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Crosslinking of the anticodon of P site bound tRNA to C-1400 of *E.coli* 16S RNA does not require the participation of the 50S subunit

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ABSTRACT

Crosslinking of the 5'-anticodon base of ribosomal P site bound AcVal-tRNA to residue C-1400 of 16S RNA or to its equivalent in 18S RNA has been shown to occur on 70S or 80S ribosomes of both prokaryotes and eukaryotes [Ciesiolka, J., Nurse, K., Klein, J. and Ofengand, J. (1985) *Biochemistry* **24**, 3233-3239]. In the present work, we show that the crosslinking rate, crosslinking yield, and site of crosslinking are all unchanged when the 50S subunit is omitted. Therefore, all of the positional features of tRNA-ribosome complexes which allow crosslinking to occur are entirely contained in the 30S subunit. Blockage of reverse transcription by crosslink formation was used to determine the site of crosslinking. This analysis revealed that RNA modifications which do not directly block base-pairing ligands sometimes allow the modified base to be transcribed, leading to doublet band formation even when there is only a single crosslink site.

INTRODUCTION

Previous work from this laboratory has established that certain tRNAs, placed in the P site of ribosomes from both prokaryotes and eukaryotes can be crosslinked to the small ribosomal subunit by irradiation with light of 310-330 nm (1-4). The crosslink is a cyclobutane dimer (5) which binds the 5'-anticodon tRNA base, mo^5U or cmo^5U in those species which can be crosslinked, to a specific C residue in the 16S or 18S rRNA (6-8). The residue involved, C-1400 in *E. coli*, is located in the center of a highly conserved 17-nucleotide sequence found in virtually unaltered form in prokaryotic, eukaryotic, and organelle ribosomes. The retention across species lines of both sequence and close proximity to the anticodon of tRNA is strongly suggestive of an important role for this sequence in some ribosomal process likely related to mRNA translation, but so far no specific functional or structural role has been assigned.

In all previous studies, 70S or 80S ribosomes were used, since A and P sites are best defined on the entire ribosomal particle. However, since the crosslink is entirely to the small ribosomal subunit, it was of interest to

determine if the 30S ribosome was sufficient for crosslinking. In this work we show that while the 50S subunit stimulates binding, the crosslinking rate and yield, and the site of crosslinking were unchanged in the absence of the 50S subunit. In addition, the ability of reverse transcription arrest to localize cyclobutane dimer-like crosslinks was characterized.

EXPERIMENTAL

Materials

E. coli 70S tight couple ribosomes, Ac[^{3}H]Val-tRNA, and poly(U_2,G) were prepared as described previously (1). pGUU and puromycin were obtained according to Ofengand and Liou (10). 50S and 30S subunits were prepared and activated before use as described by Krzyzosiak *et al.* (11) except that the activation buffer contained 60 mM NH_4Cl instead of 100 mM. 30S-4 and 30S-4A were prepared in buffer RA and stored in buffer Rec20. 30S-5A and 30S-5B were separated in buffer RE. Storage of 30S-5A and 30S-5B was in buffer Rec20 and buffer RC, respectively. The buffers are described in Krzyzosiak *et al.* (11). One A_{260} unit of 70S or 30S was assumed equal to 25 or 67 pmoles, respectively. The primer used for reverse transcription, complementary to residues 1431-1453, was made on an Applied Biosystems 381A synthesizer, manually cleaved, deprotected, and purified as described by Krzyzosiak *et al.* (11). [α-^{32}P]dATP, dideoxy nucleotides, AMV reverse transcriptase, and deoxynucleoside triphosphates were obtained as described by Nurse *et al.* (4).

P site binding and crosslinking

The reaction mixture contained 50 mM Hepes, pH 7.5, 100 mM NH_4Cl, 15 mM $Mg(OAc)_2$, 20 µg/ml poly(U_2,G) or 60 µM pGUU, 150 nM Ac[^{3}H]Val-tRNA, and 136-150 nM 30S, 30S plus 50S, or 70S *E. coli* ribosomes except as indicated. The 30S and 30S plus 50S reactions contained 0.4-0.7 mM DTT carried over from the activation reaction. The 70S reactions did not contain mercaptans except for the experiment of Fig. 1. Incubation was at 37°C for 15 min. Irradiation for 25 min (30S) or 45 min (70S) was at 0°C in the Rayonet photoreactor with the 300 nm lamps using ca. 1 cm of Pyrex glass as a low wavelength cutoff filter (1). Crosslinking analysis was by adsorption to membrane filters (Schleicher and Schuell, BA85) as described previously (1).

