Nonanucleotide Sequence from 16S Ribonucleic Acid at the Peptidyl Transfer Ribonucleic Acid Binding Site of the *Escherichia coli* Ribosome†

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ABSTRACT: When *N*-acetylvalyl-tRNA$_1^{Val}$ is bound to the P site of *Escherichia coli* 70S ribosomes and irradiated with light of 300–400 nm, the tRNA becomes covalently attached to 16S RNA in the 30S ribosomal subunit. The rRNA fragments cross-linked to the tRNA were isolated from partial RNase T_1 digests of the covalent *N*-acetylvalyl-tRNA$_1^{Val}$–16S [^{32}P]-RNA complexes. Separation of these fragments from other digestion products was accomplished by two-dimensional polyacrylamide gel electrophoresis under denaturing conditions. After the first dimension, the tRNA–rRNA cross-link was cleaved in situ by irradiation at 254 nm. As most of the rRNA fragments were unaffected by this treatment, they were located on a diagonal following the second dimension. Segments previously attached to tRNA, however, migrated more rapidly to positions below the diagonal. The appearance of the off-diagonal components was strictly dependent both upon the presence of tRNA in the initial reaction mixture and upon subsequent photoreversal of the covalent bond and did not result merely from irradiation of ribosomes or 16S RNA. Sequence analysis revealed that the smallest of three nested rRNA fragments containing the site of tRNA cross-linking comprised residues 1362–1497 of the 16S RNA. Complete RNase T_1 hydrolysis of covalent *N*-acetylvalyl-tRNA$_1^{Val}$–16S [^{32}P]RNA complexes released the cross-linked tRNA–rRNA oligonucleotide which was then isolated on a thin polyacrylamide gel. This adduct, identified by its size and susceptibility to photoreversal, contained a ^{32}P-labeled nonanucleotide component from the 16S RNA and an unlabeled pentadecanucleotide component from the tRNA that was detected by labeling its 5′ terminus with ^{32}P. The sequence of the nonanucleotide was determined to be U-A-C-A-C-A-C-C-G, which occurs at positions 1393–1401 of the 16S RNA molecule and is located within a region that has been highly conserved in small-subunit rRNAs from prokaryotes, eukaryotes, and eukaryotic organelles. We conclude that this evolutionarily conserved sequence is an integral part of the tRNA binding domain at the ribosomal P site.

Investigations of structure–function relationships in bacterial ribosomes have concentrated mainly on the contribution of ribosomal proteins to the numerous activities mediated by these particles during protein biosynthesis. Although it has long been appreciated that ribosomal RNAs play a critical role in ribosome assembly and stability by virtue of their capacity to associate with ribosomal proteins, information about their specific roles in translation has accumulated more slowly. Nonetheless, the functional significance of rRNA was inferred early from its involvement in the binding of antibiotics to the ribosome and from the fact that cleavage of some 50 bases from the 3′ terminus of 16S RNA by colicin E3 completely inactivated the treated particles (Helser et al., 1971; Lai et al., 1973; Bowman et al., 1971). More recently, improved procedures for the analysis of RNA primary and secondary structure have led to proposals that switching between alternate rRNA conformations may influence the dynamics of protein synthesis (Fox & Woese, 1975; Weidner et al., 1977; Noller, 1980; Glotz & Brimacombe, 1980). A more concrete understanding of the function of rRNA has emerged from the recognition that interaction between a pyrimidine-rich segment at the 3′ end of 16S RNA and complementary purine-rich sequences in mRNA are involved in formation of the mRNA–30S subunit complex during polypeptide chain initiation and aid in the selection of the initiator AUG codon (Shine & Dalgarno, 1974; Steitz & Jakes, 1975; Backendorf et al., 1980). Binding of tRNA to the ribosome may also be governed by RNA–RNA interaction as suggested by the complementarity between the conserved G-A-A-C sequence in prokaryotic 5S RNA and the common G-T-Ψ-C sequence in tRNA [see Ofengand (1980)]. Moreover, chemical and photochemical cross-linking studies have shown that a segment of the 23S RNA lies near the α-amino group of aminoacyl-tRNA at the ribosomal P site [see Ofengand (1980)].

The presence of 16S RNA at the peptidyl-tRNA binding site of the ribosome has also been demonstrated in the past few years. Specifically, certain unmodified AcAA[1]-tRNAs can be covalently cross-linked to 16S RNA when bound to the P site of 70S ribosomes and irradiated with near-ultraviolet light (Schwartz & Ofengand, 1978; Ofengand et al., 1979; Zimmermann et al., 1979; Prince et al., 1979). It has been proposed that the adduct results from reaction between a modified uridine residue at the 5′ position of the tRNA anticodon and a suitably positioned base in the 16S RNA (Ofengand et al., 1979; Prince et al., 1979) and that it consists of a pyrimidine–pyrimidine cyclobutane dimer (Ofengand & Liou, 1980). In these studies, the site of cross-linking was located within the 8S RNA fragment derived from the 3′ portion of the 16 S by partial digestion with RNase T_1 (Zimmermann et al., 1979; Prince et al., 1979). Although the tRNA was found to be linked to a rRNA subfragment of approximately 100–125 nucleotides, the exact position of the subfragment within the rRNA molecule was not determined. To define the attachment site more precisely, we have gen-

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[1] Abbreviations used: AcAA, *N*-acetylaminoacyl; AcVal, *N*-acetylvalyl; cmo^{5}U, 5-carboxymethoxyuridine; Tris, tris(hydroxymethyl)-aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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erated covalent AcVal-tRNA$_1^{Val}$–16S RNA complexes in which the rRNA moiety was uniformly labeled with ^{32}P and therefore amenable to detailed sequence analysis. In the present report, we describe the isolation and identification of the segment of the 16S RNA to which tRNA$_1^{Val}$ becomes cross-linked and discuss the implications of our findings for the function of rRNA in protein synthesis.

Experimental Procedures

Materials. *Escherichia coli* tRNA$_1^{VAl}$, obtained from Boehringer Mannheim, was aminoacylated and N-acetylated as described by Schwartz & Ofengand (1978). Unfractionated aminoacyl-tRNA synthetases were prepared according to Muench & Berg (1966). Poly(U_3,G) and T4 polynucleotide kinase were purchased from P-L Biochemicals, pancreatic RNase A was purchased from Worthington, and RNases T_1 and U_2 were purchased from Calbiochem-Behring. [^{3}H]Valine (50–60 Ci/mmol), [^{32}P]orthophosphoric acid (carrier free), and [γ-^{32}P]ATP (2600 Ci/mmol) were products of New England Nuclear. Nonradioactive tight-couple ribosomes were obtained from *E. coli* MRE 600 essentially as described by Debey et al. (1975). Uniformly ^{32}P-labeled tight couples were isolated by the same method except that cells were grown in the presence of [^{32}P]orthophosphoric acid (Zimmermann, 1979). [^{14}C]Uracil-labeled 16S RNA was prepared according to Muto et al. (1974).

