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Correct Codon–Anticodon Base Pairing at the 5′-Anticodon Position Blocks Covalent Cross-Linking between Transfer Ribonucleic Acid and 16S RNA at the Ribosomal P Site†

James Ofengand* and Richard Liou

ABSTRACT: The codon-dependent covalent cross-linking between the 5′-anticodon base of certain tRNAs bound in the ribosomal P site and a pyrimidine residue of 16S RNA is highly specific for codons which do not base pair with the 5′-anticodon base (c)mo^{5}U. Thus, with tRNA$_1^{Val}$ (anticodon cmo^{5}UAC), pGpUpU, GpUpU, and pGpU allowed high-yield (64–95%) cross-linking, and pGpUpC was about equivalent to poly(U_2,G) in allowing moderate (45–60%) cross-linking. No cross-linking occurred when the normal codon, GpUpA, or the "wobble" codon, GpUpG, was used, although, as expected, they were the best codons in noncovalent ribosomal binding. There was no cross-linking in the absence of codons. Oligonucleotide-induced cross-linking was like that induced by poly(U_2,G) in that only 16S RNA was cross-linked and the covalently bound acetylvaline could be released from the ribosome by reaction with puromycin. Thus all cross-linking occurred at the P site. The rate of photolysis at 254 nm of the cyclobutane dimer which has been shown to be the structure of the cross-link between the tRNA and 16S RNA [Ofengand, J., & Liou, R. (1980) *Biochemistry 19*, 4814–4822], was also the same, again indicating a similar or identical structure in all cases. Covalent cross-linking of AcAla-tRNA (anticodon cmo^{5}UGC) was dependent on the presence of poly(I,C,U) but was not stimulated (<15%) by poly(I,C,A). Thus the inhibitory effect of forming the base-pair cmo^{5}U-A was also found with this tRNA. These results provide the first direct evidence that codon–anticodon interaction is maintained at the ribosomal P site. They also provide evidence that positional changes occur in the anticodon loop of P-site bound tRNA upon interaction of the 5′-anticodon base with its cognate codon residue.

Previous studies from this laboratory have shown that certain tRNAs can be cross-linked to 70S ribosomes when irradiated with light >300 nm. The reaction only occurs when the correct codon is present and when the tRNA is bound in the ribosomal P site. Irradiation of A-site bound tRNA does not result in cross-linking. A tRNA with either a cmo^{5}U[1] or mo^{5}U residue at the 5′-anticodon position is required, and the cross-link is exclusively to 16S ribosomal RNA (Schwartz & Ofengand, 1978; Ofengand et al., 1979). The cross-link is directly between the tRNA and 16S RNA as neither a protein (Zimmermann et al, 1979) nor mRNA (Ofengand & Liou, 1980) linker is involved. The structure of the cross-link has been shown to by a cyclobutane dimer between the 5′-anticodon base, (c)mo^{5}U, and a pyrimidine of the 16S RNA, based upon a number of photochemical properties usually described as being characteristic of such dimers (Ofengand & Liou, 1980).

For most of these studies, poly(U_2,G) was used as mRNA, since it supplied the potential codons, GUU and GUG, and empirically proved to be effective. However, various preliminary experiments, performed with polymers of different base compositions and ratio as a source of codons in cross-linking experiments with different tRNAs, led us to suspect that the nature of the residue paired with the 5′-anticodon base of the tRNA influenced the ability of this base to cross-link to 16S RNA. In particular, the results seemed to suggest that either A or G base-pairing with (c)mo^{5}U strongly *decreased* the cross-linking efficiency. There was no reason to believe that correct codon–anticodon base pairing should negatively influence the ability to form a cyclobutane dimer since cyclobutane dimers can be formed between adjacent thymine residues in native DNA. These residues must have been base-paired to A residues on the complementary DNA strand before dimer formation occurred. This consideration suggested that some special aspect of the mRNA–ribosome–tRNA interaction in the decoding region of the P site might be responsible for the apparent codon influence on cross-linking. It seemed, therefore, worthwhile to investigate the effect in a more rigorous way by the use of defined codons. In this paper, we describe such a detailed study and its conclusions.

Experimental Procedures

Chemicals. Ac[^{3}H]Val-tRNA$_1^{Val}$ (*E. coli*), Ac[^{3}H]Val-tRNA^{Val} (*B. subtilis*), tight couple ribosomes, and poly(U_2,G) were prepared or obtained as described previously (Ofengand et al., 1979). Ac[^{3}H]Ala-tRNA (*E. coli*) was prepared by

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[1] Abbreviations used: mo^{5}U, 5-methoxyuridine; cmo^{5}U, 5-(carboxymethoxy)uridine; AcVal- and AcAla-, *N*-acetylvalyl- and *N*-acetylalanyl-, respectively; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; NaDodSO$_4$, sodium dodecyl sulfate.

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Table I: Structural Analysis of Oligonucleotides[a]

enzyme	oligonucleotide	molar ratio												
		pC	pU	pG	pA	Cp	Up	Gp	Ap	C	U	G	A	pGp
phosphodiesterase	GpUpA		0.87		0.97							1.00		
	GpUpG		0.90	1.17								1.00		
	GpUpC	1.09	0.83	0.10								1.00		
	pGpUpC	0.95	0.83	1.00								0.01		
	GpUpU		1.93									1.00		
	pGpUpU		1.57	1.00										
	pGpU		0.90	1.00										
pancreatic plus T_1 RNase	GpUpA						0.97	1.03					1.00	
	GpUpG						1.04	1.04				1.00		
	GpUpC						0.87	1.37		1.00				
	pGpUpC						1.02			1.00				1.10
	GpUpU						1.04	1.37	0.05		1.00	0.03		
	pGpUpU						0.97				1.00	0.04		1.17
	pGpU										1.00			1.00

[a] Enzymatic digestion conditions and method of analysis are given under Experimental Procedures. The molar ratio was obtained relative to the nucleoside produced either from the 5′ end (phosphodiesterase) or from the 3′ end (pancreatic plus T_1 RNase). Where no values are present, the residue was undetectable (<1%).

aminoacylation of $tRNA_1^{Ala}$ (Plenum Scientific) in 100 mM Hepes, pH 7.5, 10 mM KCl, 20 mM $Mg(OAc)_2$, 3 mM ATP, 2 mM dithiothreitol, 10 μM alanine, and enzyme at 30 °C for sufficient time to reach saturation (usually 10–15 min). Phenol extraction, acetylation, and gel filtration were done as described previously (Schwartz & Ofengand, 1978). The final acylation level was 1180 pmol/A_{260} unit. UpU, UpC, G>p (guanosine 2, 3′-cyclic phosphate), and pancreatic RNase were from Sigma; UpA was from Boehringer; and UpG, pG>p, poly(I,C,U), poly(I,C,A), and puromycin were from P.L. Biochemicals; GpUpU was from Miles Biochemicals. T_1 RNase was from Sankyo. Venom phosphodiesterase (VPH) was from Worthington. Glass-distilled methanol was from Burdick and Jackson.

