Conservation of RNA Sequence and Cross-Linking Ability in Ribosomes from a Higher Eukaryote: Photochemical Cross-Linking of the Anticodon of P Site Bound tRNA to the Penultimate Cytidine of the UACACACG Sequence in *Artemia salina* 18S rRNA

Jerzy Ciesiolka, Kelvin Nurse, Jonathan Klein,[‡] and James Ofengand*
Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110
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ABSTRACT: The complex of *Artemia salina* ribosomes and *Escherichia coli* acetylvalyl-tRNA could be cross-linked by irradiation with near-UV light. Cross-linking required the presence of the codon GUU, GUA being ineffective. The acetylvalyl group could be released from the cross-linked tRNA by treatment with puromycin, demonstrating that cross-linking had occurred at the P site. This was true both for pGUU- and also for poly(U_2,G)-dependent cross-linking. All of the cross-linking was to the 18S rRNA of the small ribosomal subunit. Photolysis of the cross-link at 254 nm occurred with the same kinetics as that for the known cyclobutane dimer between this tRNA and *Escherichia coli* 16S rRNA. T_1 RNase digestion of the cross-linked tRNA yielded an oligonucleotide larger in molecular weight than any from un-cross-linked rRNA or tRNA or from a prephotolyzed complex. Extended electrophoresis showed this material to consist of two oligomers of similar mobility, a faster one-third component and a slower two-thirds component. Each oligomer yielded two components on 254-nm photolysis. The slower band from each was the tRNA T_1 oligomer CACCUCCCUVACAAGp, which includes the anticodon. The faster band was the rRNA 9-mer UACACACCGp and its derivative UACACACUG. Unexpectedly, the dephosphorylated and slower moving 9-mer was derived from the faster moving dimer. Deamination of the penultimate C to U is probably due to cyclobutane dimer formation and was evidence for that nucleotide being the site of cross-linking. Direct confirmation of the cross-linking site was obtained by "Z"-gel analysis [Ehresmann, C., & Ofengand, J. (1984) *Biochemistry 23*, 438–445]. This analysis also showed that the cross-linked tRNA nucleotide was the V base and that <1% of the cross-linking could have involved the 5′-adjacent cytidine of the rRNA oligonucleotide. The cross-linking properties and sites of cross-linking are the same as those found previously for *Escherichia coli* and yeast ribosomes. Thus, this work now extends those results from prokaryote to higher eukaryotes. The close contact between the middle of an almost completely conserved sequence loop of rRNA and the anticodon of tRNA must have considerable functional importance to be preserved over such a divergent range of species.

It is now well established that the anticodon loop of tRNA at the P site of *Escherichia coli* ribosomes can make a unique contact with a specific nucleotide of the 16S rRNA (Ofengand et al., 1979). Upon irradiation of certain tRNAs, this contact results in cross-linking by formation of a cyclobutane dimer (Ofengand & Liou, 1980) between the 5′-anticodon base of the tRNA (cmo^{5}U-34)[1] and C-1400 of the 16S *E. coli* rRNA (Prince et al., 1982; Ehresmann et al., 1984). Analogous cross-linking occurs with yeast ribosomes (Ofengand et al., 1982), and the nucleotide of rRNA equivalent to C-1400 (C-1626) is the only residue to be cross-linked (Ehresmann et al., 1984; Ehresmann & Ofengand, 1984).

The cross-linking site occurs in the center of a 16-nucleotide sequence which has been virtually completely conserved in all species so far studied (Ofengand et al., 1984). This conserved region is believed to be single-stranded in the ribosome (Noller & Woese, 1981; Stiegler et al., 1981a; Zwieb et al., 1981), to be near the surface of the 30S subunit (Herr et al., 1979; Stiegler et al., 1981b; Vassilenko et al., 1981), and to lie at the interface between 50S and 30S subunits (Herr et al., 1979; Vassilenko et al., 1981). No function has been so far assigned to this region, and no ribosomal proteins are known to have any interaction with it (Brimacombe et al., 1983). It seems highly unlikely, however, that such strong sequence conservation, coupled with the functional conservation exemplified by the ability of both *E. coli* and yeast to cross-link at the same residue, would have been retained if it did not have some important role in ribosome structure or function.

In order to extend these observations, we now report that *Artemia salina* ribosomes can also be cross-linked to P site bound tRNA. Cross-link formation has the same codon dependencies and photolysis kinetics as does cross-linking to *E. coli* or yeast ribosomes, and the equivalent residue of rRNA is cross-linked. This contact site therefore appears to be common to tRNA–ribosome complexes of species ranging from prokaryotes to higher eukaryotes.

Materials and Methods

Materials. *E. coli* and yeast ribosomes, *E. coli* AcVal-tRNA, poly(U_2,G), poly(C_2,A), pGUU, and GUA were obtained as previously described (Ofengand et al., 1982). Poly(A) was from Miles Laboratories. *A. salina* ribosomes from undeveloped embryos were prepared according to Zasloff & Ochoa (1971). Buffer A contained 20 mM Mes, pH 6.0,

[‡] Present address: Columbia University, New York, NY.

[1] Abbreviations: cmo^{5}U-34, 5-(carboxymethoxy)uridine located at position 34 in $tRNA_1^{Val}$; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BB, bromphenol blue; XC, xylene cyanol FF; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; AcVal, acetylvalyl; PNK, T_4 polynucleotide kinase; TEAB, triethylammonium bicarbonate.