RESULTS

Site location of anticodon-crosslinked tRNA

In previous reports, we have shown that AcVal-tRNA crosslinked via its anticodon to the 30S subunit can still transfer the AcVal moiety to puromycin

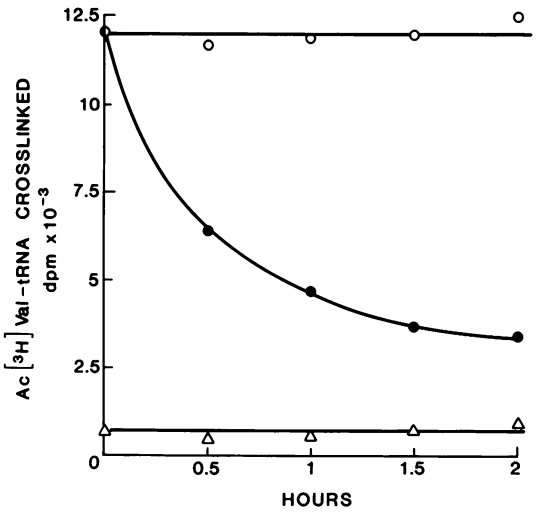

Fig. 1. Puromycin reactivity of AcVal-tRNA crosslinked to 70S ribosomes. Binding and crosslinking were as described in *Experimental* except that 38 nM Ac[^{3}H]Val-tRNA and 302 nM 70S ribosomes were used, 1 mM DTT was added, and irradiation was for 30 min. Puromycin (0.7 mM) and 5 mM mercaptoethanol were added, and incubation continued at 0°C for the indicated times. Samples were analyzed by membrane filtration at 0.1 mM Mg^{++}. At zero time, the values for poly(U_2,G)-dependent covalent and non-covalent binding were 0.078 and 0.114 pmoles tRNA/pmole ribosomes, respectively (68% crosslinking). The amount non-covalently bound was 91% of the added tRNA in this ribosome-excess experiment. O, crosslinking in the presence of poly(U_2,G); Δ, crosslinking in the absence of poly(U_2,G). Solid symbols, incubated with puromycin; open symbols, incubated without puromycin.

when incubated at 37°C (1,10). This result was interpreted to mean that the crosslinking reaction occurs from the P site. It was confirmed by the inability of Val-tRNA bound to the ribosome by EFTu to crosslink in a similar manner (1). Nevertheless, it was possible that AcVal-tRNA bound in a non-enzymatic fashion to the A site might be able to crosslink. Since the puromycin reaction was done at 37°C, the possibility existed for translocation of the 3'-end of A-site crosslinked tRNA either non-enzymatically (12) or catalyzed by traces of EFG in the ribosomes. A precedent for such a reaction exists. AcPhePhe-tRNA, crosslinked at the A site via its s^4U residue, could react with puromycin when incubated with EFG (13). Consequently, it was necessary to confirm that the anticodon crosslink really was P-site specific.

This was accomplished by carrying out the puromycin reaction at 0°C in the presence of excess functional ribosomes. Under these conditions, negligibly low levels of translocation occur (14). Fig. 1 shows that after 2 hr at 0°C, at

Table 1
Binding of AcVal-tRNA to 30S Ribosomes

Ribosomes	$[Mg^{++}]$	$[NH_4^+]$	Binding
	mM	mM	pmole/pmole
30S	10	50	0.25
30S	15	50	0.33
30S	20	50	0.34
30S	15	100	0.33
30S	20	100	0.35
70S	15	100	0.49

Reaction conditions were as described in *Experimental*, except as indicated. Values are poly(U_2,G)-dependent pmoles of tRNA bound per pmole of ribosomes added. The mRNA-independent blank was less than 5% of the reported values.

least 77% of the codon-dependent crosslinked species had reacted. We conclude that anticodon crosslinking to C-1400 of 16S rRNA is indeed a P site-specific reaction.

