Covalent Cross-Linking of Transfer Ribonucleic Acid to the Ribosomal P Site. Site of Reaction in 16S Ribonucleic Acid†

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ABSTRACT: *N*-Acetyl[^{3}H]aminoacyl derivatives of $tRNA_1^{Val}$ and $tRNA_1^{Ser}$ from *Escherichia coli*, and of $tRNA^{Val}$ from *Bacillus subtilis*, have been covalently cross-linked to the P site of 70S tight-couple ribosomes by irradiation with 310–350-nm light. The irradiated ribosomes were dissociated into 30S and 50S subunits, and ribosomal RNA was extracted from each of the particles with phenol. Sucrose gradient analysis of this material revealed that the covalently bound *N*-acetyl[^{3}H]aminoacyl-tRNA cosedimented with 16S ribosomal RNA in all three cases. Following limited digestion of the *N*-acetyl[^{3}H]aminoacyl-tRNA–16S RNA adducts with RNase T_1, the tRNA moiety remained attached to a fragment of about 8 S which encompasses the entire 3′ third of the 16S RNA with the exception of approximately 50 residues at the 3′ terminus. Polyacrylamide gel electrophoresis in 6 M urea was used to separate the 8S fragment into a number of smaller components through the exposure of "hidden breaks" introduced during RNase treatment. Under these conditions, most of the ^{3}H radioactivity from the 8S fragment complex migrated in a band which consisted of 150–200 nucleotides and was larger than any of the major subfragments arising from non-cross-linked 8S RNA. Assuming the tRNA to be intact, the main ^{3}H-labeled band should contain a sequence of 75–125 bases derived from the 16S RNA. Since the electrophoretic mobility of this component was not altered by prior incubation with proteolytic enzymes, it is unlikely that the cross-link is mediated by protein. On the basis of these results, we suggest that a short segment of the 16S RNA, located near the 3′ end but not within 50 bases of the 3′ terminus, comprises part of the ribosomal P site and is juxtaposed to the anticodon of the peptidyl-tRNA in such a way that direct photochemical cross-linking of nucleotides in the two molecules can occur.

Chemical cross-linking procedures employing bifunctional reagents and affinity labels have been used extensively to probe the regions of the *Escherichia coli* ribosome that interact with the three initiation factors, elongation factors EFTu and EFG, several mRNA analogues, and both aminoacyl- and peptidyl-tRNAs [reviewed by Pellegrini & Cantor (1977); Stöffler & Wittmann (1977); Zamir (1977); Cooperman (1978)]. As a result, a relatively large number of proteins from the 30S and 50S ribosomal subunits have been located in the vicinity of important active sites. Labeling of the ribosomal RNAs has been achieved much less frequently, however, even though these molecules comprise two-thirds of the particle mass and are also believed to make a vital contribution to ribosome function. Among the well documented cases, a photolabile derivative of the phenylalanine-specific codon UUU has been covalently bound to the 16S RNA some 450 residues from its 5′ terminus (Wagner et al., 1976), and several analogues of peptidyl-tRNA containing reactive substituents attached to the free α-amino group have been cross-linked to the 3′ half of 23S RNA in the ribosomal P site (Barta et al., 1975; Sonenberg et al., 1975, 1976, 1977; Yukioka et al., 1977).

Aside from numerous affinity labeling studies that place the aminoacyl moiety of aminoacyl-tRNA in the vicinity of four or five specific 50S subunit proteins as well as the 23S RNA [see Pellegrini & Cantor (1977) and Cooperman (1978)], there are only a few instances in which cross-linking techniques have been used to investigate ribosomal sites adjacent to other portions of the tRNA molecule (Schwartz & Ofengand, 1974; Schwartz et al., 1975; Ofengand et al., 1977). As an outgrowth of the latter studies, it was discovered that *E. coli* AcVal[1]-$tRNA_1^{Val}$ could be photochemically attached to 70S ribosomes with high efficiency in a reaction that required neither chemical modification of the tRNA nor the presence of 4-thiouridine within it. The formation of covalent cross-links was dependent upon irradiation with light of 310–350 nm and the presence of mRNA, occurred only when the tRNA was located in the ribosomal P site, and joined the tRNA exclusively to the 16S RNA of the 30S subunit (Schwartz & Ofengand, 1978; Ofengand et al., 1979). In addition, it was found that AcSer-$tRNA_1^{Ser}$ from *E. coli* as well as AcVal-$tRNA^{Val}$ and AcThr-$tRNA^{Thr}$ from *Bacillus subtilis* could also be photochemically linked to 70S ribosomes whereas a number of other tRNAs could not. Those results suggested that attachment was mediated by the modified uracil that occurs at the 5′ end of the anticodon in each of the cross-linked tRNA molecules (Ofengand et al., 1979). In the work described in the present paper, the site to which tRNA is attached in the 16S RNA has been localized to a fragment of approximately 100 bases that arises from the 3′ third of the ribosomal RNA molecule.

Experimental Section

Components. *E. coli* $tRNA_1^{Val}$, *E. coli* $tRNA_1^{Ser}$, and *B. subtilis* $tRNA^{Val}$ were obtained, charged with radioactive amino acids, and N-acetylated as described in the preceding paper (Ofengand et al., 1979). [^{3}H]Valine and [^{3}H]serine (10–30 Ci/mmol) were products of New England Nuclear or Amersham. Active 70S ribosomes were prepared from *E. coli* MRE600 as tight couples (Noll et al., 1973). Polynucleotides were purchased from Boehringer-Mannheim. [^{14}C]Uracil-

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[1] Abbreviations used: AcVal-, *N*-acetylvalyl-; AcSer-, *N*-acetylseryl-; AcThr-, *N*-acetylthreonyl-; AcAA-, *N*-acetylaminoacyl-; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; mo^5U, 5-methoxyuridine; cmo^5U, 5-(carboxymethoxy)uridine.

