promote the deamination of C to U (Liu & Yang, 1978). The results with CL_3 RNase (C specific) were less clear in this experiment due to low activity of the RNase. Nevertheless, in other experiments using the yeast rRNA oligomer, the retention of the 3′-terminal C residue after bisulfite treatment could be shown (Figure 7). The other C residues exhibited a strongly reduced susceptibility to the CL_3 enzyme. The sum of these results indicates that the C residue in the penultimate position of the nonamer is protected against C to U conversion in the covalent duplex. Thus, C_{1400} in *E. coli* 16S RNA is the residue cross-linked to both *E. coli* $tRNA_1^{Val}$ and *B. subtilis* $tRNA^{Val}$, and C_{1626} in yeast 18S RNA is the residue cross-linked to *E. coli* $tRNA_1^{Val}$. Bisulfite modification does not, however, allow one to totally exclude cross-linking at a low extent from another position of the rRNA nonanucleotide such as the adjacent C residue.

(*ii*) *Statistical Hydrolysis Followed by Two-Dimensional Gel Electrophoretic Analysis.* A new method for determining the site of cross-linking in any two oligonucleotides linked together by a cleavable cross-linking reagent has been recently developed by us. Application of this method to the covalent duplexes described above allowed us not only to confirm that C_{1400} and C_{1626} were the sites of cross-linking in 16S and 18S rRNA, respectively, but also to show that the cross-link is restricted to one unique site (Ehresmann & Ofengand, 1984). This result illustrates the remarkable specificity of the cross-linking reaction. As the new method automatically yields the site of cross-linking in both oligomers, use of this technique also allowed us to directly confirm that the site of cross-linking in the tRNA was the (c)mo^5U_{34} residue.

Discussion

In this work, we have localized the site of cross-linking between *E. coli* $tRNA_1^{Val}$ and both *E. coli* 16S RNA and yeast 18S RNA, as well as between *B. subtilis* $tRNA^{Val}$ and *E. coli* 16S RNA. In the first step, covalent oligonucleotide duplexes were isolated from total T_1 RNase digests of covalently linked tRNA–rRNA complexes. The oligonucleotide pairs could then be dissociated by photoreversal of the cross-link at 254 nm, and after separation, each moiety was identified. The precise localization of the cross-linked nucleotides was achieved by two distinct approaches. The former used bisulfite modification as a means to detect those C residues whose C_5–C_6 bonds were not involved in cyclobutane dimer formation. The latter was based on two-dimensional gel electrophoresis of a ramdom digest of the covalent oligonucleotide duplex and allowed determination of both cross-linked residues in a single step (Ehresmann & Ofengand, 1984).

A unique oligonucleotide duplex could be isolated from the total T_1 RNase digest of the *E. coli* $tRNA_1^{Val}$–*E. coli* 16S RNA complex. The tRNA pentadecamer pC-A-C-C-U-C-C-C-U-cmo^5U-A-C-m^6A-A-G was found to be cross-linked to the unique 16S RNA nonamer pU-A-C-A-C-A-C-C-G (residues 1393–1401). This sequence has also been identified by Taylor et al. (1981) using a completely different approach. The cross-link was shown to occur exclusively between cmo^5U_{34} in the tRNA and C_{1400} in the 16S RNA. This result is in complete agreement with the investigations of Prince et al. (1982), who used a totally different methodology.

When *E. coli* $tRNA_1^{Val}$ was covalently bound to yeast 18S RNA, the tRNA pentadecanucleotide containing the modified uridine was found cross-linked to the identical sequence pU-A-C-A-C-A-C-C-G in 18S RNA. This sequence (residues 1619–1627) is a unique sequence in the 18S RNA and is part of a longer highly conserved sequence. In this case also, the wobble base cmo^5U_{34} was found unambiguously cross-linked to the C residue, C_{1626}, which corresponds to C_{1400} in *E. coli*. In some experiments, a faster migrating oligonucleotide duplex was also observed but at a very low yield (ca. 1% of the major duplex). It contained the octamer pA-C-A-C-A-C-C-G instead of the complete nonamer, cross-linked to the tRNA oligonucleotide. In this particular case, cmo^5U was found to be cross-linked to the adjacent C residue, C_{1625} (Ehresmann & Ofengand, 1984). Possibly, cleavage at U_{1619} modified the ribosome conformation in this area, directing the wobble base interaction to the adjacent C residue.

Two distinct oligonucleotide duplexes could be isolated in almost identical yields from total T_1 RNase digests of the *B. subtilis* $tRNA^{Val}$–*E. coli* 16S RNA complex. They both contained the tRNA nonamer pC-C-U-mo^5U-A-C-m^6A-A-G cross-linked to the 16S RNA nonamer pU-A-C-A-C-A-C-C-G. Strikingly, only the tRNA moiety was capable of being 5′-labeled in the slower moving duplex, while only the rRNA moiety was labeled in the faster moving one. Both unlabeled oligomers could be 5′-labeled after photoreversal of the cross-link. The cross-link was the same in both duplexes A and B, being between mo^5U_{34} and C_{1400}. Therefore, the remarkable difference between their electrophoretic mobilities and 5′-labeling behavior cannot be explained either by a difference in their nucleotide length or sequence or by a different location of the cross-linking site. It appears to be related to the existence of the cross-link, since after photolysis both 5′-ends could be labeled and the subproducts from both duplexes A and B migrated identically. The effect which produces this difference in electrophoretic migration only affects the 5′-end and/or the specificity of T_4 polynucleotide kinase, since 3′-labeling with RNA ligase was unaffected, although the electrophoretic behavior of duplexes A and B was the same as when the 5′-ends were labeled.