Ribosomal subunit requirement for crosslinking

Table 1 shows that a reasonable level of binding of AcVal-tRNA to 30S ribosomes could be achieved at a moderate concentration of Mg^{++}. 15 mM Mg^{++} was optimal at either 50 or 100 mM NH_4Cl. The level of binding reached 70% of that

Table 2
Crosslinking of AcVal-tRNA to 30S and 70S Ribosomes

Ribosomes	Codon-Dependent Binding			Crosslinking			Percent Crosslinking
	+ mRNA	- mRNA	Δ (A)	+ mRNA	- mRNA	Δ (B)	
	pmol tRNA/pmol ribosomes						100 x B/A
30S	0.32	0.02	0.30	0.171	0.004	0.167	56
30S + 50S	0.50	0.03	0.47	0.250	0.004	0.246	52
70S	0.52	0.03	0.49	0.300	0.013	0.287	58

Reaction conditions were as in *Methods*. mRNA is poly(U_2,G). Percent crosslinking is 100x tRNA crosslinked/tRNA bound. Irradiation for 22 and 45 min yielded values which differed by only 2-3%. The average value is reported.

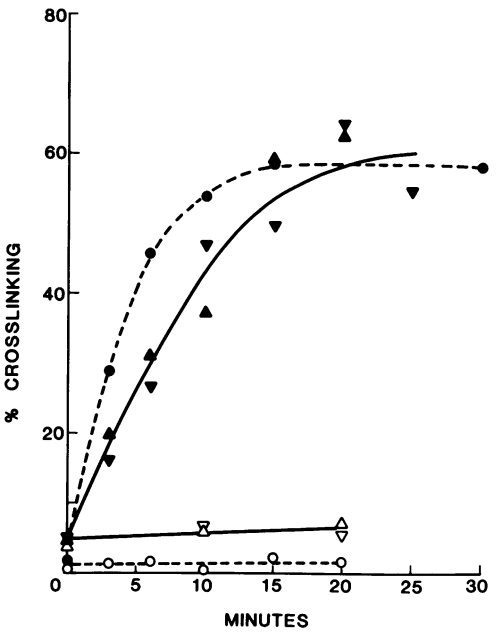

Fig. 2. Kinetics of crosslinking of 30S and 70S ribosomes. Binding and crosslinking was done as in *Experimental* except as noted. Percent crosslinking is the amount of tRNA crosslinked x100 divided by the amount of poly(U_2,G)-dependent non-covalent binding. Δ, 134 nM 30S; ∇, 268 nM 30S; O, 148 nM 70S. Solid symbols, plus poly(U_2,G); open symbols, minus poly(U_2,G).

achieved with 70S ribosomes. Table 2 compares the binding and crosslinking of AcVal-tRNA to 30S, 30S plus 50S, and 70S ribosomes. While the binding levels were like those of Table 1, the crosslinking yield of the 30S particles was equal to that for 70S or 30S plus 50S. The rate of crosslinking of 70S and 30S ribosomes (Fig. 2) also shows the mRNA dependence of crosslinking. The rate of 30S crosslinking was independent of a 2-fold change in ribosome concentration in the reaction, and was somewhat slower than 70S crosslinking in the experiment, but was similar to the rates of 70S crosslinking obtained previously (1-3). We conclude that 30S crosslinking is very similar in rate and yield to 70S crosslinking.

Analysis of the site of crosslinking by reverse transcriptase arrest

In order to determine if the site of crosslinking to 16S RNA was altered when 30S subunits alone were used, we analyzed the tRNA-rRNA covalent complexes extracted from 30S ribosomes. This had previously been a rather tedious undertaking (3,7,8) when applied to 70S or 80S ribosomes. However, the finding