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labeled 16S RNA (3000 cpm/μg) and unlabeled 16S RNA were isolated according to Muto et al. (1974). Colicin E3 was kindly donated by P. Model and N. Zinder.

Formation and Irradiation of tRNA–Ribosome Complexes. Nonenzymatic binding of Ac[^{3}H]Val-tRNA$_1^{Val}$ (*E. coli*), Ac[^{3}H]Ser-tRNA$_1^{Ser}$ (*E. coli*), and Ac[^{3}H]Val-tRNA^{Val} (*B. subtilis*) to the ribosomal P site was carried out in 3–10-mL reaction mixtures by using the conditions previously described (Ofengand et al., 1979). Following incubation, samples of the reaction mixture were removed for measurement of noncovalent Ac[^{3}H]AA-tRNA–ribosome association (Schwartz & Ofengand, 1974). From 20 to 80% of the Ac[^{3}H]AA-tRNA was complexed under these conditions, and over 90% of that bound was released by puromycin. Incubation mixtures were irradiated at 0 °C for 2–4 h in a Rayonet RPR-100 photochemical reactor equipped with either unfiltered 350-nm lamps or 300-nm lamps in conjunction with a Mylar filter (Ofengand et al., 1979). After irradiation, the amount of Ac[^{3}H]AA-tRNA covalently cross-linked to ribosomal particles was measured (Ofengand et al., 1979). This figure varied from 20 to 60% of the tRNA noncovalently bound or from 5 to 40% of the total tRNA present.

Treatment of Covalent Complexes with Colicin E3. Covalent complexes between Ac[^{3}H]Val-tRNA$_1^{Val}$ and 70S tight couples were prepared as described above except that the incubation buffer contained 50 mM Tris-HCl, pH 7.4, 15 mM $MgCl_2$, 50 mM NH_4Cl, and 5 mM dithiothreitol. Samples from the irradiated reaction mixture were treated with colicin E3 for 15 min at 38 °C by using 40 μg of colicin per A_{260} unit of ribosomes. This amount of colicin is sufficient to inactivate 10–20 A_{260} units of 70S ribosomes under the conditions employed. The reaction was stopped, and the ribosomes were dissociated by the addition of $NaDodSO_4$ and Na_2EDTA to final concentrations of 1% and 1.5 mM, respectively. This mixture was then chilled on ice and incubated a further 15 min at 0 °C with occasional stirring. The resulting extract was chromatographed on a 0.6-cm i.d. × 28 cm column of Sephadex G-100 at room temperature in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, and 0.5% $NaDodSO_4$.

Isolation and Extraction of tRNA–30S Subunit Complexes. Irradiated complexes were precipitated by the addition of 0.1 volume of 20% KOAc, pH 5, and 2 volumes of 95% ethanol at −20 °C. Ribosomes were recovered by centrifugation and suspended in 10 mM potassium phosphate, pH 6.2, 0.25 mM $MgCl_2$, and 6 mM 2-mercaptoethanol. Up to 24 A_{260} units of ribosomes were layered on a 12-mL 10–30% sucrose gradient in the same buffer and centrifuged for 4.5 h at 40000 rpm in a Spinco SW41 rotor at 2 °C. The 30S subunits were pooled, precipitated with 2 volumes of 95% ethanol after the addition of 0.1 volume of 1 M NaCl and 0.02 volume of 1 M $MgCl_2$, resuspended in PM buffer (10 mM potassium phosphate, pH 6.2, and 0.25 mM $MgCl_2$) containing 0.1% $NaDodSO_4$, and extracted with an equal volume of phenol. After removal of the first aqueous phase and reextraction of the phenol phase with PM buffer, the aqueous phases were combined and adjusted to 0.1 M NaCl, and the RNA was precipitated with ethanol. The RNA was washed and reprecipitated twice and finally dissolved in a small amount of PM buffer. The mobility of the Ac[^{3}H]AA-tRNA–16S RNA complexes was checked by sedimentation in sucrose gradients at pH 6.2 and by electrophoresis in 3% acrylamide–0.5% agarose gels at pH 5.2 (see below). From 80 to 90% of the ^{3}H radioactivity was found to migrate with the 16S peak. The specific activity of the purified complexes ranged from 400 to 1000 cpm/μg of RNA.

Digestion of Covalent tRNA–16S RNA Complexes. Sufficient Ac[^{3}H]AA-tRNA–16S RNA complex, ^{14}C-labeled 16S RNA, and unlabeled 16S RNA to yield a total of 25 μg were mixed in 100 μL of PMK buffer (50 mM potassium phosphate, pH 6.5, 20 mM $MgCl_2$, 350 mM KCl, and 5 mM 2-mercaptoethanol; final pH 6.2) containing 10 μg of bovine serum albumin, incubated for 30 min at 40 °C, and chilled on ice. After 15 min, the pH was adjusted to 7.0 with 5 μL of 1 M K_2HPO_4, and from 1 to 2.5 units (0.25 to 0.625 μg) of RNase T$_1$ (Sankyo) was added. The mixture was left at 0 °C for 15 min and then centrifuged through 3–15% sucrose gradients in PMK buffer at 32000 rpm for 16 h at 2 °C in a Spinco SW41 rotor. Gradients were collected in 20 fractions, and a portion of each was acid-precipitated for analysis of radioactivity. In preparative runs, fractions containing RNA fragments were pooled, dialyzed overnight against 500 volumes of 10 mM NaOAc, pH 5.2, concentrated to 0.2 mL with an immersible molecular separator (Millipore), and frozen at −20 °C.