We speculate that the observed effect may be due to the formation of two geometrical isomers of the cyclobutane dimer since cis, trans, syn, and anti forms are all possible (Fisher & Johns, 1976). If the mutual orientation of the two tRNA chains is influenced by the stereochemistry of the cyclobutane dimer, different and possibly structured conformations at the 5′-ends may be possible. This could conceivably affect both the electrophoretic mobility and the relative reactivity of the ends to polynucleotide kinase. Since this unusual behavior was not observed with either of the *E. coli* $tRNA^{Val}$ covalent duplexes, it might be related either to the differences in length of the two T_1 oligomers derived from tRNA or to the absence of the COOH from the wobble base in *B. subtilis* tRNA.

As the above comments illustrate, nothing is known about the correct geometry of the cyclobutane dimer formed in this cross-linking reaction, although such information would shed considerable light on the true orientation of the anticodon loop of tRNA when it is in contact with the ribosomal surface. Another important question is whether the (c)mo^5U_{34} residue of tRNA, which is located at the very tip of the anticodon loop according to the crystal structures of tRNA, is stacked on the 5′-side or the 3′-side of C_{1400}. That is, is it intercalated between C_{1399} and C_{1400} or between C_{1400} and G_{1401}? In this regard, the observation that the adjacent C_{1625} of yeast 18S RNA was found cross-linked when the rRNA chain was cleaved at U_{1619} might indicate that stacking occurs on the 5′-side of C_{1400}.

In summary, *E. coli* $tRNA_1^{Val}$ yields one homogeneous covalent complex with either *E. coli* 16S RNA or yeast 18S RNA. The stereochemistry of the cross-link occurring between cmo^5U_{34} and C_{1400} or C_{1626}, respectively, appears to be identical at least insofar as electrophoretic mobility is concerned. On the other hand, *B. subtilis* $tRNA^{Val}$, while cross-linked to *E. coli* 16S RNA at the same C_{1400}, nevertheless shows some apparent stereochemical heterogeneity, judging by its electrophoretic behavior.

The cross-linking site is identical in all three complexes studied. It is located in the center of a 17-mer whose sequence conservation extends from prokaryotes, including archaeobacteria, all the way to higher eukaryotes [cited in Ofengand et al. (1982); also see Magrum et al. (1978), Balch et al. (1979), Woese et al. (1980); Torczynski et al. (1981); Lockard et al. (1982), Michot et al. (1982), Seewaldt & Stackebrandt (1982), Graf et al. (1982), and Dron et al. (1982)]. The structure of this sequence, that of a single-stranded region connecting two base-paired stems, is also thought to be conserved (Noller & Woese, 1981; Stiegler et al., 1981a; Zwieb et al., 1981). This region is located in the 3′-terminal domain of the 16S RNA and has been shown, at least in *E. coli*, to be highly accessible on the surface of the 30S subunit to both kethoxal modification (Herr et al., 1979) and ribonuclease digestion (Stiegler et al., 1981b; Vassilenko et al., 1981). There is also evidence that this region is located at the interface of the two subunits (Herr et al., 1979; Vassilenko et al., 1981).

It is striking that the cross-linking site was completely localized to a single site. Since the reaction is pyrimidine specific by its chemistry, the adjacent C residue, C_{1399}, should have been equally reactive. The failure to detect cross-linking to C_{1399} or its equivalent C_{1625} in yeast except in one aberrant situation strongly implies that a very specific three-dimensional structure is formed between this region of the rRNA and the tRNA anticodon. The conservation of (a) the sequence surrounding the cross-link site, (b) its location in the rRNA chain, and (c) its cross-linking ability all serve to reinforce this view, implicating this region of rRNA as well as the 5′-anticodon end of P site bound tRNA in some function(s) essential to both prokaryotic and eukaryotic protein synthesis.

Recent findings of Martin et al. (1982) reinforce the view that this area is intimately involved in the decoding process of protein synthesis. They have mapped the locus for the resistance to paromomycin, an antibiotic which promotes translational errors, in the yeast mitochondrial gene coding for 15S RNA and find it at a position which is only nine nucleotide residues to the 3′-side of the cross-linking site.

Our results also show that a tertiary folding of the rRNA in the 30S particle must be superimposed on the secondary structure. The C_{1400} residue in *E. coli* is 130 residues from the 5′-terminus of the Shine-Dalgarno region, A_{1531}. C_{1400}, being cross-linked to the 5′-anticodon base of P site bound tRNA, should be equivalent in location to the 3′-G residue of the initiator AUG codon. However, in ϕXH mRNA, this G residue is only five nucleotides away from the U residue complementary to A_{1531} (Sanger et al., 1978). Furthermore, Wagner et al. (1976) have shown that residues 462–474 are in the vicinity of the decoding site; yet, this region is distant from C_{1400} in the secondary structure models.

Finally, we should note that the site corresponding to C_{1400} has been localized on the intact 30S ribosomal subunit by affinity immunoelectron microscopy. It is found deep in the cleft between the head and the large protrusion (Keren-Zur et al., 1979; Gornicki et al., 1983).

Registry No. Mg, 7439-95-4; poly(U_2,G), 26680-26-2; pGUU, 17159-38-5; cmo^5U, 28144-25-4; mo^5U, 35542-01-9; cytidine, 65-46-3.

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