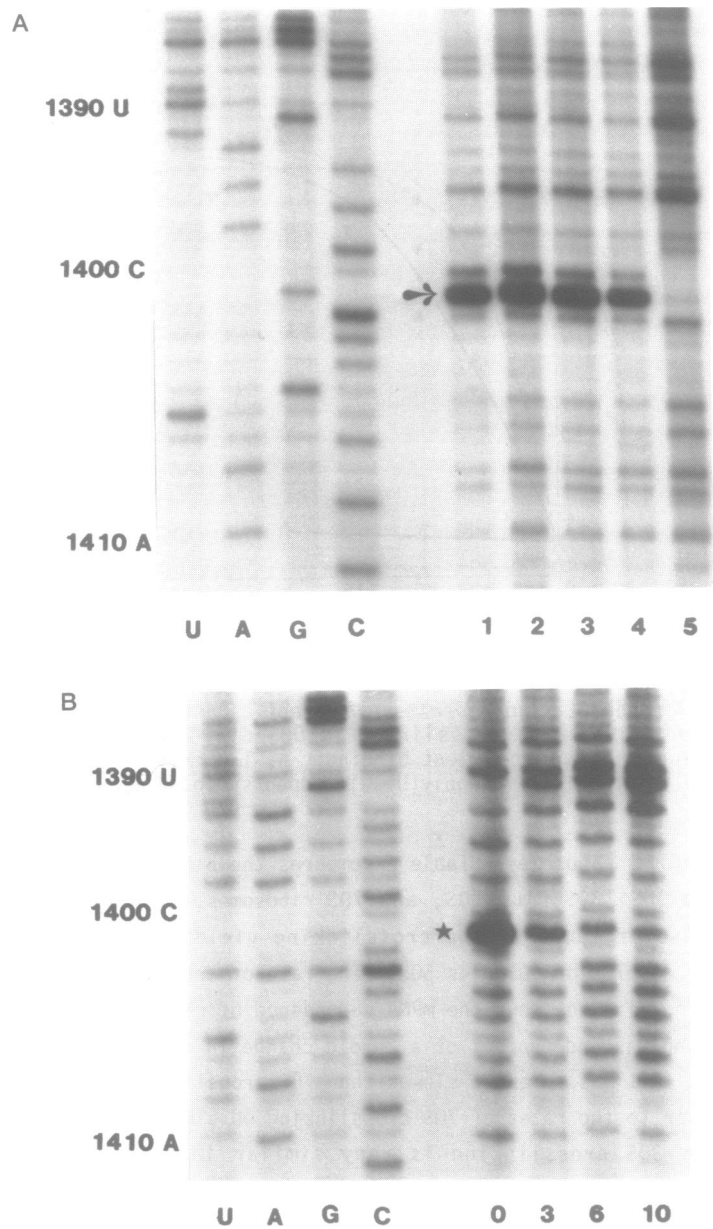

Fig. 3. Reverse transcription arrest analysis of the site and nature of crosslinking to 70S ribosomes.

Panel A. tRNA binding and crosslinking to 70S ribosomes was done as described in *Experimental*. Isolation of tRNA-rRNA covalent complexes from preparation 70S-1 (described in Table 5), reverse transcriptase extension and

sequencing, and gel analysis were as described previously (4). U,A,G,C are dideoxy sequencing lanes labeled according to the RNA template. Residue numbers are according to Noller (9). The amount of reverse transcriptase used was 1.25 units (lane 1), 2.5 units (lane 2), 5.0 units (lane 3), and 10.0 units (lanes 4, 5) with 2.2 μM dATP, 100 μM each of dCTP, dTTP, dGTP, and an extension time of 30 min. The arrow marks G-1401, the major site of arrest.

Panel B. tRNA-rRNA covalent complexes were isolated from crosslinked 70S-tRNA complexes by phenol extraction as described by Krzyzosiak *et al.* (11). After annealing of the RNA to the reverse transcriptase primer (4) a 10 μl drop was irradiated at 0°C with a set of 6 G15T8 Sylvania germicidal lamps placed 15 cm from the sample. At the indicated times, aliquots were taken for reverse transcription with 1.25 units of enzyme, 2.2 μM dATP, 100 μM each dCTP, dTTP, dGTP, and a 30 min extension time. Gel analysis, sequencing lanes, and numbering were as in panel A. The star marks G-1401.

that reverse transcriptase will pause or stop at modified bases (15-23), suggested the possibility that a similar effect might occur for this crosslink since its structure is similar to psoralen monoadducts which are known to block reverse transcription (18). Therefore, the following experiments were first carried out with crosslinked 70S ribosomes whose site of crosslinking was known to be >99% to the C-1400 residue (8).