Isolation of Covalent AcVal-tRNA$_1^{Val}$–16S RNA Complexes. Charged, acetylated tRNA$_1^{Val}$ was bound nonenzymatically to the ribosomal P site in 50 mM Hepes–NaOH, pH 7.6, 100 mM NH_4Cl, 15 mM $MgCl_2$, and 0.1 mM dithiothreitol containing 240 pmol/mL ^{32}P-labeled 70S tight-couple ribosomes, 120–240 pmol/mL Ac[^{3}H]Val-tRNA$_1^{Val}$, and 30 μg/mL poly(U_3,G). Reaction mixtures were incubated for 15 min at 37 °C and then chilled to 0 °C and irradiated for 4 h in a Rayonet RPR-100 photochemical reactor equipped with 350-nm lamps. Ribosomes were recovered by precipitation with ethanol, resuspended in 10 mM potassium phosphate, pH 6.2, 0.25 $MgCl_2$, and 6 mM 2-mercaptoethanol, and resolved into 30S and 50S subunits by centrifugation through 10–30% sucrose gradients in a Spinco SW27.1 rotor for 15 h at 24000 rpm. AcVal-tRNA$_1^{Val}$–16S RNA complexes were extracted from the 30S particles with phenol and precipitated with ethanol. Typically, 30–40% of the tRNA initially bound to ribosomes became covalently linked to 16S RNA under our conditions as inferred from parallel incubations carried out with nonradioactive ribosomes. Details of these procedures have been described elsewhere (Ofengand et al., 1979; Zimmermann et al., 1979).

Digestion of Covalent AcVal-tRNA$_1^{Val}$–16S RNA Complexes. For limited enzymatic hydrolysis, 250–500 μg of Ac[^{3}H]Val-tRNA$_1^{Val}$–16S [^{32}P]RNA complexes was resuspended in 1 mL of PMK buffer (10 mM potassium phosphate, 20 mM $MgCl_2$, 350 mM KCl, and 5 mM 2-mercaptoethanol, pH 6.2) and treated with RNase T_1 at an enzyme:substrate ratio of 1:500 (w/w). Covalent complexes containing unlabeled 16S RNA were digested at an enzyme:substrate ratio of 1:100 (w/w) together with ^{14}C-labeled 16S RNA as reported previously (Zimmermann et al., 1979). In either case, partial digestion products were separated by sucrose-gradient centrifugation in PMK buffer for 15 h at 32000 rpm in a Spinco SW41 rotor. RNA fragments were pooled, dialyzed against 10 mM NaOAc, pH 5, and concentrated with the aid of a Millipore immersible molecular separator (Zimmermann et al., 1979). When complete enzymatic digestion was required, approximately 400 μg of covalent AcVal-tRNA$_1^{Val}$–16S RNA were dissolved in 200 μL of 5 mM Tris-HCl, pH 7.4, and 0.5 mM Na_2EDTA with sufficient RNase T_1 to yield an enzyme:substrate ratio of 1:2. The mixtures were incubated at 37 °C for 60 min and then dried under vacuum.

Two-Dimensional Gel Electrophoresis of Partial Digestion Products. Covalent Ac[^{3}H]Val-tRNA$_1^{Val}$–[^{32}P]rRNA fragment complexes recovered from partial RNase T_1 digests were analyzed by two-dimensional polyacrylamide gel electrophoresis in two systems. All gels were preelectrohoresed for 1 h prior to use. (1) System I (pH 5.2): RNA samples were suspended in 6 M urea and 0.002% bromophenol blue and applied to 5 mm i.d. × 10 cm cylindrical gels containing 10% polyacrylamide and 6 M urea in 30 mM succinic acid, 20 mM $Na_2B_4O_7$, and 2.5 mM Na_2EDTA, pH 5.2. The ratio of acrylamide to *N,N'*-methylenebis(acrylamide) was 19:1 by weight. Electrophoresis was performed at 4 mA/tube and at 4 °C until the marker dye was 1 cm from the bottom of the tube. The gel was then removed to a quartz tube containing electrophoresis buffer and irradiated for 15 min at 4 °C in a Rayonet reactor equipped with four 254-nm lamps. Next, this gel was placed across the top of a 5 mm thick, 12 × 10 cm slab gel whose composition was identical with that of the first-dimension gel and polymerized in position with 3% polyacrylamide. Electrophoresis in the second dimension was carried out at 50 V and at 4 °C until the bromophenol blue dye reached the bottom of the slab. ^{32}P-Labeled RNA was visualized by autoradiography of the gel at 20 °C using Kodak XR-5 X-ray film. (2) System II (pH 8.3): RNA samples were dissolved in 7 M urea, 0.02% bromophenol blue, and 0.02% xylene cyanole and applied to 7 mm wide slots of a 0.35 mm thick, 25 × 40 cm gel containing 10% polyacrylamide and 7 M urea in 50 mM Tris–50 mM H_3BO_3, pH 8.3, and 1 mM Na_2EDTA. The weight ratio of acrylamide to *N,N'*-methylenebis(acrylamide) was 30:1 in this case. Electrophoresis was carried out at 1800 V and at 20 °C until the bromophenol blue marker was 2 cm from the bottom of the gel. The upper third of each sample track was excised, placed in a shallow dish with sufficient distilled water to keep the gel moist, and irradiated at 254 nm as described above. The gel strip was then positioned across the top of a 0.5 mm thick, 25 × 40 cm gel of identical composition and subjected to electrophoresis in the second dimension at 1800 V and at 20 °C until the bromophenol blue dye was at the bottom of the gel. After visualization of the RNA fragments by autoradiography, gel slices containing appropriate components were excised and immersed in 150 μL of 50 mM Tris–50 mM H_3BO_3, pH 8.3, 1 mM Na_2EDTA, and 500 mM KCl with 20 μg of unfractionated *E. coli* tRNA. ^{32}P-Labeled RNA was eluted from the gel slices by shaking overnight at 20 °C and recovered from the supernatant by precipitation with ethanol.

Fingerprint Analysis of rRNA Fragments. Two-dimensional fingerprints of ^{32}P-labeled rRNA fragments were prepared according to the method of Sanger and colleagues (Sanger et al., 1965; Brownlee, 1972) as modified by Uchida et al. (1974). Fragments were digested completely with RNase T_1 at an enzyme:substrate ratio of 1:5, and the resulting oligonucleotides were fractionated by electrophoresis on cellulose acetate strips (Schleicher & Schuell) in 5% HOAc and 0.5% pyridine, pH 3.5, in the first dimension and on DEAE paper (Whatman) in 75 mM pyridinium formate, pH 2.3, in the second dimension. The T_1 oligonucleotides were located by autoradiography, cut from the fingerprints, counted by Čerenkov radiation, eluted from the paper with 30% triethylammonium carbonate, pH 9.5, and dried. Each oligonucleotide was then subjected to secondary and tertiary analysis as follows. (1) Digestion with pancreatic RNase A:

RNA samples were resuspended in 6 μL of 10 mM Tris-HCl, pH 7.4, and 1 mM Na_2EDTA containing 0.1 mg/mL RNase A and incubated for 30 min at 37 °C. Digestion products were electrophoresed on DEAE paper in 5% HOAc and 0.5% pyridine, pH 3.5. (2) Digestion with RNase U_2: Oligonucleotides were dissolved in 6 μL of 50 mM NaOAc, pH 4.5, and 2 mM Na_2EDTA containing 10 units/mL RNase U_2 and incubated at 37 °C for 2 h. Products were separated by electrophoresis on DEAE paper in 75 mM pyridinium formate, pH 2.3. (3) Alkaline hydrolysis: RNA samples were resuspended in 6 μL of 0.2 N NaOH and incubated at 37 °C for 18 h in sealed capillaries. The resulting mononucleotides were fractionated by electrophoresis on Whatman 540 paper in 5% HOAc and 0.5% pyridine, pH 3.5. In all cases, the digestion products were located by autoradiography, identified by reference to known standards, and analyzed for radioactivity.