Gel Electrophoresis. RNA samples were electrophoresed through 10-cm polyacrylamide gels containing a 19:1 mixture by weight of acrylamide–*N,N'*-methylenebis(acrylamide) in a buffer consisting of 20 mM $Na_2B_4O_7$, 30 mM succinic acid, and 2.5 mM Na_2EDTA, at pH 5.2. Reservoirs contained the same buffer. High molecular weight RNA was analyzed in composite gels containing 3% acrylamide mixture and 0.5% agarose. RNA fragments and tRNA were analyzed in denaturing gels which contained 10% acrylamide and 6 M urea. Gels were preelectrophoresed for 1 h at 5 mA/tube. All samples were made up in 15% sucrose and 0.002% bromphenol blue, and samples for denaturing gels received in addition solid urea to a final concentration of 6 M and both 5S RNA and tRNA as markers. When appropriate, samples were also treated with Pronase (Calbiochem) or proteinase K (EM Biochemicals) prior to analysis. Electrophoresis was performed at 20 °C and at 5 mA/gel for 7 h (3% gels) or until the bromphenol blue dye was 1 cm from the bottom of the tube (10% gels). The gels were removed, scanned spectrophotometrically at 260 nm, and frozen on dry ice. Each gel was then sectioned at intervals of 2 mm, and the slices were placed in glass vials for radioactive counting.

Analysis of Radioactivity. Sucrose gradient fractions were precipitated with 5% trichloroacetic acid, and the precipitates were collected on glass-fiber filters (Whatman). Filters were counted in a scintillation cocktail containing 4 g of Omnifluor (New England Nuclear) per L of toluene. Gel slices were incubated in 5 mL of toluene containing 4 g/L Omnifluor and 3% (v/v) Protosol (New England Nuclear) for 24 h at room temperature before counting.

Results

It was shown in the preceding paper that unmodified AcVal-tRNA$_1^{Val}$ and AcSer-tRNA$_1^{Ser}$ from *E. coli*, and AcVal-tRNA^{Val} and AcThr-tRNA^{Thr} from *B. subtilis*, can be photochemically cross-linked to 70S ribosomes following nonenzymatic binding to the P site (Ofengand et al., 1979). In the present investigation, we have examined the properties of covalent AcAA-tRNA–16S RNA complexes isolated from cross-linked ribosomes in an effort to define the site at which the tRNA molecules are attached to the 16S RNA.

Characterization of AcAA-tRNA–16S RNA Complexes. Ac[^{3}H]Val-tRNA$_1^{Val}$ was covalently cross-linked to the P site of 70S tight-couple ribosomes as described in the preceding paper (Ofengand et al., 1979). In various experiments 50–80% of the input tRNA was initially bound to the ribosomes, and, of this, 40–60% became covalently attached upon irradiation

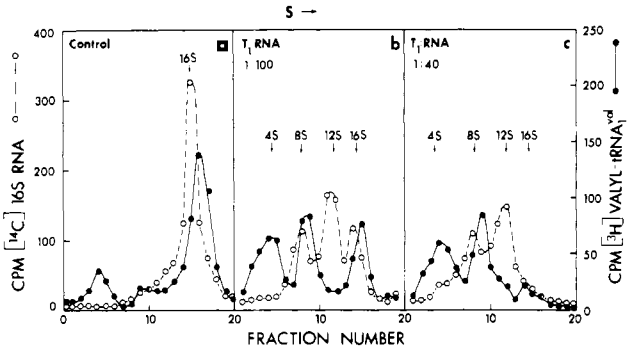

FIGURE 1: Limited hydrolysis of the AcVal-tRNA$_1^{Val}$–16S RNA complex with RNase T_1. The Ac[^{3}H]Val-tRNA$_1^{Val}$–16S RNA complex (4 μg) was mixed with ^{14}C-labeled 16S RNA (1 μg) and unlabeled 16S RNA (20 μg) in 100 μL of PMK buffer (pH 6.2) and incubated for 30 min at 40 °C. After the mixtures were chilled on ice, they received (a) 0, (b) 0.25, or (c) 0.625 μg of RNase T_1, and incubation was continued for 15 min at 0 °C. RNA was resolved by centrifugation on 3–15% sucrose gradients in PMK buffer for 16 h at 32 000 rpm. Acid-precipitable radioactivity in each fraction was measured as described under Experimental Section. (○) ^{14}C-Labeled 16S RNA; (●) Ac[^{3}H]Val-tRNA$_1^{Val}$–16S RNA complex.

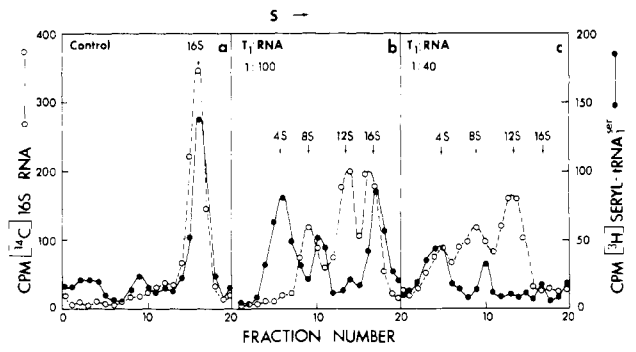

FIGURE 2: Limited RNase T_1 digestion of the covalent AcSer-tRNA$_1^{Ser}$–16S RNA complex. The Ac[^{3}H]Ser-tRNA$_1^{Ser}$–16S RNA complex (1 μg) was incubated with ^{14}C-labeled 16S RNA (1 μg) and unlabeled 16S RNA (23 μg) in 100 μL of PMK buffer (pH 6.2) for 30 min at 30 °C, chilled on ice, and treated for 15 min at 0 °C with (a) 0, (b) 0.25, or (c) 0.625 μg of RNase T_1. The mixtures were fractionated on sucrose gradients, and the acid-precipitable radioactivity in each tube was assayed. (○) ^{14}C-Labeled 16S RNA; (●) Ac[^{3}H]Ser-tRNA$_1^{Ser}$–16S RNA complex.