Fig. 3A shows a strong stop at residue G-1401, just 3' to the known crosslink site at C-1400, which is absent in the control, lane 5. Surprisingly, a second stop appeared at C-1400, corresponding to a putative second crosslink site at C-1399. When quantitated (Table 3; Fig. 3A), this amounted to 7-10% of the total, depending on the amount of reverse transcriptase used. However, such a level of crosslinking to C-1399 would have easily been detected by the procedure employed earlier, suggesting that in the case of the cyclobutane dimer structure, reverse transcriptase is able to occasionally read the crosslinked residue before halting. We refer to this process as *stuttering*. Additional experiments tend to support this interpretation. First, Fig. 3B shows that the ability to induce a stop was sensitive to UV irradiation, as are cyclobutane dimers (5), confirming that tRNA-rRNA crosslinking is the cause of reverse transcriptase arrest. Second, the right-hand lanes of Fig. 4 show that increasing the time for reverse transcription also increases the amount of stuttering. The minimum amount of stuttering in this experiment is three times the maximum in Fig. 3A, probably due to the use of 4.2 μM dATP during the primer extension step instead of 2.2 μM (Table 4). In addition to these known variables, there was also considerable fluctuation in the relative stops at C-1400 and G-1401, particularly at 2.2 μM dATP, due to unknown causes. This is indicated by the large average deviation from the mean and by the discrepancy between the values in Tables 3 and 4. Since all of these analyses were done on

Table 3
Effect of enzyme and extension time on the relative arrest at 16S rRNA residues 1400 and 1401 due to crosslinked AcVal-tRNA

Expt.	Lane	Reverse Transcriptase	Extension Time	cpm at 1400 + 1401	% cpm at 1400
		units/reaction	min		
Fig. 3A	1	1.25	30	4382	6.5
	2	2.50	30	6594	10.0
	3	5.00	30	7736	10.1
	4	10.00	30	3790	9.5
	5*	10.00	30	nd	-
Fig. 4	5	1.25	5	1397	30.4
	6	1.25	10	1994	38.0
	7	1.25	20	2502	42.6
	8	1.25	30	1983	44.8
	9*	1.25	30	nd	-

Analysis of the gels was carried out by slicing and Cerenkov counting of the ^{32}P-labeled bands. Percent C-1400 is the cpm at position 1400 x 100 ÷ by the sum of the cpm at 1400 and 1401.
nd = not detectable on the autoradiograms
*Same as the preceding line except that codons and AcVal-tRNA were omitted from the crosslinking reaction.

aliquots of the same crosslinked sample, it is clear that the variable distribution of stop sites is due to the reverse transcription assay and not to variations in the distribution of crosslinking sites. The major determinant is likely to be the dATP concentration. Since it is used at low concentrations, and is highly radioactive, it is possible that radiation-induced or hydrolytic decay could be responsible for most of the variability observed.

The left-hand portion of Fig. 4 shows that stuttering is not inherent to the C-1400 region but is determined by the chemistry of the crosslink. In the same series of reactions which produced 45% stopping atetermined by the chemistry of the crosslink. In the same series of reactions which produced 45% stopping at C-1400 with the cyclobutane dimer crosslink, a tRNA-rRNA crosslink at C-1400 via the aromatic azide SNAP (24) produced only a single stop site at G-1401.