Isolation of Covalent tRNA–rRNA T_1 Oligonucleotide. Ac[^{3}H]Val-tRNA$_1^{Val}$–16S [^{32}P]RNA complexes were digested to completion with RNase T_1 and the samples were dried. A small portion of the RNA was dissolved in 30 μL of distilled water, spotted onto Parafilm, and irradiated at 254 nm for 15 min at 4 °C. Both irradiated and unirradiated samples were then resuspended in 7 M urea, 0.02% bromophenol blue, and 0.02% xylene cyanole. Aliquots of 7 μL containing up to 50 μg of RNA were applied to 7 mm wide pockets of a 0.5 mm thick, 25 × 40 cm gel consisting of 20% polyacrylamide in 50 mM Tris–50 mM H_3BO_3, pH 8.3, 1 mM Na_2EDTA, and 7 M urea. Electrophoresis was carried out at 1800 V and at 20 °C until the bromophenol blue marker had migrated 25 cm into the gel. Following autoradiography, selected bands were excised from the gel and the oligonucleotides eluted by overnight incubation in 0.5 mL of distilled water at 20 °C with gentle agitation. Each sample was adsorbed to a small column containing approximately 0.1 cm^3 of DEAE-cellulose (Whatman) which was then washed with 10 mL of distilled water to remove urea and developed with 30% triethylammonium carbonate, pH 9.5, to release the RNA. Secondary and tertiary analysis of the oligonucleotides recovered was performed as in the preceding section.

5′-^{32}P-Labeling of RNA. Intact 16S RNA and tRNA–rRNA fragments were labeled with ^{32}P at the 5′ terminus by incubation with [γ-^{32}P]ATP in the presence of T_4 polynucleotide kinase (Silberklang et al., 1979). Chain-length markers were generated by boiling 5′-^{32}P-labeled 16S RNA in formamide for 30 min.

Analysis of Radioactivity. Aqueous radioactive samples were precipitated with 5% trichloroacetic acid and collected on glass-fiber filters or spotted directly on glass-fiber filters. The filters were dried and counted in a scintillation cocktail containing 4 g of Omnifluor (New England Nuclear) per L of toluene. ^{32}P-Labeled oligonucleotides bound to electrophoresis paper were quantitated by Čerenkov counting in the absence of external scintillants.

Results

Isolation of rRNA Fragments Cross-Linked to tRNA$_1^{Val}$. Ac[^{3}H]Val-tRNA$_1^{Val}$ was bound to the P site of uniformly ^{32}P-labeled tight-couple ribosomes in the presence of poly-(U_3,G) and irradiated for 4 h with light of 350 nm. Following dissociation of the ribosomes in 0.25 mM Mg^{2+}, the 30S subunits were isolated and extracted with phenol. Covalent AcVal-tRNA$_1^{Val}$–16S RNA complexes were then digested with RNase T_1, and the products were fractionated by sucrose-gradient centrifugation (Figure 1). Partial RNase hydrolysis yielded two major RNA fragments of 12 S and 8 S that correspond to the 5′ 60% and 3′ 40% of the parent RNA

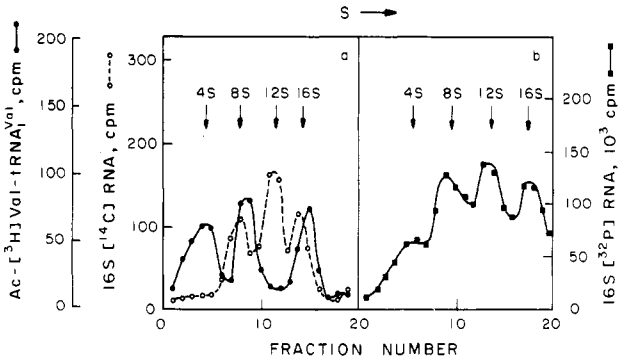

FIGURE 1: Partial digestion of covalent AcVal-tRNA$_1^{Val}$–16S RNA with RNase T_1. AcVal-tRNA$_1^{Val}$–16S RNA complexes in PMK buffer were incubated for 30 min at 40 °C, chilled on ice, and exposed to RNase T_1 for 15 min at 0 °C. The digestion mixtures were fractionated by centrifugation through 3–15% sucrose gradients in th same buffer for 15 h at 32000 rpm. Acid-precipitable radioactivity in each fraction was measured as described under Experimental Procedures. (a) Ac[^{3}H]Val-tRNA$_1^{Val}$–16S RNA was mixed with 16S [^{14}C]RNA (20 μg) and digested at an enzyme:substrate ratio of 1:100 (w/w). (b) Ac[^{3}H]Val-tRNA$_1^{Val}$–16S [^{32}P]RNA was digested at an enzyme:substrate ratio of 1:500 (w/w). The ^{32}P:^{3}H ratio was too high to permit reliable quantitation of ^{3}H radioactivity in this case.

molecule (Muto et al., 1974). Uncleaved 16S RNA and small byproducts sedimenting in the 4S region were also evident in the gradient profiles. As seen from the control digest in which nonradioactive ribosomes were substituted for ^{32}P-labeled tight couples (Figure 1a), Ac[^{3}H]Val-tRNA$_1^{Val}$ was associated with the 8S, but not the 12S, RNA fragment (Zimmermann et al., 1979). Accordingly, the 8S [^{32}P]RNA fraction, presumed to contain the Ac[^{3}H]Val-tRNA$_1^{Val}$–8S [^{32}P]RNA complexes, was recovered from the appropriate portion of the gradient (Figure 1b) and analyzed further by polyacrylamide gel electrophoresis.

When the 8S fragment is subjected to electrophoresis in the presence of 6 M urea, the RNA is resolved into numerous subfragments owing to the unmasking of hidden breaks introduced during RNase digestion (Zimmermann et al., 1974). Fractionation of covalent Ac[^{3}H]Val-tRNA$_1^{Val}$–8S RNA adducts under denaturing conditions demonstrated that the tRNA was cross-linked to one or more rRNA subfragments of 100–125 nucleotides (Zimmermann et al., 1979). To isolate these subfragments for sequence analysis, we employed a two-dimensional "diagonal" electrophoretic system which capitalized upon our ability to selectively cleave the link between tRNA and rRNA by briefly exposing the covalent complex to light of 254 nm (Ofengand et al., 1979; Ofengand & Liou, 1980). In our initial experiments, AcVal-tRNA$_1^{Val}$–8S [^{32}P]RNA was first fractionated on 10% polyacrylamide gels in succinate–borate buffer at pH 5.2 under denaturing conditions. The gels were then immersed in the same buffer, irradiated at 254 nm, and polymerized across the top of a slab gel of identical composition. When electrophoresis was carried out in the second dimension, most oligonucleotides exhibited the same mobility as in the first dimension and migrated to positions along a diagonal, while those subfragments that had been released from tRNA migrated more rapidly and appeared below the diagonal (Figure 2a). The latter products derived from locations in the first-dimension gel that corresponded in mobility to the cross-linked tRNA$_1^{Val}$–rRNA fragment complexes observed earlier (Zimmermann et al., 1979). Although only the ^{32}P-labeled rRNA moiety was visualized in the pattern, unlabeled tRNA fragments presumably occurred beneath the diagonal as well.