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100 mM NH_4Cl, 0.5 mM $Mg(OAc)_2$, 3 mM EDTA, and 0.1% SDS. RNase T_1 was from Sankyo, and RNase CL_3 was from BRL. RNases *Bacillus cereus*, Phy M, U_2, and T_4 polynucleotide kinase (PNK) were from P-L Biochemicals. Snake venom phosphodiesterase was from Worthington, and rye grass 3′-nucleotidase type IV was from Sigma. [γ-^{32}P]ATP was from Amersham.

Isolation of 5′-Labeled RNase T_1 Dimer Fragment. Cross-linked tRNA–rRNA covalent dimer was obtained by phenol extraction of 40S ribosomal subunits (27% cross-linking), isolated as in Figure 3. The ribosomes were freed of sucrose by precipitation with an equal volume of EtOH and redissolved in 1 mL of 0.1 M LiCl, 20 mM Hepes, pH 7.5, and 0.1% SDS. RNA was extracted with an equal volume of redistilled phenol equilibrated with 20 mM Hepes, pH 7.5, and 10 mM mercaptoethanol. After the phenol phase was washed, the pooled aqueous phases were precipitated with 2% KOAc (pH 5)–70% EtOH at −20 °C, dissolved in 2% KOAc (pH 5), and precipitated with 70% EtOH. The precipitate, dissolved in water, was stored at −20 °C. Reference 18S rRNA was obtained in the same way.

RNase T_1 digestion was performed in 25 mM Tris-HCl, pH 8.0, with 300–700 nM RNA and 5.5 Sankyo units of enzyme per picomole of RNA. Before addition of the enzyme, the RNA was denatured at 90 °C for 1 min and then quenched at 0 °C. Digestion was for 2–3 h at 37 °C. 5′-end labeling was done by making a 2-fold dilution of the RNase T_1 digest to contain 25 mM Tris-HCl, pH 8.0, 10 mM Mg^{2+}, 6 mM 2-mercaptoethanol, 50–200 μM [γ-^{32}P]ATP, and 100 units/mL PNK. Before addition of ATP and PNK, the RNA fragments were denatured at 90 °C for 1 min. Incubation was at 37 °C for 2–3 h. The solution was made 8 M in urea, bromophenol blue and xylene cyanol FF were added, and the mixture was electrophoresed at 600 V for 8 h at 23 °C in a 20% polyacrylamide gel. The radioactive T_1 dimer band was located by autoradiography, excised, and repurified by electrophoresis. For the experiments of Figures 7, 9, and 10, a two-step labeling procedure was used to obtain a high yield at high specific activity without the use of excessive [γ-^{32}P]-ATP. In the first step, after digestion of a 730 nM solution of the RNA–tRNA mixture, 70 units/mL PNK and 900 nM [γ-^{32}P]ATP were added, and the solution was incubated at 37 °C for 45 min; 10% of the dimer was labeled. After electrophoresis, the dimer band was localized by autoradiography and excised along with an equal-sized area immediately behind the labeled band, where the putative unlabeled dimer was expected. After elution of the gel, the eluate was diluted 5-fold with water and passed over a 0.5 × 0.5 cm column of DEAE-cellulose equilibrated in 0.1 M triethylammonium bicarbonate (TEAB), pH 8.0. After being washed with the same buffer, the dimer was eluted with ca. 0.5 mL of 1 M TEAB, pH 8.0. Buffer salts were removed by repeated evaporation to dryness in vacuo. Relabeling was accomplished by incubating 25 mM Tris, pH 8.0, 10 mM Mg^{2+}, 6 mM 2-mercaptoethanol, 950 nM [γ-^{32}P]ATP, and 160 nm 5′ ends at 37 °C for 60 min; 88% labeling was obtained. This material was isolated and repurified by electrophoresis as described above.

Isolation of Subfragments by Photolysis of T_1 Dimer. The dimer band either was photolyzed directly in the gel or was first eluted and then photolyzed. After location by autoradiography, the gel slice was placed in a quartz tube, positioned in the center of a Rayonet RPR-100 photochemical reactor equipped with four 254-nm lamps, and irradiated for 7 min at 23 °C. The gel piece was then polymerized onto a second 20% gel and electrophoresed at 600 V for 90 h. After autoradiography, the oligonucleotides were eluted from the gel and recovered by precipitation with 0.3 M NaOAc (pH 6.0), 0.5–2 A_{260} units of carrier tRNA, and 3 volumes of EtOH at −70 °C. The precipitate was dissolved in water and stored at −20 °C. In some experiments, the T_1 dimer was eluted from the gel before photolysis for 7 min as described above. The oligonucleotides were precipitated, dissolved in 8 M urea, and electrophoresed. The subfragments were located, eluted, and recovered by EtOH precipitation as above.