Oligonucleotides. GpUpA, GpUpG, GpUpU, and GpUpC were prepared by condensing G>p with the appropriate dinucleotide by the action of T_1 RNase according to the general procedure of Grunberger et al. (1968a). Similarly, pGpUpU, pGpU, and pGpUpC were prepared by reacting pG>p with either UpU, Urd, or UpC. The conditions used for each synthesis varied: (for GpUpU) 17 μmol of G>p, 4.4 μmol of UpU, 5 Sankyo units of T_1 RNase, 60 μL of buffer (50 mM Tris, pH 7.0, and 10 mM EDTA), 18 h, 0 °C; (for GpUpA) 20 μmol of G>p, 4.4 μmol of UpA, 5 units of T_1 RNase, 110 μL of buffer, 18 h, 0 °C; (for GpUpG) 4 μmol of G>p, 4 μmol of UpG, 2 units of T_1 RNase, 104 μL of buffer, 4 h, at 0 °C; (for GpUpC) 19 μmol of G>p, 9 μmol of UpC, 10 units of T_1 RNase, 102 μL, 18 h, 0 °C; (for pGpUpU) 8 μmol of pG>p, 30 μmol of UpU, 10 units of T_1 RNase, 150 μL, 5 h, 0 °C; (for pGpU) 4 μmol of pG>p, 16 μmol of Urd, 5 units of T_1 RNase, 110 μL, 5 h, 0 °C; (for pGpUpC) 8 μmol of pG>p, 17 μmol of UpC, 10 units of T_1 RNase, 100 μL, 18 h, 0 °C. Reactions were terminated by application to Whatman 3MM paper at 4 °C and the oligonucleotides purified by chromatography and electrophoresis as described by Grunberger et al. (1968a). Yields, based on the limiting reagent, were as follows: pGpUpU, 7%; GpUpU, 5%; GpUpA, 7%; GpUpG, 4%; GpUpC, 5%; pGpU, 12%; pGpUpC, 4%. Each oligonucleotide showed the expected behavior on chromatography and electrophoresis when compared with appropriate standards. In addition, the structure of each of the oligonucleotides was confirmed directly by analysis (Table I). Concentration was calculated from A_{260} measurements in water and the known extinction coefficients for the mononucleotides. A hypochromic effect of 23% was assumed for all the oligonucleotides. This value, obtained for the commercial sample of GpUpU by total phosphate analysis, was provided by the supplier.

Sequence Analysis of Oligonucleotides. Parallel digests of the oligonucleotides were done with either snake venom phosphodiesterase or a combination of pancreatic RNase and RNase T_1. The diesterase reactions, containing approximately 0.06 A_{260} unit of oligonucleotide, 50 mM Tris, pH 9.0, 15 mM $MgCl_2$, and 2.5 μg of phosphodiesterase in 50 μL, were incubated for 10 min at 23 °C. Reaction was stopped by incubation for 2 min at 100 °C. The samples were diluted to 80 μL and adjusted to contain 50 mM potassium phosphate, pH 5.5. The pancreatic plus T_1 RNase digests, containing 0.06 A_{260} unit of oligonucleotide, 50 mM Tris, pH 7.4, 15 μg of pancreatic RNase, and 100 Sankyo units of T_1 RNase in 50 μL, were incubated for 25 min at 37 °C. Reaction was stopped by boiling, and the samples were diluted to 80 μL and adjusted to contain 9.4 mM $MgCl_2$ and 50 mM potassium phosphate, buffer, pH 5.5. The nucleotide and nucleoside compositions were quantitated by high-pressure liquid chromatography using a modification of the method of Tyson & Wickstrom (1980). After centrifugation to remove any particulate matter, 20 μL (0.015 A_{260} unit) was injected into a 4.6 × 250 mm column of Ultrasphere ODS 5-μm beads (Altex) preequilibrated with filtered (0.2-μm Sybron/Nalge disposable filter) 50 mM potassium phosphate, pH 5.5 (solvent A). The column was run at 1 mL/min and ~3000 psi. The effluent was passed through a Laboratory Data Control spectromonitor III variable-wavelength absorption detector at 260 nm. The detector output was displayed on a Soltec Model 210 recorder. After 8 min with solvent A a linear gradient was started changing from 100% solvent A to solvent B (32% methanol, 30 mM potassium phosphate, pH 5.5) at a rate of 1% min^{-1} mL^{-1}. Under these conditions, pA, pG, pU, pC, A, G, U, and C were all separated from each other, as was the mixture of pGp, Ap, Gp, Up, Cp, A, G, U, and C. The elution position was reproducible and characteristic for each component. In order of elution, the positions were (in mL) pGp, 3.1; pC, 3.1; pU, 4.0; Cp, 5.3; pG, 6.6; Up 7.4; C, 8.5; Gp, 12.0; U, 13.8; pA, 16.3; Ap 22.6; G, 23.0; A, 28.4. The dead volume of the column, 2.3 mL, has not been subtracted. In the absence of Mg^{2+} in the injected sample, the elution positions showed a marked shift as well as a continual drift upon repeated runs. Quantitation was performed by integration of the peaks and comparison with a standard curve.