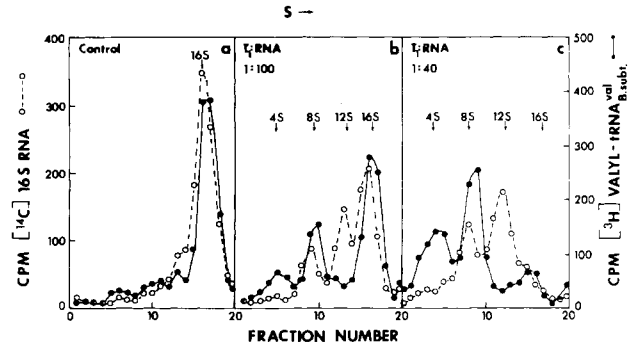

FIGURE 3: Limited digestion of the covalent complex between AcVal-tRNA^{Val} from *B. subtilis* and 16S RNA from *E. coli*. Ac-[^{3}H]Val-tRNA$^{Val}_{B.subtilis}$–16S RNA complex (1 μg), ^{14}C-labeled 16S RNA (1 μg), and unlabeled 16S RNA (23 μg) were mixed together and incubated for 30 min at 40 °C in 100 μL of PMK buffer (pH 6.2). The mixtures were chilled and exposed to (a) 0, (b) 0.25, or (c) 0.625 μg of RNase T_1 for 15 min at 0 °C. Fractionation and analysis of the RNA were performed as described in Figure 1. (○) ^{14}C-Labeled 16S RNA; (●) Ac[^{3}H]Val-tRNA$^{Val}_{B.subtilis}$–16S RNA complex.

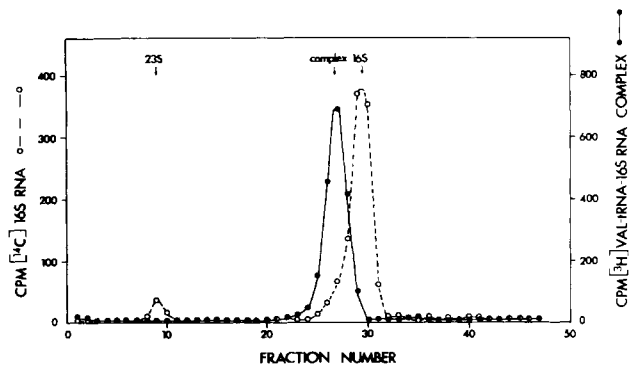

FIGURE 4: Electrophoretic mobilities of 16S RNA and the AcVal-tRNA$_1^{Val}$–16S RNA complex. A mixture of 4 μg of the Ac[^{3}H]Val-tRNA$_1^{Val}$–16S RNA complex and 12 μg of ^{14}C-labeled 16S RNA containing a small amount of ^{14}C-labeled 23S RNA in PMK buffer was heated for 30 min at 40 °C and electrophoresed through a composite 3% polyacrylamide–0.5% agarose gel at pH 5.2 for 7 h at 20 °C. The gels were sectioned at 2-mm intervals, and the radioactivity in each slice was analyzed by scintillation counting in an Omnifluor–Protosol–toluene cocktail. (○) ^{14}C-Labeled rRNA; (●) Ac[^{3}H]Val-tRNA$_1^{Val}$–16S RNA complex.

at 310 to 350 nm. More than 80% of both noncovalently and covalently bound tRNA was in the P site as shown by reaction with puromycin (Ofengand et al., 1979). Separation of covalent Ac[^{3}H]Val-tRNA$_1^{Val}$–ribosome complexes into subunits by sucrose gradient centrifugation in 0.25 mM $MgCl_2$ revealed that the tRNA was attached solely to the 30S particles (Schwartz & Ofengand, 1978). Following extraction of the subunits with phenol, 80–90% of the radioactivity was recovered in the 16S RNA fraction (Schwartz & Ofengand, 1978; see also Figure 1a).

Similar results were obtained with complexes containing *E. coli* tRNA$_1^{Ser}$ and *B. subtilis* tRNA^{Val}. Formation of the complexes and cross-linking of the Ac[^{3}H]AA-tRNAs were dependent upon the presence of the appropriate polynucleotide, and 20–40% of the ribosome-bound tRNA became covalently attached during irradiation with 310–350-nm light. As in the case of *E. coli* tRNA$_1^{Val}$, most of the cross-linked tRNA$_1^{Ser}$ and *B. subtilis* tRNA^{Val} was found to be associated with the 16S RNA (see Figures 2a and 3a).

For determination of whether the Ac[^{3}H]Val-tRNA$_1^{Val}$–16S RNA complex could be resolved from non-cross-linked 16S RNA, the two species were electrophoresed together in a composite 0.5% agarose–3.0% polyacrylamide gel at pH 5.2. The low pH was chosen to retard chemical deacylation of the tRNA moiety which contained the radioactive amino acid. Figure 4 shows that the complex migrates as a discrete component and that it can be distinguished from free 16S RNA by this procedure.