General Methods. Ribosomal binding, irradiation, and assay for cross-linking by filtration were performed as described previously (Ofengand et al., 1982). Codon-independent reaction in the presence of poly(C_2,A) or poly(A) and zero-time values for cross-linking have been subtracted. *A. salina* ribosomes were used at 6 A_{260} units/mL of reaction mixture. One A_{260} unit was assumed equal to 25 pmol of *E. coli* 70S ribosomes, 20 pmol of yeast or *A. salina* 80S ribosomes, and 54 pmol of *A. salina* 40S subunits or 18S rRNA. Separation of cross-linked *A. salina* 80S ribosomes into subunits was performed by loading 1.3-mL reaction mixtures directly on a 10–30% isokinetic sucrose gradient containing 0.7 M KCl, 50 mM Tris, pH 7.5, 11 mM Mg^{2+}, and 2 mM dithiothreitol. Centrifugation at 4 °C was in an SW40 rotor at 28 400 rpm for 15 h. Non-cross-linked reference 40S ribosomal subunits were prepared identically, including irradiation, except that pGUU and AcVal-tRNA were omitted from the incubations. Electrophoresis was carried out on polyacrylamide slab gels as described by Ehresmann et al. (1984). Preparative gels were 29 × 14 × 0.15 cm, and sequencing gels were 30 × 40 × 0.04 cm. Bands were located by autoradiography on Kodak XAR-5 film using intensifying screens. The selected oligomers were eluted from the gel with 0.3–0.5 mL of 0.5 M NH_4OAc, pH 7.0, 0.1% SDS, 10 mM Mg^{2+}, and 0.1 mM EDTA (Tanaka et al., 1980) at 37 °C for 4 h. Two to three elutions were normally sufficient for >80% recovery.

Sequencing Procedure. Enzymatic cleavage methods were used to sequence the 5′-labeled oligonucleotides. The procedures were as described by Ehresmann et al. (1984) except that 10-μL volumes and 2 μg of unlabeled tRNA were used and the following enzyme to substrate ratios (units/μg) were employed: U_2, 1.0; Phy M, 0.25 and 0.5; *B. cereus*, 0.5; CL_3, 1.0. Before addition of enzyme, the mixture was heated at 90 °C for 1 min and then quenched in ice to denature the RNA fragment. Electrophoresis in 25% gels was at 900 V for 12 h. Nucleotide length determination and 5′-nucleotide analysis were as described previously (Ehresmann & Ofengand, 1984).

Results and Discussion

Properties of the Cross-Linking Reaction. Irradiation of complexes of *E. coli* AcVal-tRNA with *A. salina* ribosomes led to cross-linking which was almost as efficient in both rate and yield as that to *E. coli* ribosomes, even though the binding efficiency of the *A. salina* ribosomes was only 64% that of *E. coli* (Figure 1A). On the other hand, the *A. salina* ribosomes were more effective in both binding and cross-linking than yeast ribosomes. The codon dependence of cross-linking is shown in Figure 1B. In agreement with previous findings with *E. coli* (Ofengand & Liou, 1981) and yeast (Ofengand et al., 1982) ribosomes, cross-linking occurred with pGUU as codon but not with GUA, although both codons supported binding almost equally as well. The cross-linking yield with poly(U_2,G) was more like that with *E. coli* than with yeast ribosomes.

The ribosomal site of cross-linking was shown by treatment of the cross-linked tRNA with puromycin, reactivity being

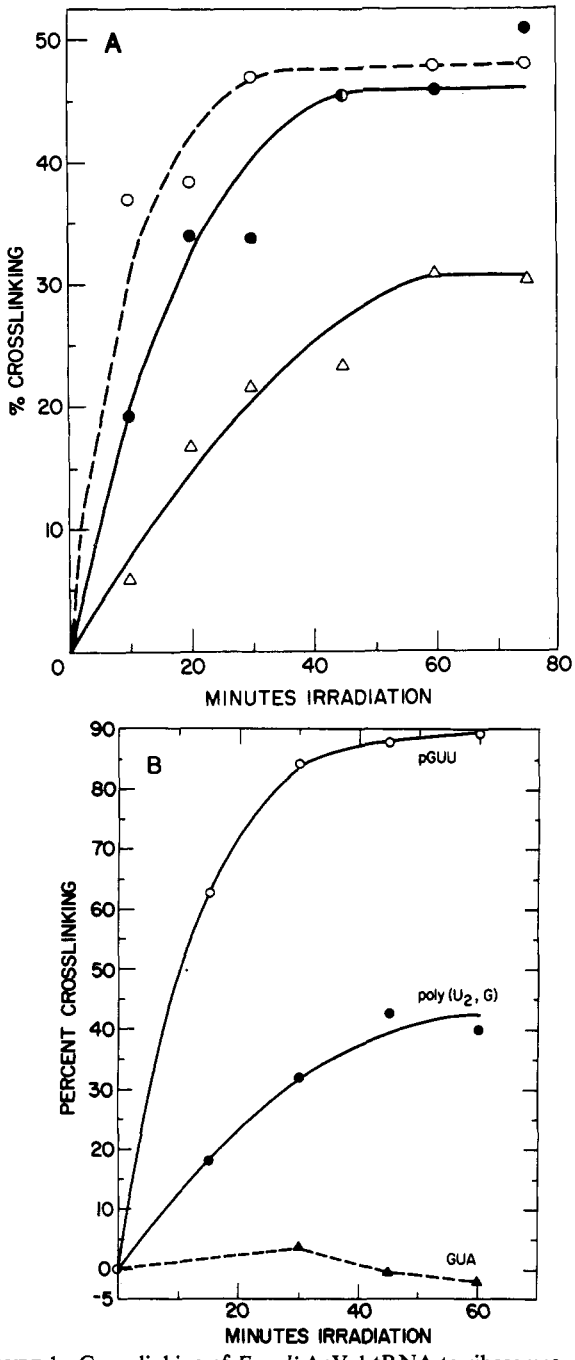

FIGURE 1: Cross-linking of *E. coli* AcVal-tRNA to ribosomes. (A) Cross-linking to ribosomes from different species. Ribosomal P site binding and cross-linking in the presence of 50 μg/mL poly(U_2,G) were carried out at 7.5 mM Mg^{2+} for 20 min at 37 °C. Binding efficiency (equivalent to 100% cross-linking) was 0.28, 0.12, and 0.44 pmol of tRNA/pmol of ribosome for *A. salina* (●), yeast (△), and *E. coli* (○), respectively. (B) Cross-linking to *A. salina* ribosomes in the presence of different codons. Reaction was as in panel A except that 15 mM Mg^{2+} was used with 60 μM pGUU or GUA. 100% cross-linking corresponds to 0.29, 0.23, and 0.22 pmol of tRNA/pmol of ribosome for pGUU (○), GUA (△), and poly(U_2,G) (●), respectively.