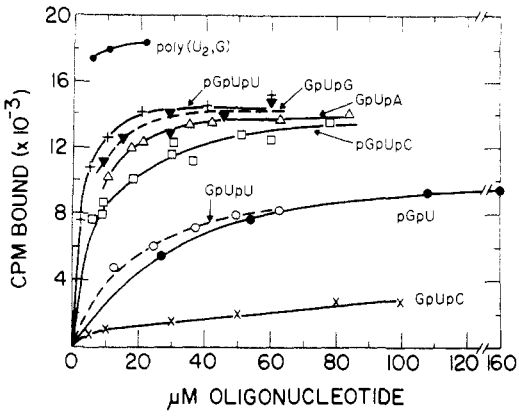

FIGURE 1: Noncovalent binding of *E. coli* AcVal-tRNA to ribosomes in the presence of different codons. Ribosomal binding at 20 mM Mg^{2+} was performed in 50-μL incubations as described under Experimental Procedures; 20000 cpm corresponds to 3.3 pmol of AcVal-tRNA bound (67% of input tRNA, 44% of ribosomes active). (+) pGpUpU, (●, upper) poly(U_2G), (▼) GpUpG, (△) GpUpA, (□) pGpUpC, (○) GpUpU, (●, lower) pGpU; (×) GpUpC. The micromolar concentration of poly(U_2,G) was calculated on the basis of its GpUpU content. Binding in the absence of added codons (3200 cpm) has been subtracted.

Ribosomal P-Site Binding. Noncovalent binding mixtures contained 50 mM Hepes, pH 7.5, 50 mM NH_4Cl, 20 mM $Mg(OAc)_2$, 6 A_{260} units/mL of tight couple ribosomes, 98 nM Ac[^{3}H]Val-tRNA, and mRNA codons as indicated. After incubation at 37 °C for 15 min, samples were assayed by cellulose nitrate membrane filtration at 20 mM Mg^{2+} (Ofengand et al., 1979). For the experiment of Figure 6, conditions were adjusted to 50 nM Ac[^{3}H]Ala-tRNA, 10 mM Mg^{2+}, and 100 μg/mL poly(I,C,U) or poly(I,C,A). For the AcVal-tRNA control for Figure 6, the conditions were 125 nm AcVal-tRNA (*E. coli*), 7 mM Mg^{2+}, and 20 μg/mL poly(U_2,G). Polynucleotide was saturating in each case.

Cross-Linking Assay. Ribosomal P-site binding mixtures containing poly- or oligonucleotide codons as specified in each experiment were incubated for 15 min at 37 °C and then irradiated for the indicated times at 0 °C in the Rayonet RPR photoreactor equipped with 300-nm lamps plus a Mylar plastic filter (Ofengand et al., 1979). The samples were assayed by membrane filtration at 20 or 0.1 mM Mg^{2+} and percent cross-linking was calculated, all as previously described (Ofengand et al., 1979).

Irradiation at 254 nm. Samples were irradiated in the Rayonet RPR-100 photochemical reactor by using a single 254-nm lamp (instead of the usual 16). The sample was contained in a 12-mm i.d. quartz tube mounted in the center of the reactor and irradiated at room temperature. No heating occurred at the low light fluences used. The sample was stirred with a magnetic flea during irradiation, and the tube was masked off at the liquid meniscus. In this configuration, the number of photons incident on the sample, 0.22 μeinstein min^{-1} mL^{-1}, was proportional to volume as well as to irradiation time. The light flux was determined by potassium ferrioxalate actinometry (Jagger, 1967).

Results

Codon Dependence of Noncovalent Binding. For investigation of the effect of base pairing at the 5′-anticodon position of P site bound tRNA, it was necessary to establish the optimal noncovalent binding conditions. Figure 1 shows the effect of the concentration of the different codons used in this work on the ability of *E. coli* AcVal-tRNA to bind to ribosomes at 20 mM Mg^{2+}. As expected from the anticodon, cmo^{5}UAC, GpUpG, and GpUpA were excellent codons. GpUpU was less

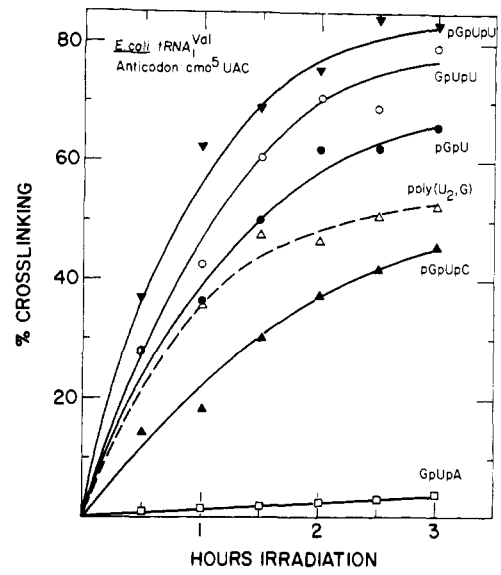

FIGURE 2: Covalent cross-linking of *E. coli* AcVal-tRNA (anticodon cmo^{5}UAC) to ribosomes in the presence of different codons. Noncovalent binding and cross-linking were performed and assayed as given under Experimental Procedures. (▼) 31 μM pGpUpU, (○) 50 μM GpUpU, (●) 54 μM pGpU, (△) 25 μM (10 μg/mL) poly(U_2,G), (▲) 30 μM pGpUpC, (□) 35 μM GpUpA.

effective, and GpUpC was essentially inactive. These results agree with earlier studies (Kellogg et al., 1966). Note, however, that adding a 5′-phosphate to GpUpU or GpUpC made it a much more active codon and that pGpU was now as active as GpUpU, whereas GpU was reported to be unable to stimulate tRNA binding (Grunberger et al., 1968b; Holy et al., 1970). This effect of 5′-phosphorylation of codons is well-known [see Grunberger et al. (1968b) for earlier references) and has been studied in detail by Grunberger and his colleagues (Grunberger et al., 1968b; Holy et al., 1970). The only difference from our work is that we found GpUpU and pGpU to be about equally active while Holy et al. (1970) consistently found GpUpU to be 2.5 times more active. This may be due to the fact that these authors used a mixture of $tRNA_1^{Val}$ and $tRNA_2^{Val}$ while we have used only the $tRNA_1^{Val}$ species. $tRNA_2^{Val}$ responds to GpUpC and GpUpU codons. If this tRNA fails to respond to 5′-phosphorylation, like $tRNA^{Asp}$ and $tRNA^{Glu}$ (Grunberger et al. 1968b), it could account for the greater response to GpUpU than to pGpU.