Localization of the Cross-Link in the 16S RNA. In order to define the position of the cross-linked tRNA in the 16S RNA, we subjected covalent AcAA-tRNA–16S RNA complexes to partial ribonuclease hydrolysis. Digestion of 16S RNA with RNase T_1 in neutral buffers containing 20 mM Mg^{2+} has been found to generate two large fragments of roughly 12 S and 8 S that can be easily distinguished from each other and from intact 16S RNA by sucrose gradient sedimentation (Zimmermann et al., 1972). Sequence analysis demonstrated that the 5′ end of the 12S RNA occurs within a few bases of the 5′ terminus of the 16S RNA and that the fragment spans some 900 residues. The 8S fragment is contiguous with the 12S fragment and encompasses roughly 600 nucleotides from the 3′ portion of the 16S RNA although it does not contain the 50 3′-terminal bases (Muto et al., 1974).

The covalent Ac[^{3}H]Val-tRNA$_1^{Val}$–16S RNA complex was mixed with ^{14}C-labeled 16S RNA and heated in the presence of 20 mM $MgCl_2$ to ensure the conformational homogeneity

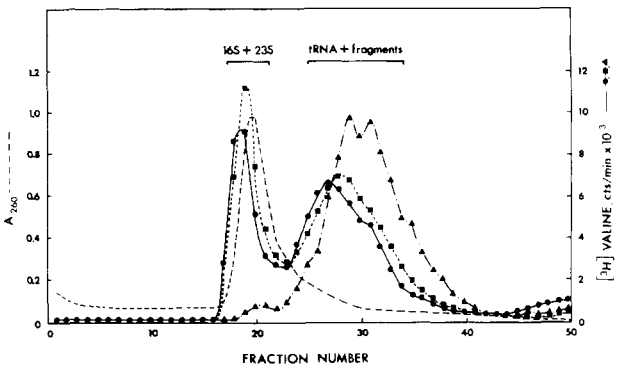

FIGURE 5: Gel filtration of colicin E3 treated AcVal-tRNA$_1^{Val}$–16S RNA complexes. A reaction mixture containing 40 pmol of Ac-[^{3}H]Val-tRNA$_1^{Val}$, 50 μg of poly(U_2,G), and 4.5 A_{260} units of 70S tight couples per mL was prepared and irradiated as described under Experimental Section. All of the tRNA was noncovalently bound in this experiment, and 25% was photochemically cross-linked to ribosomes. A 200-μL aliquot was withdrawn and incubated with 44 μg of colicin E3. After the addition of NaDodSO$_4$ and Na_2EDTA, the chilled reaction mixture was fractionated by chromatography on Sephadex G-100. (---) A_{260}; (●) plus colicin E3; (■) minus colicin E3; (▲) unirradiated sample, minus colicin E3.

of the RNA (Schulte et al., 1974; Muto & Zimmermann, 1978). Samples were treated with different concentrations of RNase T_1 at 0 °C, and the products were fractionated on sucrose gradients. The results, presented in Figure 1, clearly show that the AcVal-tRNA moiety is associated with the 8S fragment, but not the 12S fragment, of the 16S RNA. At an enzyme/substrate ratio of 1:100, the cross-linked tRNA is about equally distributed between the fragment and undissociated 16S RNA whereas higher RNase concentrations lead to almost complete elimination of the 16S peak and conversion of approximately 30% of the total ^{3}H radioactivity to the acid-soluble form (compare parts b and c of Figure 1).

Parallel experiments were carried out on complexes of 16S RNA with Ac[^{3}H]Ser-tRNA$_1^{Ser}$ and *B. subtilis* Ac[^{3}H]-Val-tRNA^{Val}. In both cases, the covalently bound tRNAs cosedimented with the 8S fragment of the 16S RNA (Figures 2 and 3).

On the basis of these experiments, we infer that *E. coli* tRNA$_1^{Val}$, *E. coli* tRNA$_1^{Ser}$, and *B. subtilis* tRNA^{Val} can be photochemically cross-linked to one or more nucleotides within a segment of 600 residues arising from the 3′ portion of the 16S RNA. However, this segment does not contain the 3′-terminal fragment of the 16S RNA that is released from intact ribosomal particles upon treatment with colicin E3 (Bowman et al., 1971). For confirmation that the cross-link does not occur in the colicin fragment, 70S ribosomes carrying covalently bound Ac[^{3}H]Val-tRNA$_1^{Val}$ were incubated with colicin E3, dissociated with 1% NaDodSO$_4$ and 1.5 mM EDTA, pH 7, and then analyzed by gel filtration (Figure 5). Under these conditions, the radioactivity remained stably attached to the undigested rRNA and was not liberated as a small RNA fragment.

In order to be sure that cross-linking of tRNA to 16S RNA did not alter the cleavage pattern of the 16S RNA, we examined the behavior of purified tRNA–^{32}P-labeled 16S RNA covalent complexes. The highly purified complexes, freed of unreacted ^{32}P-labeled 16S RNA by a modification of the antibody method previously described (Keren-Zur et al., 1979), were digested with RNase T_1 as described above. Both 8S and 12S ^{32}P peaks appeared just as they did in control 16S RNA, demonstrating that covalent attachment of tRNA does not alter the digestion pattern (data not shown). It is therefore unlikely that the ^{3}H-labeled 8S fragment seen in Figure 1 was derived from the 5′ portion of the 16S RNA by aberrant RNase T_1 cleavage.

Analysis of the AcAA-tRNA–8S RNA Fragment Complex in Denaturing Gels. The 8S RNA fragment derived from free 16S RNA is known to harbor a number of internal scissions or "hidden breaks" that are apparently masked in the presence of Mg^{2+} by secondary or tertiary interactions within the nucleic acid chain (Zimmermann et al., 1974). When the fragment is electrophoresed in polyacrylamide gels containing high concentrations of urea, however, such breaks are revealed by the appearance of eight to ten smaller, yet discrete, RNA components. Accordingly, we have isolated the three AcAA-tRNA–8S RNA fragment complexes and analyzed them on denaturing gels, once again using the pH 5.2 buffer to minimize spontaneous release of *N*-acetylamino acids from the tRNAs.