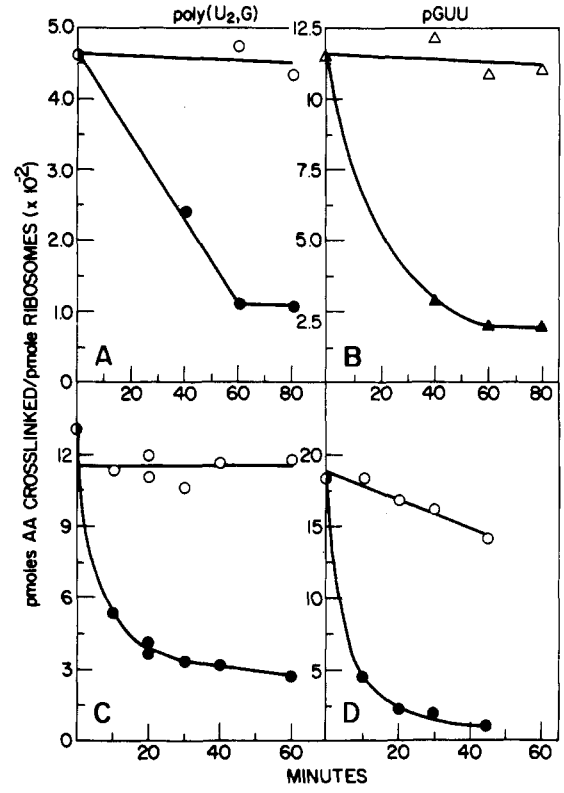

FIGURE 2: Puromycin reactivity of cross-linked tRNA. Cross-linking and puromycin treatment were as described under Materials and Methods except that the reaction was performed at 25 mM Mg^{2+} (panels A and B), 7 mM Mg^{2+} (panel C), and 15 mM Mg^{2+} (panel D) and irradiation was for 45 min. For panels A and B, 1.8 mM puromycin was added to the reaction mixture, and incubation was at 37 °C. For panels C and D, 2.3 mM puromycin was added to a reaction mixture diluted 2-fold with reaction buffer lacking Mg^{2+}, and incubation was at 25 °C. Panels A and B, yeast ribosomes; panels C and D, *A. salina* ribosomes. Panels A and C, 50 μg/mL poly(U_2,G); panels B and D, 60 μM pGUU. Minus puromycin, open symbols; plus puromycin, closed symbols. Noncovalent binding was 0.18, 0.13, 0.25, and 0.21 pmol of tRNA/pmol of ribosomes for panels A, B, C, and D, respectively.

taken as an indication of P site occupancy. The same test has been used previously for both *E. coli* and yeast ribosomes (Ofengand et al., 1979, 1982). The results of such an experiment using both poly(U_2,G) and pGUU as codons are shown in Figure 2. In panels A and B, cross-linked yeast ribosomes were used as a control. Panels C and D show the results with *A. salina* ribosomes. Clearly, the acetyl[^{3}H]valine cross-linked to ribosomes in the presence of either poly(U_2,G) or pGUU is releasable by puromycin treatment. Thus, at least 80% of the cross-linking takes place at the P site.

Localization of the Site of Ribosomal Cross-Linking. Previous work with *E. coli* and yeast ribosomes has shown that this cross-link is exclusively to the 16S or 18S rRNA component of the ribosome. Similar results were obtained for *A. salina* ribosomes. Figure 3 shows that all of the cross-linking was to the 40S subunit, with no detectable cross-linking to the 60S subunit, while Figure 4 shows that >92% of the codon-dependent cross-linking was to 18S rRNA. In the latter experiment, cross-linked ribosomal complexes free of non-cross-linked tRNA were prepared by gel filtration (Figure 4A) before disruption with SDS and centrifugation on SDS–sucrose gradients. Although the radioactivity peak was rather broad, it was clearly centered on the 18S RNA peak detected by UV absorption and did not overlap with the 28S RNA peak. A small amount of radioactivity was detected at the top of the gradient. When the amount found in the presence of poly(C), used as a codon-dependence control, was subtracted, the residual radioactivity amounted to >8% of the total. This could be due to some cross-linking to 40S protein but is more likely a result of some deacylation of the AcVal-tRNA or of nuclease cleavage of the 18S rRNA–tRNA complex.