From a practical standpoint, 5′-phosphorylation allows one to test the cross-linking ability of a full range of codons, namely, those with the correct (A) or wobble (G) base pairing, those with U-U or U-C pairing, and those with nothing available to interact with the 5′-anticodon base.

Cross-Linking in the Presence of Different Codons. In our previous publications (Schwartz & Ofengand, 1974, 1978; Ofengand et al., 1979), we had used poly(U_2,G) as mRNA since it was a source of the expected valine codon, GUU, and gave excellent noncovalent binding as well as cross-linking. In preliminary experiments, poly(A,U,G) was less effective and was not used further at that time. However, this observation remained puzzling since the GUA codon should have been even better than GUU. The experiments described here were performed in order to resolve this apparent paradox. When defined codons were used to bind AcVal-tRNA to ribosomes for subsequent cross-linking, the results were quite startling. As shown in Figure 2, the correct codon, GpUpA, blocked cross-linking while codons which do not base pair with the 5′-anticodon base were active. The codon GpUpG, not shown in the figure, behaved like GpUpA. pGpUpU, GpUpU, and pGpU were even more active than poly(U_2,G), presumably

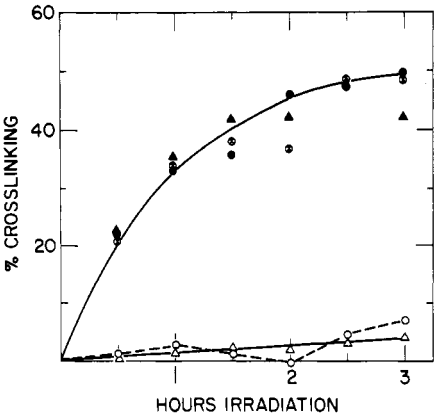

FIGURE 3: Lack of inhibition by GpUpA or GpUpG of poly-(U_2,G)-stimulated cross-linking. Cross-linking was performed and assayed as under Experimental Procedures except that incubation was for 20 min at 37 °C followed by 20 min at 0 °C before irradiation. *E. coli* AcVal-tRNA was used. (⊗) 17 μM poly(U_2,G), (○) 10 μM GpUpG, (△) 21 μM GpUpA, (●) 17 μM (U_2G) plus 10 μM GpUpG, (▲) 17 μM poly(U_2,G) plus 21 μM GpUpA.

because the polynucleotide supplied some inactive GUG codons in addition to active GUU codons. Even pGpUpC, in which a C residue is paired with the 5′-anticodon base, cmo^5U, was moderately active.

There was little or no cross-linking in the absence of codons. In some experiments, where a low level of cross-linking with GpUpA was observed (as in Figure 2), cross-linking in the absence of codons occurred to about the same extent. In other experiments (for example, Figure 3), where there was no GpUpA- or GpUpG-dependent cross-linking, there was also no cross-linking above the zero time value in the absence of codons.

In order to sure the GpUpA and GpUpG codons were not inactive because of some contaminant, a mixing experiment was performed with added poly(U_2,G). The experiment was done in this way since at the relative concentrations used it was expected that the triplets would not be able to compete with the polynucleotide for ribosomal binding sites, and hence could not inhibit in this manner. The purpose of the experiment was to test for inhibitory substances in the triplet preparations that could affect the outcome of a binding experiment but which were unrelated to codons. As shown in Figure 3, the addition of GpUpA or GpUpG to a poly(U_2,G) directed reaction had no inhibitory effect, while the codons themselves were completely inactive. Thus the expectation was borne out, and the triplets GpUpA and GpUpG did not contain nonspecific inhibitors of tRNA binding or of cross-linking.

In order to see if the nature of the modification of the 5′-anticodon base, namely, cmo^5 vs. mo^5, made a difference, *B. subtilis* AcVal-tRNA (anticodon mo^5UAC) was used in a parallel series of experiments (Figure 4). The results were essentially the same as before, except that the efficiency of all of the oligonucleotide-dependent cross-linking reactions were slightly higher than for the analogous *E. coli* case, although the poly(U_2,G)-dependent reaction was the same. Possibly the absence of the COOH group in the mo^5U of the *B. subtilis* series allows for a better orientation when single codons are used.

Are the Oligonucleotide-Dependent Cross-Links to the Same Site as the Polynucleotide-Dependent Cross-Link? It was earlier shown that all of the polynucleotide-dependent cross-linking was to 16S RNA (Schwartz & Ofengand, 1978). In order to see if the pGpUpU-, GpUpU-, and pGpU-dependent cross-linking was also only to 16S RNA, the tRNA–

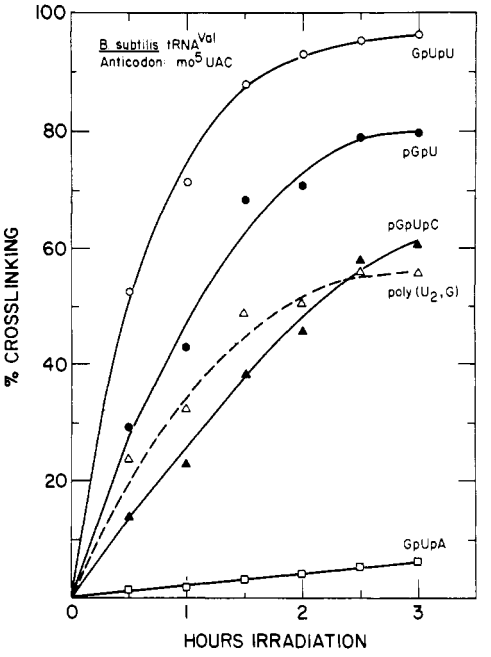

FIGURE 4: Covalent cross-linking of *B. subtilis* AcVal-tRNA (anticodon mo^5UAC) to ribosomes in the presence of different codons. Conditions were as given in the legend to Figure 2. (○) GpUpU, (●) pGpU, (▲) pGpUpC, (□) GpUpA, (△) poly(U_2,G).