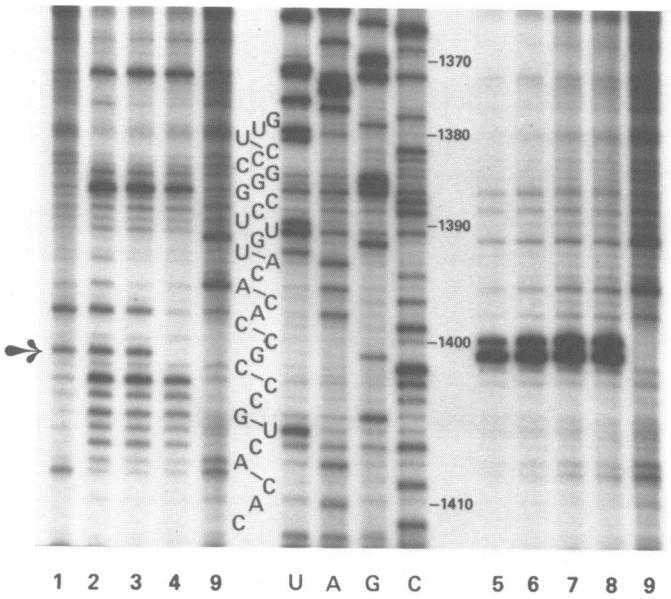

Fig. 4. Effect of extension time and nature of the crosslink on the site(s) of reverse transcription arrest. tRNA-RNA covalent complexes from preparation 70S-1 were made and analyzed as in Fig. 3, with 1.25 units of reverse transcriptase, 4.2 μM dATP, and 100 μM each dCTP, dTTP, dGTP. Lanes 1-3, tRNA crosslinked in the presence of EFTu to C-1400 via the SNAP probe (24). Each lane is a separate crosslinking reaction. Lane 4, as in lane 3 but in the absence of EFTu. Lanes 1-4 were extended for 30 min. Lanes 5-9, preparation 70S-1 extended for 5 min (lane 5), 10 min (lane 6), 20 min (lane 7), and 30 min (lanes 8, 9). Sequencing lanes and numbering as in Fig. 3. Arrow marks G-1401.

Table 4
Effect of dATP concentration on reverse transcription arrest

dATP (μM)	Percent C-1400	No. of Analyses
2.2	22 ± 8	5
4.2	49 ± 4	3
6.2	50 ± 3	2
12.2	48 ± 1	2

Reverse transcription arrest of 70S-1 rRNA-tRNA was performed with 1.25 units reverse transcriptase, dATP as indicated, 100 μM dCTP, dTTP, dGTP, and an extension time of 30 min. Gels were sliced and counted to determine the percent C-1400 as in Table 3. Values are given ± the average deviation from the mean.