Structure of the Cross-Link. The cross-link in *E. coli* and yeast was shown to be a pyrimidine–pyrimidine cyclobutane dimer (Ofengand & Liou, 1980; Ofengand et al., 1982). One property of such dimers is photoreversal by 254-nm light with

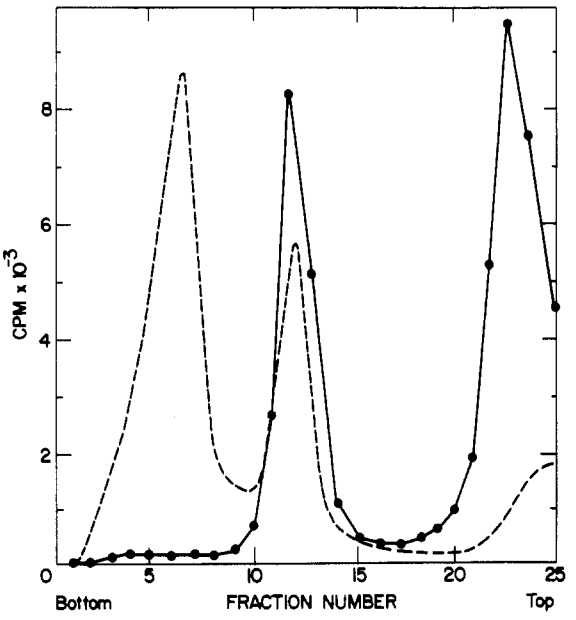

FIGURE 3: Subunit localization of the site of cross-linking to *A. salina* ribosomes. Binding at 15 mM Mg^{2+} in the presence of 60 μM pGUU was performed at 240 nM *A. salina* ribosomes and 200 nM *E. coli* Ac[^{3}H]Val-tRNA. Cross-linking was at 0 °C for 45 min. The ribosomes were separated into subunits by sucrose gradient centrifugation. ^{3}H, solid circles; A_{260}, dashed line.

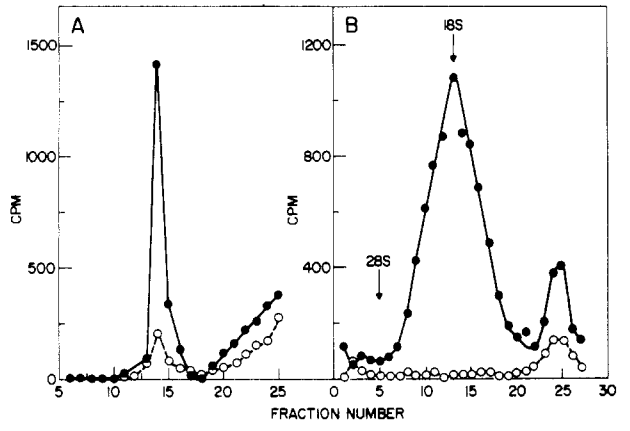

FIGURE 4: Cross-linked component in *A. salina* 40S ribosomal subunit. (A) Separation of cross-linked from non-cross-linked tRNA by gel filtration. Binding and cross-linking were done in the presence of 60 μM pGUU (●) or 60 μg/mL poly(C) (○) at 25 mM Mg^{2+}. Of the bound tRNA, 82% was cross-linked. The reaction mixture (450 μL) was placed on a 0.9 × 52 cm column of Sephadex G-100 equilibrated with 25 mM Hepes, pH 7.5, 25 mM KOAc, and 0.1 mM $Mg(OAc)_2$ at 4 °C. One-milliliter fractions were collected at 0.4 mL/min. (B) SDS–sucrose gradient centrifugation analysis of the cross-linked complex. The ribosome-containing regions of the eluate in panel A (fractions 13–16) were pooled, precipitated with 2% KOAc–67% EtOH, dissolved in 0.5 mL of buffer A, incubated at 37 °C for 5 min, and then centrifuged at 15 °C through a 10–30% isokinetic sucrose gradient in buffer A plus 100 mM LiCl in an SW40 rotor at 38 000 rpm for 15 h. The locations of the 28S and 18S rRNA peaks (shown by arrows) were determined by A_{260} absorption.

defined kinetics. This was shown previously by comparing photolysis of the *E. coli* and yeast complexes (Ofengand et al., 1982). Figure 5 shows the kinetics of 254-nm photolysis of *A. salina* and *E. coli* complexes. The rates of photolysis were the same with a rate constant of 0.80 min^{-1} under these conditions. This compares favorably with a value of 0.65 min^{-1} obtained previously under similar conditions (Ofengand et al., 1982). We conclude that the *A. salina* cross-link is also a cyclobutane dimer.

Identification of the Cross-Linked rRNA Nucleotide. Since

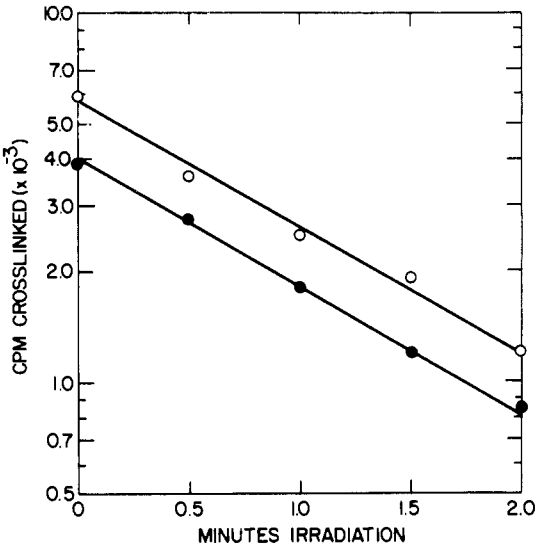

FIGURE 5: Photolysis kinetics of the cross-linked product. Cross-linked tRNA–ribosomes were prepared in the presence of pGUU at 15 mM Mg^{2+} by irradiation for 75 min. The reaction mixtures were diluted in reaction buffer minus Mg^{2+} to a ribosome A_{260} value of 0.36 and a concentration of 0.38 mM Mg^{2+}. They were then photolyzed at 254 nm and assayed as described previously (Ofengand et al., 1982). *A. salina* ribosomes, open circles; *E. coli* ribosomes, closed circles. $T_{1/2}$, 0.87 min; rate constant, 0.80 min^{-1}.