ribosome covalent complexes formed by irradiation were first separated from un-cross-linked tRNA by sucrose gradient centrifugation at 0.5 mM Mg^{2+} (Figure 5A). Clearly, cross-linking was highly dependent on the presence of the codons, as was already demonstrated above: 80, 67, and 55% of the input tRNA was cross-linked when pGpUpU, GpUpU, or pGpU were used, respectively, but only 3% in the absence of codons. There was no detectable cross-linking without irradiation in the presence of either pGpUpU or pGpU (not shown). In every case, only the 30S subunit was cross-linked. The tRNA–30S complexes were then treated with $NaDodSO_4$ to dissociate the rRNA from the ribosomal proteins, and centrifuged on $NaDodSO_4$–sucrose gradients (Figure 5B). All of the cross-linked tRNA was found attached only to 16S RNA when either pGpUpU, GpUpU, or pGpU was used. Even the small amount of codon-independent cross-linking was found only in the 16S RNA region of the gradient.

It was also previously shown that all of the 16S RNA cross-linking dependent on poly(U_2,G) was from the ribosomal P site since all of the Ac[^{14}C]valine bound to the covalently attached tRNA could be released upon incubation with puromycin (Ofengand et al., 1979). Reaction with puromycin is generally assumed to be diagnostic for a P-site bound tRNA. The same results were obtained for the oligonucleotide-dependent cross-linking. After cross-link formation with each of the four active codons or with poly(U_2,G), incubation in the presence of puromycin resulted in the release of 80% of the bound Ac[^{3}H]valine from association with the ribosome (Figure 6). In this assay, non-cross-linked complexes are released to the same extent (Ofengand et al., 1979). Incubation in the absence of puromycin had no effect.

Lastly, the structure of the cross-link formed in all cases appears to be the same, or at least very similar. Recent studies of the poly(U_2,G)-induced cross-link have established that the structure of the cross-link between tRNA and 16S ribosomal RNA is very probably that of a cyclobutane dimer between the 5′-anticodon base, cmo^5U, of the tRNA and a pyrimidine base of the 16S RNA (Ofengand & Liou, 1980). Such dimers are readily photolyzed by 254-nm irradiation and it is known

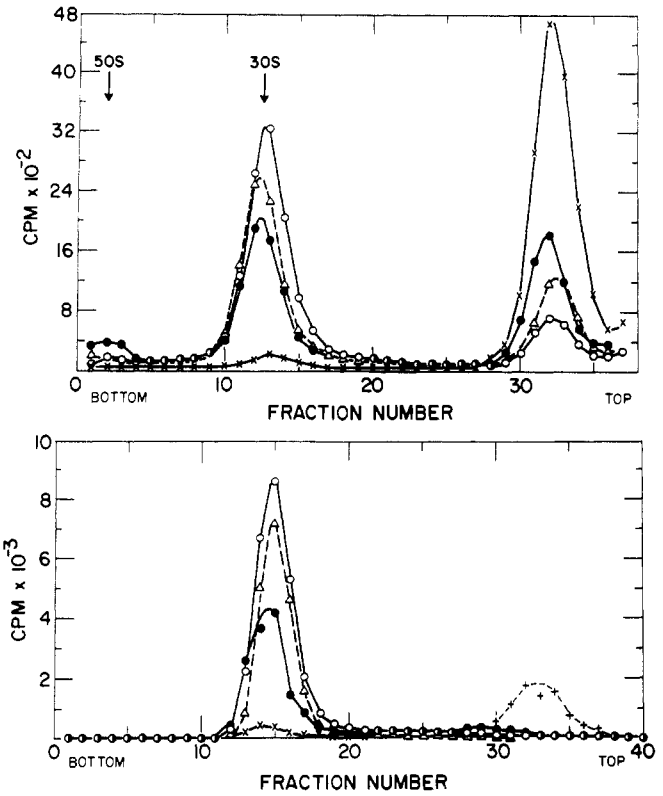

FIGURE 5: Analysis of site of oligonucleotide-induced cross-linking. (A, upper) Separation of ribosomal subunit–tRNA covalent complexes from free tRNA by sucrose gradient centrifugation. Ribosome–tRNA covalent complexes were prepared as described under Experimental Procedures by irradiation at 300 nm for 3 h in the presence of 31 μM pGpUpU, 40 μM GpUpU, 63 μM pGpU, or in the absence of codons. 0.5 mL reaction mixtures, precipitated by 67% EtOH–2% KOAc, pH 5, at 0 °C, were dissolved in 0.5 mL of 20 mM MES, pH 6.0, 100 mM NH_4Cl, and 0.5 mM Mg^{2+} and layered on 10–30% isokinetic sucrose gradients in the same buffer. Centrifugation in the SW40 rotor was at 29000 rpm, 40 °C, for 17 h. (○) pGpUpU, (●) pGpU, (△) GpUpU, (×) no codon. The positions of the UV-absorbing peaks are indicated by arrows. (B, lower) Separation of 16S RNA from ribosomal protein by $NaDodSO_4$–sucrose gradient centrifugation. Fractions 9–14 from the gradients of part A were pooled, adjusted to 10 mM Mg^{2+}, precipitated with 2% KOAc, pH 5, and 67% EtOH, dissolved in 525 μL of 15 mM MES, pH 6, 76 mM NH_4Cl, 0.38 mM Mg^{2+}, 9.5 mM EDTA, and 1.9%$NaDodSO_4$, and incubated at 37 °C for 2 min. to completely dissociate 16S RNA from ribosomal proteins (Noll & Stutz, 1968). The samples were then centrifuged through 5–27% isokinetic sucrose gradients in the same buffer as in part A plus 5 mM EDTA and 0.1% $NaDodSO_4$ in the SW40 rotor at 29000 rpm, 20 °C, for 16.5 h. (○) pGpUpU, (●) pGpU, (△) GpUpU, (×) no codon, (+) added marker Ac[^{14}C]Val-tRNA. In each case, the radioactivity peak at fraction 15 coincided with the UV-absorbing peak of 16S RNA (UV trace not shown).