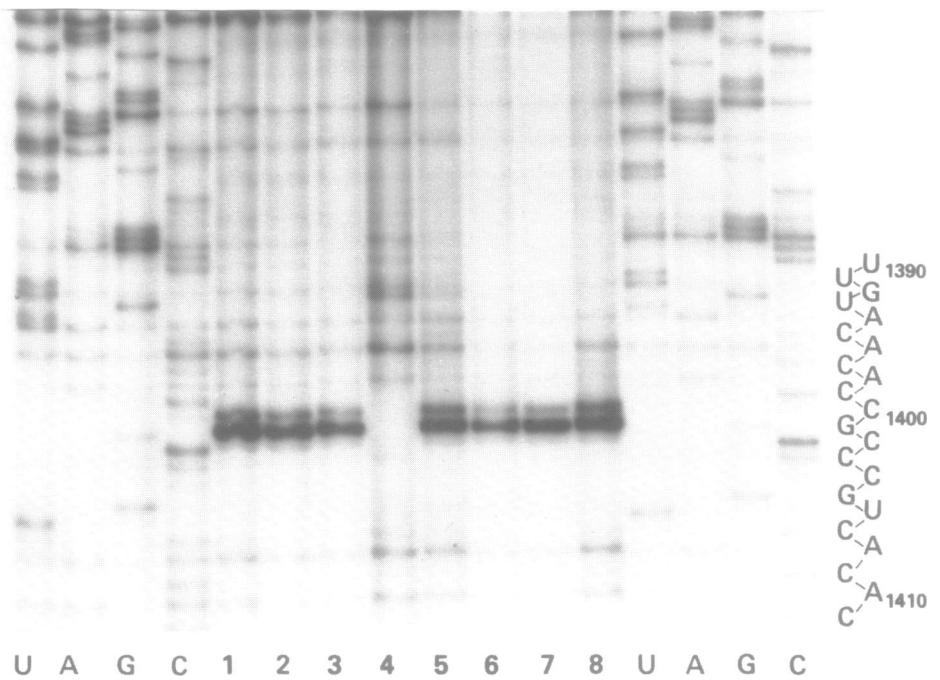

Fig. 5. Site of crosslinking to 30S ribosomes. tRNA-rRNA covalent complexes were prepared and analyzed as in Fig. 3. Extension was for 30 min with 1.25 units of reverse transcriptase, 2.2 μM dATP, and 100 μM each dCTP, dTTP, dGTP. Sequencing lanes and numbering were as in Fig. 3. Lane 1, 30S-4; lane 2, 30S-5A; lane 3, 30S-5B; lane 4, 30S-4 in the absence of mRNA or tRNA during crosslinking; lane 5, 70S-1; lane 6, 70S-2; lane 7, 70S-3; lane 8, 70S-4. See Table 5 for a description of the different crosslinked preparations.

Site of crosslinking to 30S ribosomes

When crosslinking to 30S and 70S ribosomes was analyzed by reverse transcription arrest, both classes of ribosomes showed stops at C-1400 and G-1401 (Fig. 5). These stops were not present when crosslinking was done in the absence of mRNA and tRNA (see Figs. 3A and 4 for the equivalent 70S control). The results are quantitated in Table 5 which also lists the differences in the various preparations. Analysis of the data indicated that none of the various conditions used for either the 30S or 70S particles caused a distinctive distribution of stops between positions 1400 and 1401. The overall ratio for the 30S particles was 20 ± 3 percent at C-1400, while the corresponding 70S ratio was 18 ± 8 percent. The clear conclusion is that no difference in the stop distribution exists between 30S and 70S crosslinks. Since 70S, known to be crosslinked only at C-1400, gives the same distribution as 30S, we conclude that

Table 5
Percent transcription arrest at C-1400 after crosslinking under various conditions

Ribosome				Percent C-1400		
Type	nM	mRNA	NH_4^+/Mg^{++}	Expt. 1	Expt. 2	Expt. 3
30S - 4	268	poly(U_2,G)	100/15	28	20	-
30S - 5A	268	poly(U_2,G)	100/15	21	-	-
30S - 5B	268	poly(U_2,G)	100/15	17	20	-
30S - 4A	268	pGUU	100/15	-	14	-
70S - 1	311	pGUU	50/15	28	15	7
70S - 2	156	poly(U_2,G)	50/7	18	12	-
70S - 3	156	poly(U_2,G)	50/15	17	10	-
70S - 4	156	pGUU	50/15	30	8	-

Binding and crosslinking was as in *Experimental* except as indicated. Reverse transcription arrest analysis was as in Fig. 3 with 1.25 units of reverse transcriptase/reaction, 30 min extension, and 2.2 μM dATP. Gel slices were analyzed by Cerenkov counting of the ^{32}P. Percent C-1400 is the cpm at position 1400 times 100 divided by the sum of the cpm at position 1400 and 1401. Expt. 1 is shown in Fig. 5; expt. 2 is not shown; and expt. 3 is lane 1 of Fig. 3A.

30S particles are also crosslinked only at C-1400. For example, if crosslinking to C-1399 had also occurred, arrest would have been predicted at position C-1399 as well as at C-1400, but no stop at position C-1399 was detected on the gel of Fig. 5.

DISCUSSION

Crosslinking of tRNA to C-1400 of 16S RNA.

Two new aspects of the crosslinking of the anticodon of tRNA to 16S RNA have been revealed by this work. First, we have verified that the crosslinked tRNA is truly at the P site. Previous claims from our laboratory (1,10) were based on the (erroneous) assumption that tRNA crosslinked via its anticodon to the A site would be incapable of translocation to the P site and subsequent reaction with puromycin. However, studies with AcPhePhe-tRNA crosslinked via s^4U_8 to the A site showed that EFG-catalyzed translocation could occur (13). This issue has now been settled by the demonstration that >74% of the crosslinked AcVal-tRNA could react with puromycin in 1.5 hr at 0°C (Fig. 1).

Under similar conditions of ribosome excess, >10 mM Mg^{++}, and absence of added EFG, Geigenmüller *et al.* (14) showed that even after 4 hr at 0°C, only 4% of A-site bound AcPhe-tRNA had reacted with puromycin.