previous work from our laboratory had established that the tRNA cross-linking base in both *E. coli* and yeast complexes was cmo^{5}U-34 (Ofengand et al., 1979; Prince et al., 1982; Ehresmann & Ofengand, 1984), a complete RNase T_1 digestion of the cross-linked complex was expected to produce an RNA 15-mer linked to an rRNA oligomer. Such a complex should be larger than any 18S rRNA T_1 oligomer, on the basis of our prior experience with *E. coli* and yeast rRNA. As shown in Figure 6, this expectation was realized. After complete RNase T_1 digestion of the cross-linked tRNA–rRNA, and 5′-end labeling, electrophoresis (lane XL) revealed an oligomer of slower mobility (arrow) than the bulk of the reference rRNA oligomers (lane R) and different in position from any of the reference RNA band. Moreover, if the digest was photolyzed at 254 nm before electrophoresis, this new band disappeared (data not shown). Upon excision of the band, photolysis, and reelectrophoresis (lane D), the original band disappeared and three faster moving bands (T, R1, and R2) appeared.

As shown in Figure 7, R1 and R2 arise from two different dimer bands with closely similar mobilities (lanes 1 and 2), the differences in mobility only being noticeable when the isolated dimer band was electrophoresed much further during repurification. Each dimer band was associated with only one of the two R bands, but both contained the apparently identical T band (lanes 3 and 4). The greater amount of R2 in D1 as compared to R1 in D2 is in line with the greater contamination of D1 by D2 as expected from their relative positions on the purification gel (not shown). Unexpectedly, the faster dimer D1 yielded the slower monomer R1 and vice versa. Since R1 and R2 are derived from the same oligonucleotide (see below), various methods were tried to convert one into the other. Neither additional UV photolysis nor T_1 RNase digestion had any effect, but R2 could be converted into R1 by treatment with either rye grass 3′-nucleotidase (Shuster & Kaplan, 1955) or PNK under 3′-phosphatase incubation conditions (Cameron & Uhlenbeck, 1977) (data not shown). R1 was unaffected by phosphatase treatment. R2 and R1 could be further distinguished by treatment with snake venom phosphodiesterase.

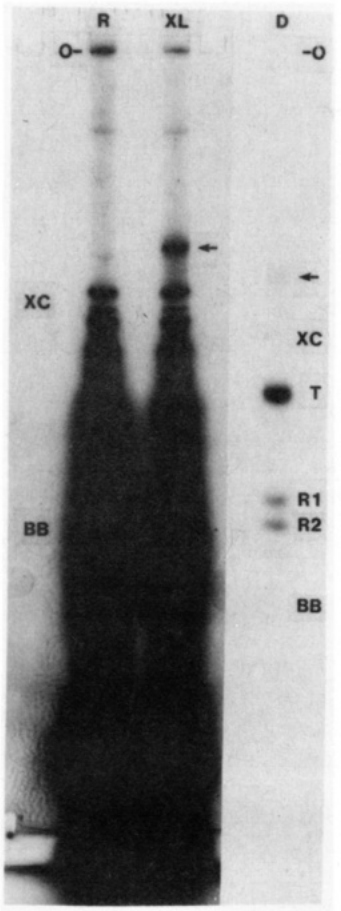

FIGURE 6: Detection of the cross-linked RNase T_1 oligonucleotide and its subfragments on polyacrylamide gels. The complete RNase T_1 digest of cross-linked (lane XL) and reference (lane R) 16S RNA was 5′-^{32}P labeled and electrophoresed on a 20% gel. The cross-linked dimer band (arrow) was eluted, photolyzed, and reelectrophoresed (lane D). The arrow marks the location of the unphotolyzed dimer, T is the tRNA-derived oligonucleotide, and R1 and R2 are the 16S RNA derived oligomers. XC and BB mark the location of the tracking dyes xylene cyanol FF and bromphenol blue, respectively, and O marks the origin.

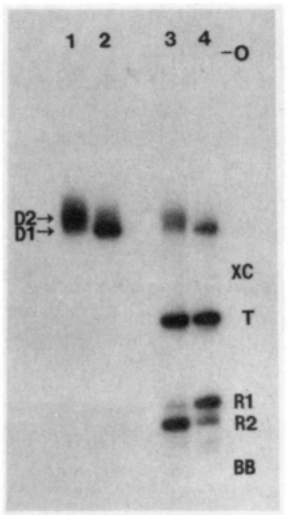

FIGURE 7: Derivation of R1 and R2 from different dimer bands. Cross-linked dimer like that of Figure 6 was isolated, relabeled, and purified as described under Materials and Methods. Long electrophoresis yielded a slower (D2) component and a faster (D1) component in a 1:2 ratio. Lanes 1 and 2, D2 and D1, respectively, before photolysis. Lanes 3 and 4, D2 and D1, respectively, after UV photolysis. T, R1, R2, XC, BB, and O are as in Figure 6.