The following control experiments were performed to ensure that the off-diagonal spots arose from cross-linked tRNA–

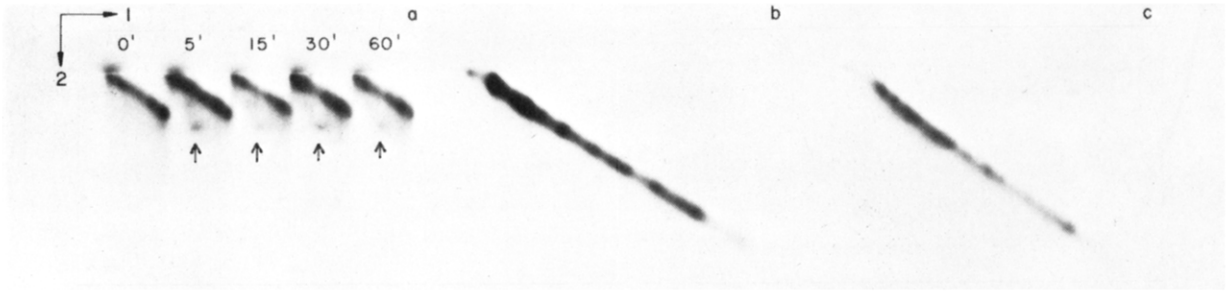

FIGURE 2: Two-dimensional gel electrophoresis of covalent AcVal-tRNA$_1^{Val}$–8S [^{32}P]RNA and other [^{32}P]rRNA fragments at pH 5.2. RNA fragments were subjected to electrophoresis at 4 °C in cylindrical gels containing 10% polyacrylamide and 6 M urea at pH 5.2. The gels were removed from their tubes, irradiated at 254, and polymerized across the top of a slab gel of identical composition. Electrophoresis in the second dimension was carried out at 4 °C until the bromophenol blue marker reached the bottom of the slab. Autoradiography was performed immediately after electrophoresis. (a) AcVal-tRNA$_1^{Val}$–8S [^{32}P]RNA, isolated as in Figure 1b, was divided into five portions and fractionated on separate cylindrical gels. Two-centimeter segments were excised from the top of each gel and irradiated at 254 nm for 0, 5, 15, 30, or 60 min, as indicated, before polymerization onto the second-dimensional gel. Arrows denote off-diagonal components. (b) 12S [^{32}P]RNA obtained from cross-linked ribosomal complexes as in Figure 1b. The first-dimension gel irradiated was at 254 nm for 15 min. (c) 8S [^{32}P]RNA was prepared from ribosomes exposed to 350-nm light as for the complete system but in the absence of AcVal-tRNA$_1^{Val}$. After electrophoresis in the first dimension, the gel was irradiated at 254 nm for 15 min.

rRNA complexes and did not represent artifacts engendered by any of the preparative procedures. First, the gel pattern was highly reproducible even when the times at which the first-dimension gel exposed to 254-nm light varied from 5 to 60 min, but if the irradiation step was omitted, no spots were noted below the diagonal (Figure 2a). Second, when 12S RNA was isolated from covalent AcVal-tRNA$_1^{Val}$–16S RNA complexes, as in Figure 1b, and treated as the 8S fraction, none of its subfragments gave rise to off-diagonal components (Figure 2b). Finally, to demonstrate that the off-diagonal spots did not in some way stem from the initial irradiation at 350 nm, we isolated 8S material from ribosomes that had been prepared in the same manner as the covalent complexes but in the absence of tRNA. Analysis of 8S RNA from this control by the two-dimensional gel procedure also showed that no spots occurred off the diagonal (Figure 2c).

Although the appearance of rRNA subfragments beneath the diagonal was reproducible and specific, the resolution of these oligonucleotides was generally poor in the pH 5.2 gel system. As a result, our first attempts to determine their sequence by fingerprint analysis was impeded by the presence of contaminating material from the diagonal band. To improve the separation of the off-diagonal spots, we utilized a second electrophoresis system consisting of long, thin polyacrylamide gels in Tris–borate buffer at pH 8.3. After one-dimensional fractionation of the AcVal-tRNA$_1^{Val}$–8S [^{32}P]RNA fragment complex, the upper third of the sample track was excised and irradiated at 254 nm for 15 min in doubly distilled water. Electrophoresis in the second dimension, which was carried out in a slightly thicker slab, once again yielded a characteristic pattern of off-diagonal spots. In this case, three major horizontal pairs of fragments were resolved (Figure 3, 1–3). As in the first system, the appearance of oligonucleotides below the diagonal was strictly dependent on the presence of tRNA in the original incubation mixture as well as on subsequent photoreversal of the covalent tRNA–rRNA complex with 254-nm light (data not shown).

Sequence of rRNA Fragments Cross-Linked to tRNA$_1^{Val}$. The sequence of each of the major off-diagonal rRNA subfragments illustrated in Figure 3 was determined by standard fingerprint analysis (Sanger et al., 1965; Brownlee, 1972; Uchida et al., 1974) after elution from the gel. Because the horizontal pairs of off-diagonal components were believed to encompass identical segments of the rRNA, both spots of each pair were pooled prior to fingerprinting. This assumption was subsequently verified by the sequencing results. Of greatest

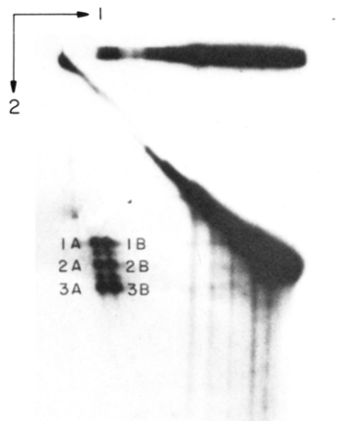

FIGURE 3: Two-dimensional gel electrophoresis of the covalent AcVal-tRNA$_1^{Val}$–8S [^{32}P]RNA fragment at pH 8.3. The 8S fraction, prepared as in Figure 1b, was analyzed by two-dimensional electrophoresis at 20 °C in gels composed of 10% polyacrylamide and 7 M urea at pH 8.3. Irradiation of the first-dimension sample track was carried out at 254 nm for 15 min at 4 °C. Details are provided under Experimental Procedures. 1A + 1B, 2A + 2B, and 3A + 3B designate the major pairs of off-diagonal components.

interest is the primary structure of component 3 which represents the smallest major subfragment containing the site originally cross-linked to tRNA$_1^{Val}$. The fingerprint of a complete RNase T_1 digest of RNA from spots 3A + 3B is presented along with an explanatory scheme in Figure 4. The sequence of each of the 21 main T_1 oligonucleotides was ascertained by secondary hydrolysis with pancreatic RNase or RNase U_2 in conjunction with base composition analysis. When ambiguities arose, they were easily resolved by comparison with the published fingerprint (Uchida et al., 1974) or primary structure (Brosius et al., 1978; Carbon et al., 1978) of the intact 16S RNA.