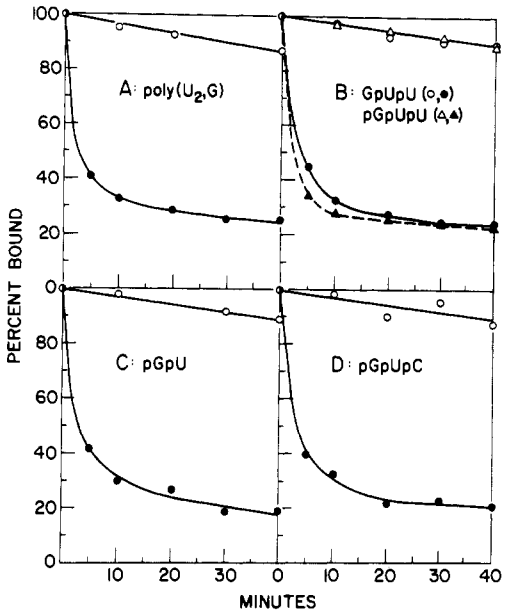

FIGURE 6: Puromycin reactivity of covalent AcVal-tRNA–ribosome complexes after cross-linking in the presence of different codons. Covalent complexes were prepared as in Figure 2 by 3-h irradiation. Puromycin was then added to 1 mM and the mixtures incubated at 37 °C for the indicated times. Samples were assayed by membrane filtration at 0.1 mM Mg^{2+}. (○ or △) Incubation without puromycin; (● or ▲) incubation with puromycin. Panel A, poly(U_2,G); panel B, GpUpU (circles), pGpUpU (triangles); panel C, pGpU; panel D, pGpUpC.

Table II: Rate of 254-nm Photolysis of tRNA-30S Covalent Complex Formed in the Presence of Different Codons[a]

codon	rate constant (min^{-1})
poly(U_2,G)	0.82
GpUpU	1.09
pGpU	1.10
pGpUpC	1.03

[a] Covalent complexes were prepared as in Figure 2 by 3-h irradiation; 0.2-mL samples were then diluted 20-fold to 1 mM Mg^{2+} with 50 mM Hepes, pH 7.5, and 50 mM NH_4Cl and photolyzed at 254 nm as described under Experimental Procedures. Samples (0.5 mL) were removed for assay by cellulose nitrate filtration at 0.1 mM Mg^{2+} to determine the amount of tRNA–ribosomal complexes remaining. The rate constant was evaluated from a semilogarithmic plot (Wang, 1962) of the data after correction for the residual blank value.

that even the different geometrical isomers of the cyclobutane dimer of thymine will photolyze at different rates (Herbert et al., 1969). When the rate of photolysis of the cross-linked complex made in the presence of different codons was examined, they all showed a common rate constant for photolysis (Table II). Although the rate constant for poly(U_2,G) did appear to be significantly lower than the others, the difference was quite small, while even the rates described by Herbert et al. (1969) differed by larger amounts, 1.5–4 times at 254 nm. It is likely, therefore, that all of the cross-linked complexes involve the same 16S RNA nucleotide.

Codon Specificity in the Cross-Linking of Other tRNAs. The codon specificity effect described above has also been detected with other cross-linkable tRNAs. As noted previously, other tRNAs with (c)mo^{5}U at the same position, namely, $tRNA_1^{Ser}$ of *E. coli* and $tRNA^{Val}$ and $tRNA^{Thr}$ of *B. subtilis*, can also be cross-linked to the ribosome by UV irradiation (Ofengand et al., 1979). *E. coli* $tRNA_1^{Ala}$ also possesses a cmo^{5}U residue at the 5′-anticodon position, but had not been tested previously. Its anticodon, cmo^{5}UGC, was more suitable than that of $tRNA_1^{Ser}$, cmo^{5}UGA, for testing the codon specificity effect with random copolymers. Thus the copolymer, poly(I,C,A), could be used to test the effect of cmo^{5}U-A base pairing with $tRNA_1^{Ala}$ without the risk of supplying ICU codons but the analogous copolymer, poly(U,C,A), needed for $tRNA_1^{Ser}$ would also supply UCU codons. $tRNA^{Thr}$ of *B. subtilis* was not tested because insufficient material was available.

The cross-linking activity of AcAla-$tRNA_1^{Ala}$ in the presence of poly(I,C,U) and poly(I,C,A) is shown in Figure 7. For comparison, the cross-linking of AcVal-$tRNA^{Val}$ with poly(U_2,G) is also shown. The main point to note is that while a polynucleotide capable of supplying the codon ICU supported cross-linking, that which could only supply the codon ICA was less than 15% as active. This result is in full agreement with

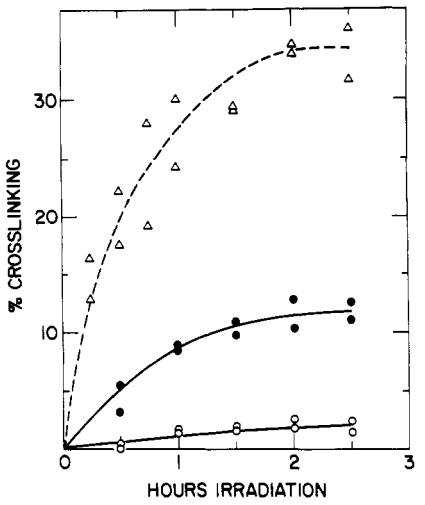

FIGURE 7: Covalent cross-linking of *E. coli* AcAla-tRNA (anticodon cmo^{5}UGC) to ribosomes in the presence of different codons. Noncovalent binding and cross-linking were as given under Experimental Procedures except that the Mylar filter was not used. Solid lines, AcAla-tRNA with poly(I,C,U) (●), with poly(I,C,A) (○). Dashed line, AcVal-tRNA with poly(U_2,G).

that obtained above with $tRNA_1^{Val}$. Note also that while $tRNA_1^{Ala}$ could be cross-linked, as expected for a cmo^{5}U-containing tRNA, the efficiency of cross-linking was less than that of $tRNA_1^{Val}$. Similar results were noted previously for *E. coli* $tRNA_1^{Ser}$ and *B. subtilis* $tRNA^{Thr}$ (Ofengand et al., 1979). At the moment, there is no clear explanation for the variation in level of cross-linking observed for different (c)mo^{5}U-containing tRNAs.