Figure 6 shows that the major ^{3}H-labeled tRNA–rRNA subfragments liberated from the 8S complexes by urea migrate to distinctive positions in the gel that do not coincide with any of the main ^{14}C-labeled subfragments which arise from free 8S RNA. Essentially similar gel patterns were obtained in all three cases. Retardation of the ^{3}H-labeled components relative to free tRNA is not due to the presence of aminoacyl or *N*-acetylaminoacyl ligands since both of the corresponding tRNA derivatives are only slightly displaced from the 4S peak at pH 5.2 (R. A. Zimmermann and S. M. Gates, unpublished experiments). If we assume that the cross-linked complexes possess normal electrophoretic properties, the main tRNA–rRNA fragment is estimated to consist of 150–200 nucleotides from its mobility relative to the 4S and 5S RNA standards. Minor bands to the left and right of the main component are presumed to represent partially overlapping digestion products. The recovery of ^{3}H radioactivity in all of these bands requires that the tRNA molecules be intact, at least between the point of linkage and the 3′ terminus. Segments of the 16S RNA in the fragment complexes must therefore be from 75 to 125 bases in length.

The 4S material from RNase T_1 digests of the Ac[^{3}H]-Val-tRNA$_1^{Val}$–16S RNA complex (see Figure 1) was also isolated and analyzed by electrophoresis. This peak contained only a small amount of intact tRNA and much larger quantities of short aminoacyl oligonucleotides that migrate with the dye front (data not shown). The slowly sedimenting RNA cannot therefore have been derived by spontaneous cleavage of the bond joining tRNA to the rRNA.

Although we favor the hypothesis that tRNA and 16S RNA are joined by a direct chemical cross-link (Ofengand et al., 1979), we considered the possibility that attachment was mediated by protein. Accordingly, the Ac[^{3}H]Val-tRNA$_1^{Val}$–8S RNA fragment complex was incubated for 1 h at 25 °C with either Pronase or proteinase K. The gel profiles illustrated in Figure 7 demonstrate that neither enzyme altered the mobility of the tRNA–rRNA fragment bands. It is therefore unlikely that the tRNA is cross-linked to the 16S RNA via a protein "bridge". If, on the other hand, attachment of the two components is mediated by mRNA, the intervening sequence cannot exceed the length of a single codon (Ofengand et al., 1979). Our current view of the tRNA linkage site in the 16S RNA is depicted in Figure 8.

Discussion

We have demonstrated that irradiation at wavelengths between 310 and 350 nm of three different AcAA-tRNAs bound at the P site of 70S ribosomes results in direct and efficient cross-linking of tRNA to the 3′ third of the 16S RNA. As shown in the preceding paper (Ofengand et al., 1979),