A catalog of the T_1 oligonucleotides and their pancreatic and U_2 RNase products is presented in Table I. The results clearly indicate that the RNA in spots 3A + 3B consists of a single, continuous segment spanning residues 1362–1497 in the 16S RNA molecule (see Figure 6). A comparison of the experimentally derived copy number of each T_1 oligonucleotide, based on Čerenkov counting of spots excised from the fingerprint, with the expected frequency of each oligonucleotide in this rRNA segment is also provided in Table I. The agreement is generally quite close, although the smaller oligonucleotides appear to have been recovered in somewhat higher yield. Fingerprints of the off-diagonal pairs 2A + 2B

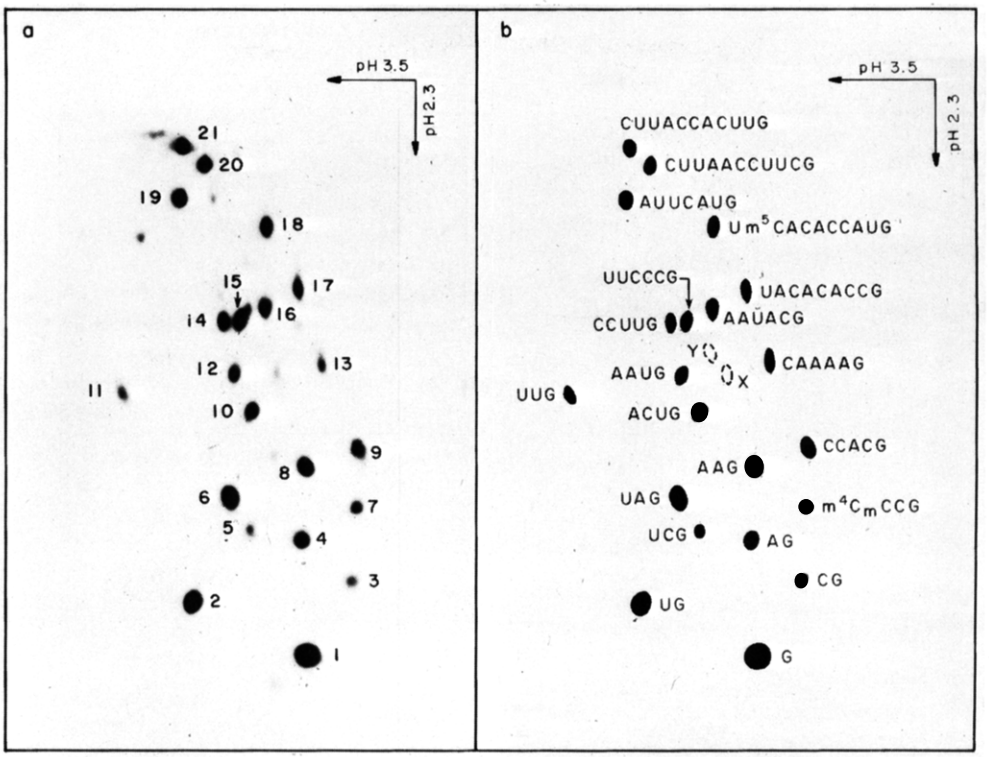

FIGURE 4: Fingerprint of subfragments 3A + 3B. Components 3A + 3B were eluted from the gel depicted in Figure 4 and completely hydrolyzed with RNase T_1. The ^{32}P-labeled digestion products were fractionated by high-voltage electrophoresis on cellulose acetate at pH 3.5 in the first dimension and on DEAE paper at pH 2.3 in the second. (a) Autoradiograph of fingerprint. Oligonucleotides are numbered as in Table I. (b) Schematic diagram of fingerprint. Sequences of the oligonucleotides are specified according to the analyses reported in Table I. Spot X is additional oligonucleotide observed in fingerprints of subfragments 2A + 2B and 1A + 1B; spot Y was found only in the fingerprint of subfragments 1A + 1B (see the text).

Table I: Sequence Analysis of RNase T_1 Digestion Products from the Main Off-Diagonal Subfragments of the 16S RNA[a]

fingerprint spot	RNase A products	RNase U_2 products	proposed sequence	molar frequencies: subfrag 1A/1B	subfrag 2A/2B	subfrag 3A/3B	expected
1	G	G	G	16.8	12.0	15.4	15
2	U,G	U-G	U-G	4.9	5.0	5.2	4
3	C,G	C-G	C-G	2.8	1.8	1.6	1
4	A-G	G,A	A-G	2.9	1.5	2.9	2
5	U,C,G		U-C-G	1.1	1.8	0.7	1
6	U,A-G	U-A,G	U-A-G	3.6	3.7	3.3	2
7	C,G,m^4C_m-C[b]		m^4C_m-C-C-G	0.8	1.0	0.9	1
8	A-A-G	G,2A	A-A-G	1.8	2.3	2.7	2
9	2C,G,A-C	C-G,C-C-A	C-C-A-C-G	0.9	1.2	1.0	1
10	U,G,A-C		A-C-U-G	1.1	1.2	1.5	1
11	2U,G	U-U-G	U-U-G	2.0	1.3	1.1	1
12	G,A-A-U	U-G,2A	A-A-U-G	1.1	1.3	1.1	1
13	C,A-A-A-A-G	G,C-A-A-A-A	C-A-A-A-A-G	0.4	0.8	0.5	1
14	2U,2C,G		C-C-U-U-G	1.0	0.9	1.1	1
15	2U,3C,G		U-U-C-C-C-G	1.1	1.1	1.4	1
16	G,A-C,A-A-U	U-A,C-G,2A	A-A-U-A-C-G	1.0	2.1	0.9	1
17	U,C,G,3A-C	U-A,2C-A,C-C-G	U-A-C-A-C-A-C-C-G	0.4	0.5	0.5	1
18	U,2C,G,2A-C,A-U	(U,G,A,C), (U,2A,3C)	U-m^5C-A-C-A-C-C-A-U-G	0.4	1.0	0.7	1
19	U,C,G,2A-U	U-G, (2U,C)A,A	A-U-U-C-A-U-G	0.9	0.9	1.2	1
20	3-4U,3-4C,G,A-A-C	(2U,C)A, (2U,2-3C)G	C-U-U-A-A-C-C-U-U-C-G	0.6	0.4	0.7	1
21	5U,2-3C,G,2A-C	(2U,C)A, (3U,C)G	C-U-U-A-C-C-A-C-U-U-U-G	0.6	0.6	0.7	1
X	U,A-A-C	U-A,C-A-A	mU-A-A-C-A-A-G[c]	0.6	0.8		1
Y	U,C,G,A-A-C		U-A-A-C-C-G[c]	0.9			1