Discussion

The use of defined codons of the series GUX where X is A, G, U, C, or nothing has made it possible to determine directly the effect of base pairing with the (c)mo$^5U_{34}$ residue on the cross-linking efficiency between this base and a residue of the 16S RNA. The result was very clear. Base pairing either in the normal way with A or by "wobble" pairing with G completely blocked cross-linking. The presence of U was equivalent to the absence of a residue showing either that U–U interaction does not occur in the P site or that it leads to the same positioning of cmo$^5U_{34}$ as no interaction. There was a definite decrease in the cross-linking efficiency when C was opposed to the (c)mo^{5}U residue, but the effect was much smaller than when A or G was present. This suggests that some C-(c)mo^{5}U interaction occurs, in contrast to the proposal of Grosjean et al. (1978). These results seem to directly rule out a "two out of three" type of codon recognition scheme for the P site of the sort that has been proposed for the A site by Lagerkvist (1978) for tRNAs such as $tRNA^{Val}$, since then there should have been no effect of changing the third codon base. The carboxyl group of the (c)mo^{5}U residue was not in any way related to the effects described in this paper, since *B. subtilis* $tRNA^{Val}$ with the analogous mo^{5}U residue, lacking the carboxyl group, showed exactly the same behavior (compare Figures 2 and 4).

The effect of base pairing cannot be due simply to some effect of H-bonding on the chemical reactivity of the (c)mo^{5}U heterocyclic ring, since as pointed out above, if base pairing blocked cyclobutane dimer formation, dimers could never form in UV-irradiated DNA. However, it is worth noting that the reverse is not the case. Cyclobutane dimer formation is known to cause disruption of the DNA helix (Rahn, 1973; Lang, 1974) for approximately four base pairs (Hayes et al., 1971), and it is thus possible that cross-link formation similarly disrupts the P-site codon–anticodon interaction once cross-linking has occurred.

The fact that it is the pairing properties of the 5′-anticodon base that influences cross-link formation is additional evidence that the 5′-anticodon base itself is part of the cross-link structure. In our previous work, this was demonstrated by an indirect, deductive approach (Ofengand et al., 1979), although it is supported by recent sequencing studies (J. Ofengand, unpublished results). The present findings further support this conclusion since it is difficult to imagine how a base change in only one of three residues of a codon could completely block cross-linking while still allowing noncovalent binding, if some other site were involved.

The work described here provides the first *direct* experimental evidence that codon–anticodon recognition (CAR) is maintained in the ribosomal P site. Although it is well-known that nonenzymatic codon-dependent binding of tRNA to ribosomes usually occurs at the puromycin-reactive site (the P site), it has been suggested (Ofengand, 1980) that this may occur via transient binding to the A site where CAR occurs, followed by a translocation to the P site with subsequent disruption of CAR. The disruption could occur either upon entering the P site so that CAR does not occur in the P site itself or only when the A site is refilled with a new aminoacyl-tRNA. This proposal that tRNAs in both A and P sites do not simultaneously base pair was put forward in order to eliminate the constraints (Rich, 1974; Sundaralingam et al., 1975) caused by simultaneous CAR of tRNAs in both P and A sites.

The evidence for CAR in the P site has up to now been indirect. Pongs et al. (1975, 1979) have shown that a codon covalently attached to the ribosome could direct P-site binding of fMet-tRNA. However, codon attachment could have occurred at the A site, after which the tRNA moved to the P site without the obligate requirement that the codon move as well. Alternatively, the site of attachment could have been such as to allow the codon to shift with the tRNA from A to P site. Wurmbach & Nierhaus (1979) used tetracycline and viomycin in an attempt to show direct P-site CAR. Tetracycline was used to block the A site and viomycin was used to inhibit translocation, but neither affected P-site CAR, implying that transient A-site binding does not occur. However, since one cannot be sure that these antibiotics inhibit the postulated transient A-site binding and movement in the same way that they act on stable A-site binding and translocation, the conclusion is open to qualification. Lührmann et al. (1979) showed that codon-dependent binding of tRNA to the P site was required for EFTu-dependent A-site binding. However, it was not shown that CAR persisted in the P site once the tRNA was stably located there. Peters & Yarus (1979) measured the dissociation constants of different tRNAs for poly(U)-coded ribosomes in the presence of tetracycline and noted that the relative affinities followed the order expected on the basis of codon–anticodon base-pairing possibilities. They concluded that CAR occurs in the P site, but their interpretation requires the same assumption about the mode of action of tetracycline as does the work of Wurmbach and Nierhaus. Fairclough et al. (1979) observed fluorescence changes at the anticodon of P-site bound $tRNA^{Phe}$ upon adding poly(U). They interpreted the change in environment signalled by the fluorescence change as a direct codon–anticodon interaction, but it might also have been due to an altered ribosomal environment induced by mRNA binding.

A more direct approach was used by Kuechler (Matzke et al., 1980), who cross-linked the Y base of yeast $tRNA^{Phe}$, at

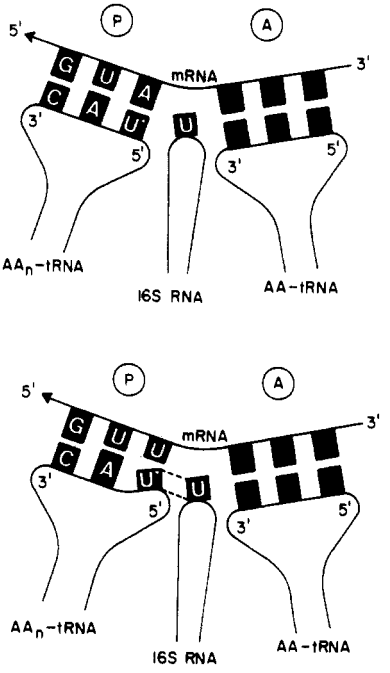

FIGURE 8: Schematic diagram of codon translation in the P site and its effect on the interaction with 16S RNA. AA_n-tRNA, peptidyl-tRNA in the P site; AA-tRNA, aminoacyl-tRNA in the A site. The arrangement of mRNA and tRNA is dictated by the direction of mRNA translation (5′ to 3′) and antiparallel base pairing. The arrow shows the direction of mRNA movement past a stationary ribosome. A loop of 16S RNA is inserted such that a U (or C) residue is sufficiently close to the 5′-anticodon base (U*) of the P site bound tRNA to allow cyclobutane dimer formation (dashed lines) when the opposite codon residue is a uridine, cytidine, or nothing (lower diagram). Correct (A) or wobble (G) base pairing prevents dimer formation (upper diagram). See text for discussion.