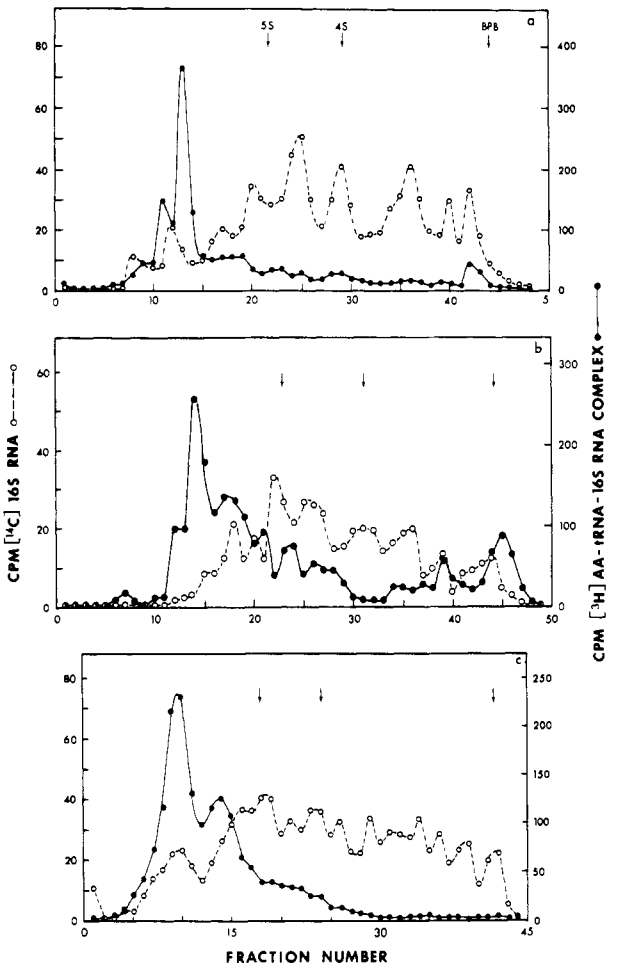

FIGURE 6: Polyacrylamide gel electrophoresis of 8S fragments from AcAA-tRNA–16S RNA complexes in the presence of 6 M urea. The 8S fragments were obtained as follows. (a) AcVal-tRNA$_1^{Val}$–8S RNA. The Ac[^{3}H]Val-tRNA$_1^{Val}$–16S complex (13 μg) was incubated with ^{14}C-labeled 16S RNA (4 μg) and unlabeled 16S RNA (8 μg) in PMK buffer (pH 6.2) for 30 min at 40 °C, treated with 0.5 μg of RNase T_1 for 15 min at 0 °C, and separated on sucrose gradients as described in Figure 1. (b) AcSer-tRNA$_1^{Ser}$–8S RNA. A mixture of the Ac[^{3}H]Ser-tRNA$_1^{Ser}$–16S RNA complex (18 μg) and ^{14}C-labeled 16S RNA (7 μg) was incubated at 40 °C, treated with 0.25 μg of RNase T_1 at 0 °C, and fractionated as described in (a). (c) AcVal-tRNA$^{Val}_{B.\ subtilis}$–8S RNA. The Ac[^{3}H]Val-tRNA$^{Val}_{B.\ subtilis}$–16S RNA complex (20 μg) and ^{14}C-labeled 16S RNA (5 μg) were incubated, chilled, and digested with 0.5 μg of RNase T_1. The 8S fragment was isolated in the usual way. In each case, the pooled 8S material was dialyzed and concentrated. Sample preparation was carried out as described under Experimental Section and included the addition of 7 μg of an unlabeled tRNA–5S RNA mixture as well as sucrose, urea, and bromphenol blue. Electrophoresis was performed at 20 °C in 10% polyacrylamide gels containing 6 M urea, pH 5.2, at 5 mA/tube. At the end of the run, gels were scanned at 260 nm and processed for radioactive counting. Arrows marked 5 S, 4 S, and BPB indicate the final positions of the 5S RNA and tRNA standards and bromphenol blue dye, respectively. (○) Fragments derived from ^{14}C-labeled 16S RNA; (●) fragments derived from Ac[^{3}H]AA-tRNA–16S RNA complexes.

covalent attachment was mRNA dependent in all three cases. A common feature of the cross-linked tRNAs that is not shared by most other tRNA molecules is the presence of the similar uracil derivatives, cmo^{5}U or mo^{5}U, in the 5′ or wobble position of the anticodon [see Ofengand et al. (1979), Table IV]. We believe that the tRNA–rRNA bond is mediated by this residue since tRNAs of nearly the same sequence, but with other bases at the wobble site, fail to establish covalent links to the ribosome under comparable conditions of incubation and irradiation (Ofengand et al., 1979).

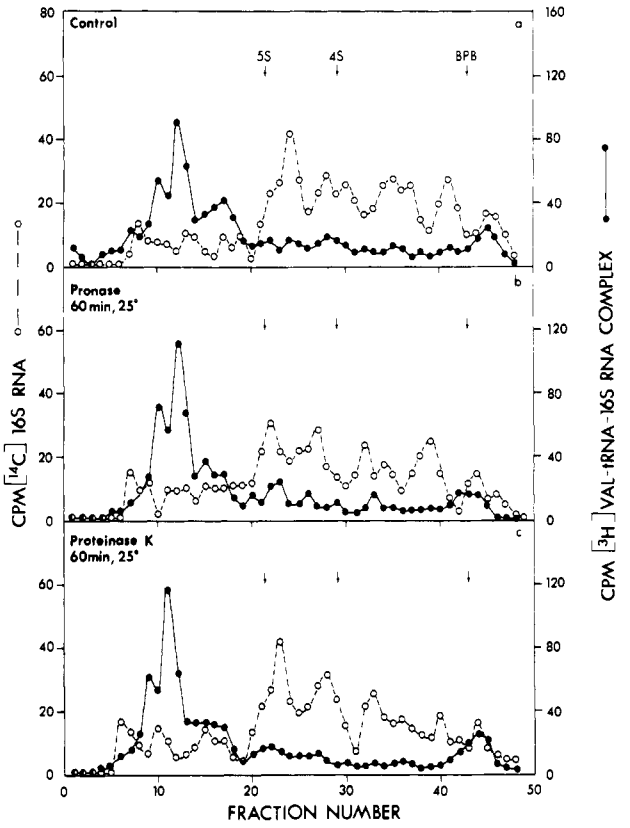

FIGURE 7: Gel electrophoresis of proteinase-treated 8S tRNA–rRNA fragments under denaturing conditions. The 8S fragment was prepared from the Ac[^{3}H]Val-tRNA$_1^{Val}$–16S RNA complex and ^{14}C-labeled 16S RNA as described in Figure 6a. Prior to electrophoresis, the concentrated 8S pool in 10 mM NaOAc·HOAc, pH 5.2, and 4 M urea was divided into three portions and treated with (a) no addition, (b) 10 μg/mL Pronase, or (c) 10 μg/mL proteinase K for 60 min at 25 °C. These samples were subjected to electrophoresis in 10% polyacrylamide gels containing 6 M urea by the standard procedure. Arrows designate the positions of 5S RNA, tRNA, and bromphenol blue at the end of the run. (○) Fragments derived from ^{14}C-labeled 16S RNA; (●) fragments derived from the Ac[^{3}H]Val-tRNA$_1^{Val}$–16S RNA complex.

The 3′ region of the 16S RNA has already been implicated in the mechanism of protein synthesis by several other functional studies. In particular, it is thought to play an important role in the binding of mRNA and IF3 during the initiation of polypeptide assembly (Shine & Dalgarno, 1974; Steitz & Jakes, 1975; van Duin et al., 1975). These interactions apparently occur within the 50 or so 3′-terminal residues of the ribosomal RNA which can be released from the 70S particle by treatment with colicin E3 (Bowman et al., 1971). We believe that the site of tRNA–16S RNA attachment described in the present report does not coincide with the functional sites in the colicin fragment since the 50 3′-terminal bases were previously shown to be absent from the 8S RNA (Muto et al., 1974). Moreover, no AcAA-tRNA is liberated from the covalent ribosomal complexes by colicin E3 under our conditions. Nevertheless, our results do not entirely preclude the possibility that the tRNA is linked to a base sufficiently near the nuclease cleavage site to block hydrolysis of a normally vulnerable bond in the rRNA chain.