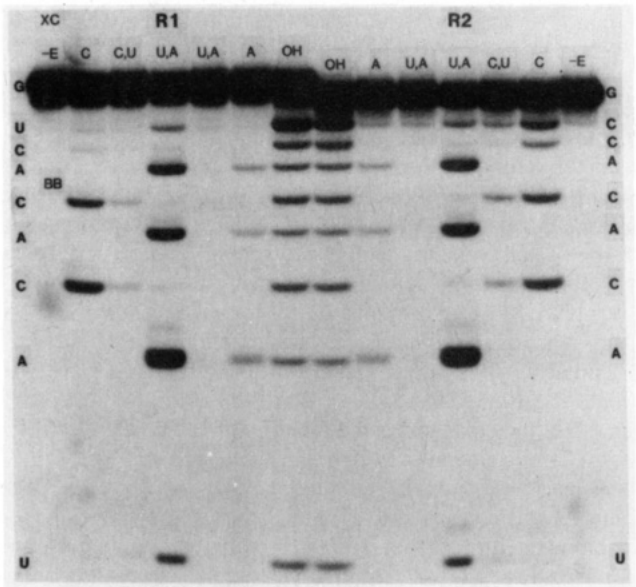

FIGURE 8: Sequence analysis of R1 and R2 on polyacrylamide gels. Enzymatic sequencing was performed as described under Materials and Methods: (−E) no enzyme; (C) CL3; (U,C) *B. cereus*; (A,U) Phy M; (A) U_2; (OH) limited alkaline digestion. XC and BB denote the positions of the tracking dyes. The deduced sequence for each oligomer is also shown.

R2 was resistant, as expected for a 3′-phosphorylated oligomer, while R1 was readily digested, showing that the 3′-terminus was not esterified. Presumably, the 3′-phosphate activity of PNK was responsible for the partial removal of the 3′-phosphate from the R moiety of the dimer. It is not clear, however, why the T fragment was apparently unaffected and why the dephosphorylated dimer D1 had a slightly greater electrophoretic mobility than the phosphorylated dimer D2 when the reverse would have been expected.

All three of the monomer bands (T, R1, and R2) were sequenced by partial nuclease digestion. Band T corresponded to the tRNA T_1 15-mer containing cmo^{5}U-34 (data not shown). The sequencing analysis of R1 and R2 is illustrated in Figure 8. Although R1 and R2 were quite distinct on the 20% gel (Figures 6 and 7), the 25% sequencing gel showed less of a mobility difference between the uncleaved oligomers. The sequences of the two oligomers were virtually identical, and completely so for the first seven residues. As no such sequence exists in *E. coli* $tRNA_1^{Val}$, but does occur in all small subunit rRNAs examined (Ofengand et al., 1984), these two bands must come from the rRNA.

Although the sequence of *A. salina* 18S rRNA is not known, sequencing of *Saccharomyces cerevisiae* (Rubtsov et al., 1980), *Xenopus laevus* (Salim & Maden, 1981), *Dictyostelium discoideum* (McCarroll et al., 1983), rat (Torczynski et al., 1983; Chan et al., 1984), mouse (Raynal et al., 1984), and rabbit (Connaughton et al., 1984) 18S rRNA reveals that the sequence UACACAC occurs only once in these rRNAs as part of the longer sequence GUACACACCGCCCGUG. This 16-mer has been detected, with only minor variations, in every small subunit rRNA examined so far (Ofengand et al., 1984). Even the shorter sequence, UACACA, only occurs once in the sequenced eukaryotic 18S rRNAs and does not occur at all in the sequences of *S. cerevisiae* (Georgiev et al., 1981), *Saccharomyces carlsbergensis* (Veldman et al., 1981), *X. laevus* (Ware et al., 1981), *Physarum polycephalum* (Otsuka et al., 1983), rat (Chan et al., 1983; Hadjiolov et al., 1984), and mouse (Hassouna et al., 1984) large subunit rRNA. Interestingly enough, UACACA *is* found in intron 1 and in the 3′-flanking sequence of the *P. polycephalum* 26S rRNA

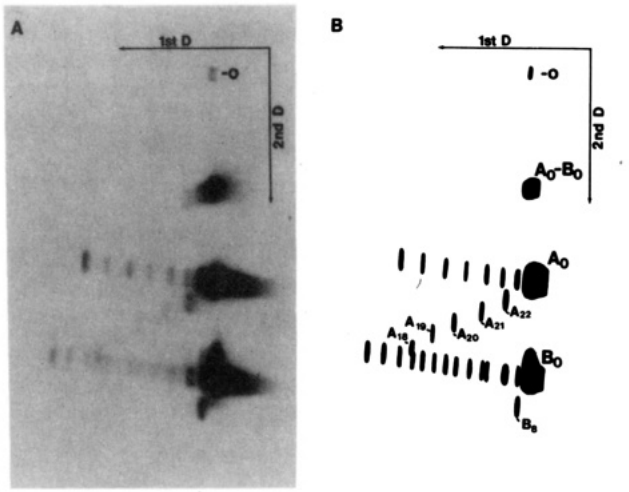

FIGURE 9: "Z"-gel analysis of the covalent *E. coli* $tRNA^{Val}$–*A. salina* 18S RNA oligonucleotide dimer. After random digestion of the dimer with alkali, the first dimension was run until the BB dye had migrated 36 cm. After photoreversal of the cross-link, the second dimension was run until the BB dye had moved 17 cm. The cleavages, fragment numbers, and procedures were as described by Ehresmann & Ofengand (1984). (A) Autoradiogram; (B) key diagram.

gene. This single occurrence in organisms which span the eukaryotic kingdom from yeast through slime molds in amphibians to mammals is likely to be true for *A. salina* also. Thus, despite the slight apparent ambiguity at the 3′ end of the sequencing gels of Figure 7, R1 should correspond to UACACACUG, R2 should correspond to UACACACCG, and R1 should be derived from R2 by conversion of the 3′-penultimate C to U. Such a deamination is readily explained if, by analogy to the situation in *E. coli* and yeast, that residue is the site of cyclobutane dimer formation since saturation of the cytidine ring is known to markedly increase the rate of deamination (Liu & Yang, 1978; Green & Cohen, 1957). Nevertheless, it is surprising that deamination should be associated only with that dimer band whose rRNA moiety is dephosphorylated at the 3′ end. Although this anomaly may be related to the unexpected mobility of D1 vs. D2 discussed above, the connection is by no means clear.