the 3′ side of the anticodon, to poly(U) by irradiation and then showed that the cross-linked tRNA was in the P site by reaction with puromycin. This result shows that codon and anticodon are close to each other in the P site, but does not prove CAR occurs there. However, the same rate and yield for A-site cross-linking, where CAR presumably does occur, suggests that CAR occurs also at the P site. Moreover, the A-site cross-linked complex could fit in the P site since translocation of the cross-linked complex was possible.

The present experiments, however, provide the most direct evidence for P-site CAR. Base pairing of the 5′-anticodon base (c)mo^{5}U$_{34}$ with A or G blocked cross-linking while pairing with U, C, or nothing allowed it. The tRNA was clearly in the P site when this occurred since *after* cross-linking the acetyl amino acid could react with puromycin. If CAR occurred as a transient reaction in the A site followed by translocation to the P site, there should be no difference between the cross-linking capability of any of the codons since by the time the tRNA reached the P site, all CAR should have been disrupted. The differential codon effect, coupled with the fact that the cross-linked tRNA is clearly in the P site, proves P-site CAR.

These results also suggest that some conformational modification of the anticodon loop of P-site bound tRNA occurs when the third (5′) anticodon base becomes paired with its cognate codon base. Evidence for an undefined codon-dependent conformational change in either the ribosomal vicinity of the anticodon loop of ribosome-bound tRNAs or in the acticodon loop itself has been previously described (Wintermeyer et al., 1979; Fairclough et al., 1979).

A scheme which summarizes our findings is shown in Figure 8. In this figure, the anticodons of two tRNAs are shown simultaneously interacting with adjacent codons, but as noted above, this may not normally occur. In any case, our experiments were performed with an empty A site. In the upper figure, standard antiparallel base pairing is illustrated. We postulate that normal A–cmo^{5}U pairing alters the position of the cmo^{5}U$_{34}$ residue with respect to the U or C residue of the 16S RNA so that cyclobutane dimer formation cannot occur. On the other hand (lower figure), in the absence of a base-pairing driving force, that is, when U or nothing is present at the 3′ position of the codon, the anticodon loop assumes an alternative configuration such that the cmo^{5}U$_{34}$ residue and the 16S pyrimidine are suitably juxtaposed for cross-linking upon irradiation. There is almost a zero probability that the conformation of the anticodon loop of the lower figure is occasionally attained by the tRNA in the upper figure since if this were the case, irradiation should still produce cross-links but at a lower rate determined by the lower probability of attaining the appropriate spatial configuration. In fact, there were no detectable cross-links found when A or G was present in the codon.

A variation of the above scheme postulates that the anticodon loop in the lower figure is conformationally flexible in the absence of a third base pair rather than possessing a single configuration. Even if only occasionally the 5′-anticodon base is suitably oriented toward the 16S RNA pyrimidine, upon continuous irradiation, the equilibrium will be continually perturbed so that eventually all of the tRNA will become cross-linked. This is possible since irradiation in the absence of a reaction partner does not destroy the excited molecule (Ofengand et al., 1979). Thus it is not necessary to postulate that the alternate conformation of the anticodon loop be one in which the cross-linking residues are juxtaposed, as above, but only that they are occasionally brought together. It is only required that pairing of the third base locks the anticodon loop into a conformation where no cross-linking can occur. Evidence for a more rigid anticodon loop in the presence of mRNA does exist (Wintermeyer et al., 1979).

In the above schemes, codon–anticodon interaction was envisaged as the perturbing force. A third possibility is that the codon 3′ base interacts with the 16S RNA loop itself. That is, in some way A or G residues perturb the location of the pyrimidine of the 16S RNA so that it cannot contact the cmo^{5}U$_{34}$ residue, while this does not occur when the codon residue is U, C, or nothing. In this scheme, the tRNA anticodon loop remains unchanged. We have no evidence against this scheme, but the purely ad hoc requirement for some unspecified mechanism for codon–16S RNA interaction makes it seem less likely.

Does the proximity of a loop of 16S RNA to the 5′ side of the P-site anticodon have any functional rationale? We have previously suggested (Ofengand et al., 1979) that this interaction could serve to augment or stabilize P-site codon–anticodon base pairing for all tRNAs, the tRNA specificity observed for cross-linking being simply due to the chemical requirements for cyclobutane dimer formation. In light of the present results, this hypothesis needs modification to explain why more complete P-site base-pairing inhibits cross-linking. We can speculate that in the P site only the first two letters are base paired, in order to free the tRNA–mRNA–ribosome complex from the stereochemical constraints introduced by requiring all three codon bases of both tRNAs to base pair simultaneously (see above discussion). In this situation, the 5′ base of the P-site anticodon would not be stereochemically in a position to base pair irrespective of its *potential* for base pairing. Stabilization of the anticodon in the P site would then be the function of the 16S RNA interaction. Only when the

A-site bound tRNA is absent would all three bases of the codon be able to base pair in the P site. This would alter the position of the 5′-anticodon base and block cross-linking. In this scheme, the lower part of Figure 8 reflects the situation in vivo even with GUA and GUG codons, while that in the upper part of the figure only occurs with an empty A site.

Possibly the only virtue of this speculative scheme is that it can be tested. In any event, the existence of a residue of 16S RNA within 4 Å of the tRNA anticodon strongly implies *some* functional significance even though one can only guess about it at this time.

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