[a] Oligonucleotides were eluted from fingerprint such as that illustrated in Figure 4 and hydrolyzed with pancreatic RNase, RNase U_2, and alkali, as described under Experimental Procedures. Products from the individual digestion mixtures were separated by paper electrophoresis and, in the case of fragments released by the two RNases, further analyzed for base composition by alkaline hydrolysis. Radioactivity was measured by Čerenkov or liquid scintillation counting at each step to establish the relative stoichiometries of the oligonuceotides. [b] The alkali-resistant dinucleotide m^4C_m-C exhibits an electrophoretic mobility very similar to that of A at pH 3.5 (Fellner, 1969). [c] Identification of these oligonucleotides is tentative as the expected products of secondary digestion were not all recovered.

and 1A + 1B revealed that these subfragments differed from subfragment 3 in containing one or two additional T_1 oligonucleotides (Table I). The position of the supplementary oligonucleotides is indicated in Figure 4. Although we were unable to determine an unambiguous sequence for these T_1 oligonucleotides, the data suggest that subfragments 2 and 1

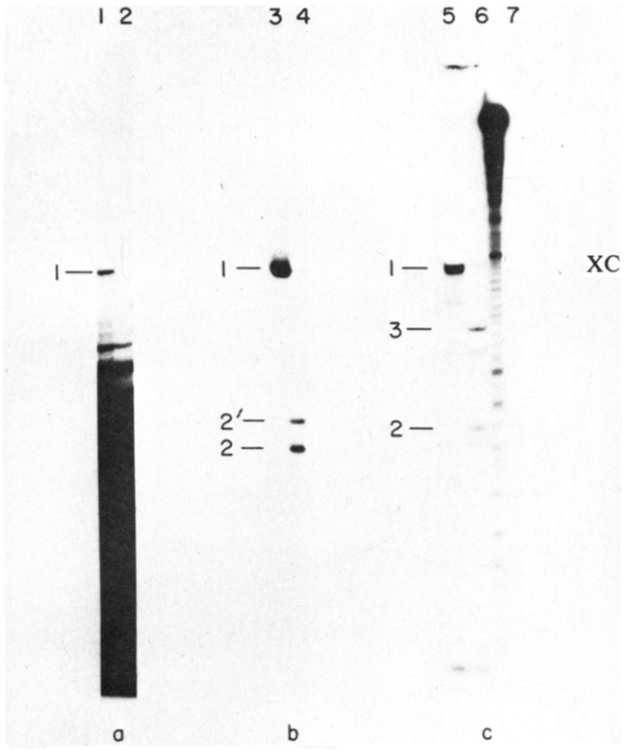

FIGURE 5: Electrophoretic analysis of cross-linked oligonucleotide from complete RNase T_1 digest of the AcVal-$tRNA_1^{Val}$–16S RNA complex. (a) Covalent AcVal-$tRNA_1^{Val}$–16S [^{32}P]RNA was digested to completion with RNase T_1 and subjected to electrophoresis through a 20% polyacrylamide gel in Tris–borate, pH 8.3, containing 7 M urea before (lane 1) and after (lane 2) irradiation for 15 min at 254 nm. (b) RNA was extracted from band 1 in (a) and analyzed by electrophoresis before (lane 3) and after (lane 4) irradiation as above. (c) RNA was extracted from band 1 in (b) and 5′-end-labeled with ^{32}P as described under Experimental Procedures. Electrophoresis of the oligonucleotides was performed before (lane 5) and after (lane 6) irradiation together with a random digest of 5′-^{32}P-labeled 16S RNA (lane 7). XC indicates position of the xylene cyanole marker dye.

both contain mUAACAAG (residues 1498–1504), while subfragment 1 probably contain UAACCG (residues 1506–1511) as well. In any case, residues 1362–1497 of the 16S RNA are common to all three subfragments, and the site to which $tRNA_1^{Val}$ becomes cross-linked must therefore occur within this region.

Identification of T_1 Oligonucleotide Cross-Linked to $tRNA_1^{Val}$. A comparison of the sequences of those tRNAs which can or cannot be cross-linked directly to 16S RNA at the ribosomal P site suggested that the cross-linked residue in all reactive tRNAs is the 5′ or wobble base of their respective anticodons (Ofengand et al., 1979; Prince et al., 1979). If this were true, then complete digestion of the covalent AcVal-$tRNA_1^{Val}$–16S RNA complex with RNase T_1 should yield a cross-linked oligonucleotide whose tRNA component is 15 nucleotides long (Kimura et al., 1971). Assuming that the rRNA component consists of at least two residues, this oligonucleotide should be the largest product arising from digestion of the tRNA–16S RNA adduct and thus readily distinguishable from non-cross-linked oligonucleotides by electrophoresis.

For isolation of the cross-linked T_1 oligonucleotide, covalent AcVal-$tRNA_1^{Val}$–16S [^{32}P]RNA complexes were hydrolyzed completely with RNase T_1, and a portion of the digest was irradiated for 15 min at 254 nm. Unirradiated and irradiated samples were then electrophoresed in adjacent lanes of a 20% polyacrylamide gel under denaturating conditions. The largest digestion product from the unirradiated sample, band 1, migrated to the position of the xylene cyanole marker (Figure 5a, lane 1), indicating an overall size of approximately 25 nucleotides, some 10 residues greater than the largest T_1 oligonucleotide expected from 16S RNA alone. Furthermore, the corresponding band was absent from the irradiated sample, presumably due to UV-induced cleavage of the tRNA–rRNA cross-link (Figure 5a, lane 2). The photolabile oligonucleotide was next eluted from the gel and divided into two portions, one of which was irradiated at 254 nm while the other remained untreated. After a second round of electrophoresis, the unirradiated oligonucleotide migrated as a single band with the same mobility as in the first gel (Figure 5b, lane 3). In the irradiated sample, however, most of the radioactivity was associated with an oligonucleotide of roughly 10 residues, designated band 2 (Figure 5b, lane 4). The latter result is precisely that anticipated upon photoreversal of a cross-link between an unlabeled pentadecanucleotide derived from tRNA and a ^{32}P-labeled oligonucleotide derived from 16S RNA.

Table II: Secondary Digestion of rRNA Oligonucleotide from Band 2[a]

enzyme	product	base composition	deduced sequence	molar frequency
RNase A	1	U	U	0.9
	2	C	C	1.1
	3	G	G	1.2
	4	A,C	A-C	3.0
RNase U_2	1	U,A	U-A	1.0
	2	A,C	C-A	2.2
	3	C	C-C	0.8

[a] ^{32}P-Labeled RNA extracted from band 2 of the gel depicted in Figure 5b was divided into two portions and digested with pancreatic RNase and RNase U_2. Products were resolved by paper electrophoresis, eluted, and analyzed for base composition. Relative stoichiometries were determined as in Table I.

The tRNA fragment was next visualized by extracting purified band 1 RNA and labeling its 5′ termini with ^{32}P by using [γ-^{32}P]ATP and polynucleotide kinase. Following reisolation of the end-labeled RNA, unirradiated and irradiated samples were once again compared by electrophoresis on a 20% polyacrylamide gel along with a random digest of 5′-labeled 16S RNA to provide a set of chain-length markers. While the unirradiated band retained its characteristic mobility (Figure 5c, lane 5), irradiation produced two main fragments (Figure 5c, lane 6). The more rapidly migrating product, which contained nine residues, corresponded in mobility to band 2 from endogeneously labeled 16S RNA. The second oligonucleotide, band 3, which consisted of 15 residues, must therefore represent the tRNA moiety of the cross-linked material in band 1.