Second, we have shown that the 50S subunit does not stimulate either the rate or yield of crosslinking (Tables 1, 2, and Fig. 2) nor does it perturb the site of crosslinking (Fig. 5). The only effect of 50S addition is a 1.5-fold stimulation of AcVal-tRNA binding. Manderschied *et al.* (25) found that 0.4 pmole AcVal-tRNA could be bound per pmol of 30S subunits in the presence of GUA or GUAA as mRNA. In this work, using poly(U_2,G) and lower concentrations of ribosomes and tRNA, the value was 0.3 (Tables 1 and 2).

Previous studies have shown that the tRNA binding site on the 30S subunit corresponds to the P site of a 70S ribosome (26, and references therein). Our results confirm this with respect to the precise positioning of the tRNA anticodon since the same site of crosslinking to 16S RNA was found using 30S ribosomes as when the 70S P site was occupied.

Reverse transcriptase arrest at cyclobutane dimers

During the course of this work, it became necessary to characterize in more detail the nature of reverse transcriptase arrest when cyclobutane dimers are formed. We have shown here that cyclobutane dimer formation which crosslinks an intact tRNA to an intact rRNA does not completely arrest reverse transcription at the base 3' to the modification. Instead some of the time the enzyme also reads the modified residue, resulting in a characteristic doublet band. The doublet is not due to two adjacent crosslinking sites as an independent sequencing approach had previously established that less than 1% of such a second site could occur (8).

Two possible explanations suggest themselves. In the first, steric hindrance due to the cyclobutane dimer structure and/or the linked tRNA blocks the polymerase. Most of the time, stops at the base 3' to the crosslink occur, but occasionally the crosslinked base itself is read. When this occurs, only the base complementary to the crosslinked one would be expected to be inserted. Alternatively, the ring pucker introduced by saturation of the 5,6-double bond of the pyrimidine ring (27) may be sufficient to disrupt base pairing. Thus, the stop 3' to the crosslink could be explained solely as the inability to base-pair. Stops at the next residue could then arise from the occasional insertion of any base opposite the crosslinked one without base-pairing, which might then disrupt the proper orientation of the nascent 3'-end so that no further residues could be added. We favor the first explanation for several reasons. First, in model experiments dihydrouracil was shown to retain 30% of

the base-pairing ability of uracil (28), although it must be noted that the effect of the cyclobutane ring on base-pairing was not investigated. Second, nicked double-stranded DNA containing thymine-psoralen monoadducts on the template strand was found to allow read-through by DNA polymerase I holoenzyme (29). Structurally, the thymine-psoralen adduct closely resembles our crosslinked product in that both contain a fused pyrimidine-cyclobutane ring. Third, and most convincingly, Kim and colleagues (30) have obtained direct evidence by NMR for base-pairing between a thymine-psoralen adduct in one deoxyoligonucleotide and an adenine in a second oligomer. We have referred to stops rather than kinetic pauses in the above discussion since stops were shown to occur with the structurally similar psoralen monoadducts (18). In our case, this point could not be investigated since only 25-50% of the RNA molecules were crosslinked.

Doublet bands upon psoralen mono-adduct formation have been reported earlier but were ascribed to modification of two adjacent residues (18). Doublets were also seen by Barta *et al.* (19) and ascribed to crosslinking of adjacent U residues. However, as the chemistry of that crosslink is unknown, the result could be another example of stuttering. Stuttering may also have been seen by Moazed *et al.* (22) upon diethylpyrocarbonate (DEP) modification to the N7 of A-1398 of 16S RNA. A weaker stop was also detected at the adjacent C residue despite the lack of reactivity of C to DEP (31). Finally, doublet bands were seen upon crosslinking of the anticodon of tRNA to Tetrahymena ribosomes (4), but were not detected when an aryl azide was used to crosslink the 5'-anticodon tRNA residue to the C-1400 of 16S rRNA (Fig. 4).

From the data presented above, a tentative conclusion can be drawn that conditions which force extension by reverse transcriptase such as more enzyme, longer times, and increased amounts of the limiting deoxynucleoside triphosphate tend to increase stuttering. It follows that a systematic variation of transcription conditions could be used to discriminate true adjacent modifications from stuttering.

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