While these features remain to be explained, the fact that only C_8 was converted to U, but not C_3, C_5, or C_7, provides stron inferential evidence that cross-linking occurred at that residue. The failure to detect U cleavage at the other cytidine sites, coupled with the almost complete conversion of C_8 to U_8, further indicates that *only* that residue was cross-linked.

Confirmation of the Cross-Linked Nucleotides by Diagonal Gel Analysis. A technique involving random alkaline hydrolysis of two cross-linked oligonucleotides, electrophoretic separation in one dimension, cleavage of the cross-link, and second-dimension electrophoresis has been described by Ehresmann & Ofengand (1984). It was used for analysis of the site of cross-linking in the *E. coli* and yeast systems. The method has the advantage over most other procedures in being able to detect even minor amounts of cross-linking to other sites. Application to the *A. salina* system is shown in Figure 9.

The autoradiogram is shown on the left, and the key diagram is on the right. In this analysis, the number of diagonal bands, A_{18}–A_{22} and B_8 in the figure, indicates the number of residues from the 3′ end of the tRNA oligomer (A) or rRNA oligomer (B) to the cross-link (Ehresmann & Ofengand, 1984). Since five A bands from the tRNA oligomer were detected, the cross-linking nucleotide cannot be more than six nucleotides from the 3′ end. As no 10-mer or greater was found along

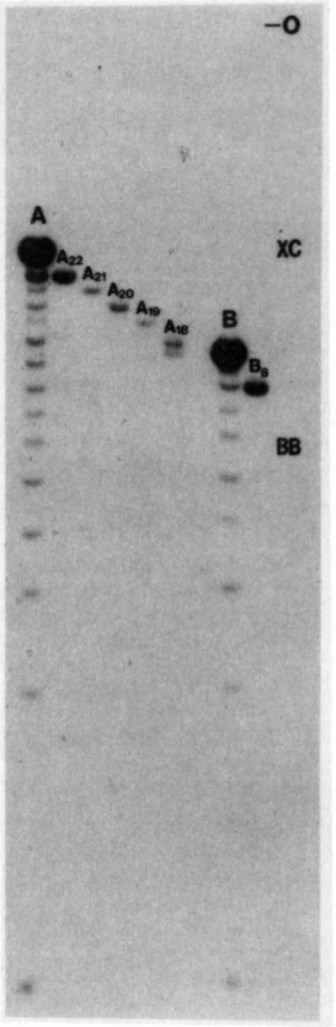

FIGURE 10: Nucleotide length determination of products derived from the gel shown in Figure 9. Bands A_0 and B_0 were digested with alkali to generate the reference ladders. Electrophoresis was on 0.4-mm 25% polyacrylamide gels. O, BB, and XC were as in Figure 6.

the diagonal (data not shown), only the tenth nucleotide was cross-linked, in agreement with previous results obtained with *E. coli* or yeast ribosomes. The finding of only one B band from the rRNA oligomer, despite extensive overexposure of the autoradiogram, shows conclusively that cross-linking is only to the 3′-terminal G or 3′-penultimate C residue. Since the cross-link is known to be a pyrimidine–pyrimidine cyclobutane dimer (see above), it must be to the 3′-penultimate C only. In order to be sure that no other bands were hidden or displaced, it was necessary to confirm the expected length of each of the oligomers A_{18}–A_{22} and B_8 as well as their origin. The origin was simply determined by 5′-nucleotide analysis since the rRNA fragment ends in pU while the tRNA fragment ends in pC. B_8 contained only pU. Length was determined by sizing against a reference ladder generated from the uncleaved fragment B_0. As shown in Figure 10, B_8 was indeed eight nucleotides long. A_{18}–A_{22} were also the expected length. The sum of these results conclusively shows that cross-linking in *A. salina* 16S RNA is to the same single cytidine residue which corresponds to C-1400 in *E. coli* or C-1626 in yeast, and to no other site.

CONCLUSIONS

The preservation of the sequence of this 16-nucleotide segment of the small subunit rRNA in all species so far studied strongly implies some important role for this region in ribosome

structure or function. The functional constancy implied by the ability to cross-link the anticodon of tRNA to the same nucleotide, and only that nucleotide, in species as disparate as *E. coli* and *A. salina* reinforces the view that this sequence of the rRNA is importantly involved in some ribosomal function, such as tRNA binding and/or codon recognition. The close approach of a conserved sequence loop of rRNA to the anticodon of tRNA which is shown by these cross-linking results may well be a universal characteristic of the protein synthesis machinery.

ADDED IN PROOF

After the manuscript was submitted, the sequence of *A. salina* 18S RNA was reported (Nelles et al., 1984). It confirms that the sequence UACACAC only occurs once as part of the conserved sequence GUACACACCGCCCGUC.

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Registry No. pGUU, 17159-38-5; poly(U_2,G), 76054-51-8; C, 71-30-7.

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