The nonanucleotide derived from uniformly ^{32}P-labeled 16S RNA was isolated from gel band 2 (see Figure 5b) and analyzed further by secondary enzymatic hydrolysis. The products that resulted from pancreatic RNase digestion were found to be U, C, G, and A-C in a molar ratio of 1:1:1:3 (Table II). In a separate experiment, material from band 2 was hydrolyzed with RNase U_2 and the products were identified as U-A, C-A, and C-C in a molar ratio of 1:2:1 (Table II). The 3′-terminal G residue was not detected in the latter analysis, either because it was cleaved from the nonanucleotide or because its 3′-phosphate group was lost at some stage of the preparation. More recently, however, we have recovered stoichiometric amounts of C-C-G in RNase U_2 digests similar to that described here. Investigation of the oligonucleotide from band 2′ (Figure 5b) by hydrolysis with RNase U_2 revealed a

structure essentially identical with that of the material from band 2; it is not known at present precisely how these two oligonucleotides differ. In any event, the results of the secondary analyses establish unambiguously that the sequence of the rRNA component of the cross-linked T_1 product is U-A-C-A-C-A-C-C-G. This nonanucleotide, which is also found in subfragment 3 (see Table I), occurs only once in the 16S RNA molecule and corresponds to residues 1393–1401 (Brosius et al., 1978; Carbon et al., 1978).

Discussion

A number of unmodified AcAA-tRNAs can be cross-linked directly to the 30S subunit when bound to the ribosomal P site and irradiated with near-ultraviolet light (Schwartz & Ofengand, 1978; Ofengand et al., 1979; Zimmermann et al., 1979; Prince et al., 1979; Ofengand & Liou, 1981). The reactive species include $tRNA_1^{Val}$, $tRNA_1^{Ser}$, $tRNA_1^{Ala}$, $tRNA^{Lys}$, and $tRNA_2^{Glu}$ from *E. coli*, and $tRNA^{Val}$ and $tRNA^{Thr}$ from *Bacillus subtilis*. In the five cases examined, the tRNAs were found to be covalently attached to a segment of 100–125 nucleotides that originated from within a fragment encompassing the 3′ third of the 16S RNA molecule (Zimmermann et al., 1979; Prince et al., 1979). By taking advantage of the lability of the tRNA–rRNA bond to irradiation with light of 254 nm (Ofengand et al., 1979; Ofengand & Liou, 1980), we developed a two-dimensional gel electrophoresis system that enabled us to cleave the covalent complex between the first and second dimensions and thereby isolated the previously cross-linked rRNA subfragments as off-diagonal components. The appearance of such components depended strictly upon photoreversal, required the presence of tRNA in the initial incubation, and was specific to the 8S RNA. Sequence analysis revealed that the smallest of three overlapping rRNA subfragments obtained by this method consisted of residues 1362–1497 of the 16S RNA. The secondary structure proposed for this region by Woese, Noller, and coworkers (Woese et al., 1980; Noller, 1980) is shown in Figure 6.

Further localization of the site of cross-linking was accomplished by isolation of the covalent tRNA–rRNA oligonucleotide from a complete RNase T_1 digest of the AcVal-$tRNA_1^{Val}$–16S RNA adduct. The cross-linked T_1 product was found to contain a pentadecanucleotide derived from $tRNA_1^{Val}$ and a nonanucleotide derived from the 16S RNA. Following dissociation of these two moieties by exposure to light of 254 nm, the sequence of the rRNA portion was determined to be U-A-C-A-C-A-C-C-G. As expected, this oligonucleotide lies within the rRNA subfragments isolated by the diagonal gel method, occupying positions 1393–1401 of the 16S RNA chain. Interestingly, these nine residues are part of a longer, highly conserved sequence that occurs in a single-stranded region of the proposed secondary structure adjacent to the base-paired stem (boldface letters, Figure 6). The conserved segment, which spans residues 1392–1407 of 16S RNA from *E. coli*, has been found with slight or no differences in virtually all small-subunit rRNAs, including 25 prokaryotic 16S RNAs (Woese et al., 1975), several eukaryotic 18S RNAs (Samols et al., 1979; Rubtsov et al., 1980; Hall & Maden, 1980; Jordan et al., 1980), and 12–16S RNAs from both chloroplasts and mitochondria (Schwarz & Kössel, 1980; Eperon et al., 1980; Van Etten et al., 1980; Baer & Dubin, 1980). Furthermore, this sequence does not reside within any known ribosomal protein binding site, at least in *E. coli* 16S RNA (Zimmermann, 1980), and although it appears to be located within the subunit interface (Herr et al., 1979), no specific functional role has heretofore been ascribed to it. We now propose that

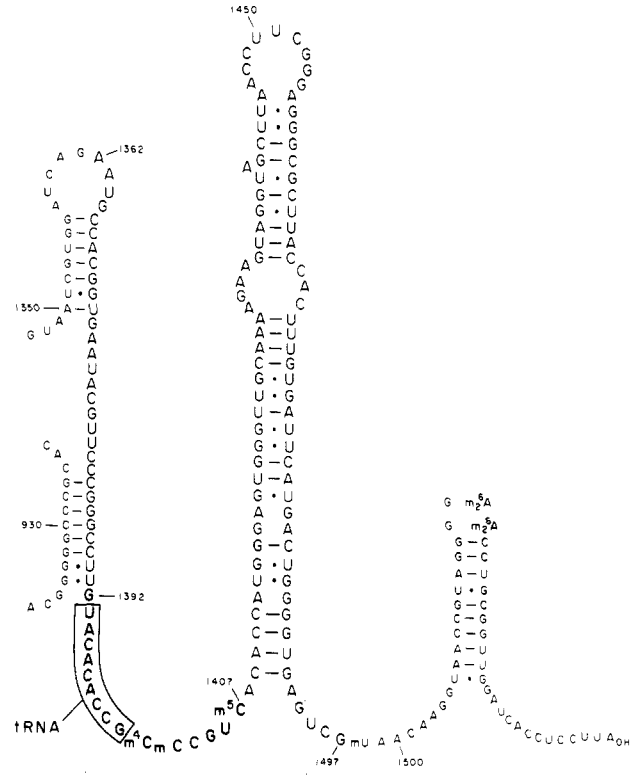

FIGURE 6: Proposed secondary structure of the 3′-terminal segment from *E. coli* 16S RNA. Large letters designate sequence of subfragments 3A + 3B (residues 1362–1497). Large bold letters indicate highly conserved region of RNA (residues 1392–1407). The box encloses T_1 oligonucleotide that is cross-linked to $tRNA_1^{Val}$ at the ribosomal P site (residues 1393–1401). The secondary-structure model was adapted from Woese et al. (1980).

the conserved sequence is an integral part of the tRNA anticodon binding domain of the ribosomal P site.