Our experiments provide presumptive evidence that a 100-nucleotide segment from the 3′ third of the 16S RNA is located in the immediate neighborhood of the anticodon of peptidyl-tRNA. This finding is wholly consistent with immune electron microscopic investigations of the 30S subunit which place the proteins of the mRNA binding region and the proteins associated with the 3′ end of the 16S RNA in the

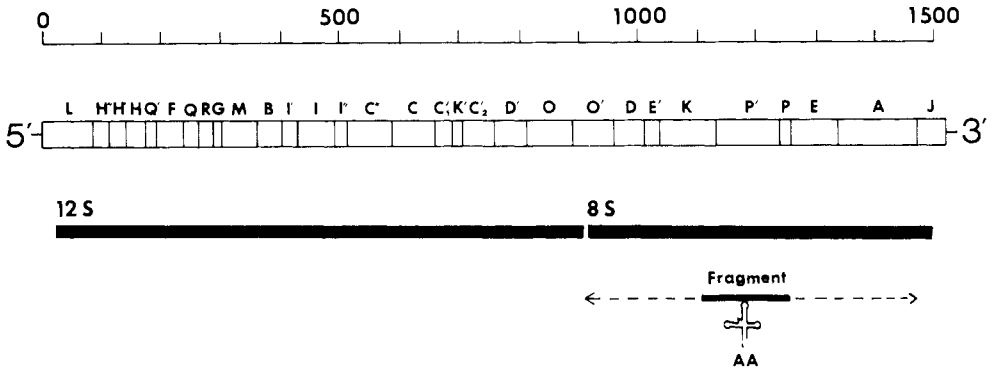

FIGURE 8: Approximate position of the cross-linked tRNA in the 16S RNA molecule. Upper case letters designate sections of the 16S RNA primary structure as characterized by Ehresmann et al. (1975). The scale at the top of the illustration shows approximate distances in nucleotide residues from the 5′ terminus of the molecule. The extent and placement of the 12S and 8S fragments have been discussed by Muto et al. (1974). The segment of 16S RNA to which the tRNA is attached, represented by the solid bar, is estimated to consist of a continuous sequence of 75–125 bases from the mobility of the tRNA–rRNA fragment complex in denaturing gels. The dashed arrows indicate the uncertainty in the position of the cross-linked segment within the 8S fragment. The tRNA molecule is thought to be attached through its anticodon loop (Ofengand et al., 1979).

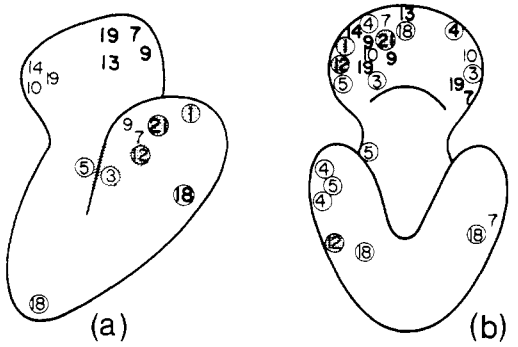

FIGURE 9: Proteins on the surface of the *E. coli* 30S ribosomal subunit that are in the vicinity of mRNA and of the 3′ segment of the 16S RNA. Location of the proteins and overall shape of the particles are from (a) Lake (1978) and Winkelmann & Kahan (1978) and (b) Brimacombe et al. (1978) as derived by immune electron microscopy. Proteins near to mRNA (circled numbers) have been identified as S1, S3, S4, S5, S12, S18, and S21 by affinity labeling (Pongs et al., 1975, 1976; Pongs & Rossner, 1976; Lührmann et al., 1976; Fiser et al., 1975, 1977; Towbin & Elson, 1978). Proteins associated with the 3′ third of the 16S RNA (plain numbers) have been identified as S1, S7, S9, S10, S12, S13, S14, S19, and S21 on the basis of protein–RNA binding studies (Zimmermann, 1974), analysis of ribonucleoprotein fragments (Morgan & Brimacombe, 1972; Rinke et al., 1977), and both chemical and photochemical cross-linking [Kenner (1973); Czernilofsky et al. (1975); Rinke et al. (1976); see also Stöffler & Wittmann (1977)]. Stippled circles represent proteins belonging to both groups. Boldface type signifies proteins on the surface toward the viewer, and light type signifies those on the surface away from the viewer. The approximate location of the m_2^6A residues near the 3′ end of the 16S RNA (Politz & Glitz, 1977) is shown by shading.

same vicinity (Figure 9). Covalent attachment of a mRNA analogue to the 5′ third of the 16S RNA (Wagner et al., 1976) suggests that this segment of the rRNA is also present in the ribosomal decoding site. Figure 9 shows in addition that both sets of marker proteins are clustered in the "upper" half of the 30S subunit in both the Lake and Stöffler models. In agreement with this localization, a recent electron microscopic visualization of the site of covalent attachment of tRNA bound to the P site implicates the same region of the 30S subunit (Keren-Zur et al., 1979).

Delineation of the decoding region of the ribosome has relied up to now on the use of mRNAs coupled to chemically reactive or photoreactive substituents which can be covalently cross-linked to nearby ribosomal components under appropriate conditions. A drawback of such techniques is that the reactive groups are usually removed at some distance from the point of functional interaction. Since most of the substituents are 10–15 Å in length, the precision with which the site in question can be located is diminished accordingly. For this reason, it is potentially more informative to employ methods that lead to direct or "zero-length" cross-linking between functionally juxtaposed molecules. Our efforts to characterize a covalent tRNA–ribosome complex, as described in the present and preceding papers (Schwartz & Ofengand, 1978; Ofengand et al., 1979), are illustrative of the latter approach. It remains now to identify precisely the cross-linked oligonucleotides and bases in both tRNA and 16S RNA by means of nucleic acid sequencing techniques, as well as to characterize the structure of the covalent link between tRNA and rRNA.

Acknowledgments

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