All of the tRNAs that can be directly attached to 16S RNA are distinguished by the presence of modified uridines at the 5′ or wobble position of their anticodons, and it has been argued that these residues mediate the cross-linking reactions (Ofengand et al., 1979; Prince et al., 1979; Ofengand & Liou, 1981). In $tRNA_1^{Val}$, the T_1 oligonucleotide that contains the wobble base, cmo^5U, is 15 nucleotides in length (Kimura et al., 1971). Since we have identified the tRNA moiety of the cross-linked fragment produced by RNase T_1 as a pentadecanucleotide, our results are consistent with the above proposal. More recently, we have verified by sequence analysis that the pentadecanucleotide is in fact the anticodon-containing T_1 product from *E. coli* $tRNA_1^{Val}$ (unpublished results). The characteristics of the photochemical cross-linking and photoreversal reactions indicate that the tRNA and rRNA components of the complex are joined via a pyrimidine–pyrimidine cyclobutane dimer (Ofengand et al., 1979; Ofengand & Liou, 1980). It thus seems likely that covalent attachment occurs between cmo^5U in the anticodon of $tRNA_1^{Val}$ and one of the five pyrimidines in the 16S RNA derived nonanucleotide, U-A-C-A-C-A-C-C-G. If binding of tRNA to the P site is stabilized by the stacking of the wobble base upon a similarly exposed base in the nonanucleotide, such an arrangement could facilitate pyrimidine dimerization between suitably positioned residues (Ofengand et al., 1979). Work now in progress is directed toward the positive identification of the bases in both tRNA and rRNA that participate in cross-link formation.

Added in Proof

The cross-linking of *E. coli* AcVal-$tRNA_1^{Val}$ and *B. subtilis* AcVal-$tRNA^{Val}$ to U-A-C-A-C-A-C-C-G at the P site of *E.*

coli ribosomes has been independently confirmed by C. Ehresmann, R. Millon, J. Ofengand, and B. Ehresmann (unpublished results).

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References

Backendorf, C., Overbeek, G. P., van Boom, J. H., van der Marel, G., Veeneman, G., & van Duin, J. (1980) *Eur. J. Biochem. 110*, 559–604.

Baer, R., & Dubin, D. T. (1980) *Nucleic Acids Res. 8*, 4927–4941.

Bowman, C. M., Dahlberg, J. E., Ikemura, T., Konisky, J., & Nomura, M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 964–968.

Brosius, J., Palmer, M. L., Kennedy, P. J., & Noller, H. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4801–4805.

Brownlee, G. G. (1972) *Determination of Sequences in RNA*, North-Holland/Elsevier, Amsterdam.

Carbon, P., Ehresmann, C., Ehresmann, B., & Ebel, J. P. (1978) *FEBS Lett. 94*, 152–156.

Debey, P., Hoa, G. H. B., Douzou, P., Godefroy-Colburn, T., Graffe, M., & Grunberg-Manago, M. (1975) *Biochemistry 14*, 1553–1559.

Eperon, I. C., Anderson, S., & Nierlich, D. P. (1980) *Nature (London) 286*, 460–467.

Fellner, P. (1969) *Eur. J. Biochem. 11*, 12–27.

Fox, G. E., & Woese, C. R. (1975) *J. Mol. Evol. 6*, 61–76.

Glotz, C., & Brimacombe, R. (1980) *Nucleic Acids Res. 8*, 2377–2395.

Hall, L. M. C., & Maden, B. E. H. (1980) *Nucleic Acids Res. 8*, 5993–6005.

Helser, T. L., Davies, J. E., & Dahlberg, J. E. (1971) *Nature (London), New Biol. 233*, 12–14.

Herr, W., Chapman, N. M., & Noller, H. F. (1979) *J. Mol. Biol. 130*, 433–449.

Jordan, B. R., Latil-Damotte, M., & Jourdan, R. (1980) *FEBS Lett. 117*, 227–231.

Kimura, F., Harada, F., & Nishimura, S. (1971) *Biochemistry 10*, 3277–3283.

Lai, C. J., Weisblum, B., Fahnestock, S. R., & Nomura, M. (1973) *J. Mol. Biol. 74*, 67–72.

Muench, K., & Berg, P. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., & Davies, D. R., Eds.) Vol. 1, pp 375–383, Harper and Row, New York.

Muto, A., Ehresmann, C., Fellner, P., & Zimmermann, R. A. (1974) *J. Mol. Biol. 86*, 411–432.

Noller, H. F. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 3–22, University Park Press, Baltimore, MD.

Ofengand, J. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 497–529, University Park Press, Baltimore, MD.

Ofengand, J., & Liou, R. (1980) *Biochemistry 19*, 4814–4822.

Ofengand, J., & Liou, R. (1981) *Biochemistry 20*, 552–559.

Ofengand, J., Liou, R., Kohut, J., III, Schwartz, I., & Zimmermann, R. A. (1979) *Biochemistry 18*, 4322–4332.

Prince, J. B., Hixson, S. S., & Zimmermann, R. A. (1979) *J. Biol. Chem. 254*, 4745–4749.

Rubtsov, P. M., Musakhanov, M. M., Zakharyev, V. M., Krayev, A. S., Skryabin, K. B., & Bayev, A. A. (1980) *Nucleic Acids Res. 8*, 4779–4794.

Samols, D. R., Hagenbüchle, O., & Gage, L. P. (1979) *Nucleic Acids Res. 7*, 1109–1119.

Sanger, F., Brownlee, G. G., & Barrell, B. G. (1965) *J. Mol. Biol. 13*, 373–398.

Schwartz, I., & Ofengand, J. (1978) *Biochemistry 17*, 2524–2530.

Schwarz, Zs., & Kössel, H. (1980) *Nature (London) 283*, 739–742.

Shine, J., & Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1342–1346.

Silberklang, M., Gillum, A. M., & RajBhandary, U. L. (1979) *Methods Enzymol. 59*, 58–109.

Steitz, J. A., & Jakes, K. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4734–4738.

Uchida, T., Bonen, L., Schaup, H. W., Lewis, B. J., Zablen, L., & Woese, C. (1974) *J. Mol. Evol. 3*, 63–77.

Van Etten, R. A., Walberg, M. W., & Clayton, D. A. (1980) *Cell (Cambridge, Mass.) 22*, 157–170.

Weidner, H., Yuan, R., & Crothers, D. M. (1977) *Nature (London) 266*, 193–194.

Woese, C. R., Fox, G. E., Zablen, L., Uchida, T., Bonen, L., Pechman, K., Lewis, B. J., & Stahl, D. (1975) *Nature (London) 254*, 83–86.

Woese, C. R., Magrum, L. J., Gupta, R. Siegel, R. B., Stahl, D. A., Kop, J., Crawford, N., Brosius, J., Guttell, R., Hogan, J. J., & Noller, H. F. (1980) *Nucleic Acids Res. 8*, 2275–2293.

Zimmermann, R. A. (1979) *Methods Enzymol. 59*, 551–583.

Zimmermann, R. A. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 135–169, University Park Press, Baltimore, MD.

Zimmermann, R. A., Muto, A., & Mackie, G. A. (1974) *J. Mol. Biol. 86*, 433–450.

Zimmerman, R. A., Gates, S. M., Schwartz, I., & Ofengand, J. (1979) *Biochemistry 18*, 4333–4339.