Organization of the 16S rRNA around its 5' terminus determined by photochemical crosslinking in the 30S ribosomal subunit

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ABSTRACT

The organization of the 5' terminus region in the 16S rRNA was investigated using a series of RNA constructs in which the 5' terminus was extended by 5 nt or was shortened to give RNA molecules that started at positions –5, +1, +5, +8, +14, or +21. The structural and functional effects of the 5' extension/truncations were determined after the RNAs were reconstituted. 30S subunits containing 16S rRNA with 5' termini at –5, +1, +5, +8 and +14 had similar structures (judged by UV-induced crosslinking) and exhibited a gradual reduction in tRNA binding activity compared to that seen with 30S subunits reconstituted with native 16S rRNA. To create the 5' terminal site-specific photocrosslinking agent, the reagent azidophenacylbromide (APAB) was attached to the 5' terminus of 16S rRNA through a guanosine monophosphorothioate and the APA-16S rRNAs were reconstituted. Crosslinking carried out with the APA revealed sites in six regions around positions 300–340, 560, 900, 1080, the 16S rRNA decoding region, and at 1330. Differences in the pattern and efficiency of crosslinking for the different constructs allow distance estimates for the crosslinked sites from nucleotide G9. These measurements provide constraints for the arrangement of the RNA elements in the 30S subunit. Similar experiments carried out in the 70S ribosome resulted in a five- to tenfold lower frequency of crosslinking. This is most likely due to a repositioning of the 5' terminus upon subunit association.

Keywords: 16S rRNA three-dimensional structure; intramolecular RNA–RNA cross-links; reverse transcription arrest assay

INTRODUCTION

The central part of the 30S ribosomal subunit that faces the 50S subunit in the working 70S ribosome contains the region essential for tRNA binding and movement. Recent cryoelectron microscopy experiments have clearly shown electron density attributable to the tRNAs in the space between the 30S and 50S subunits (Frank et al., 1995; Agrawal et al., 1996; Malhotra et al., 1998). The 30S subunit is responsible for mRNA association and the decoding process, and this requires a placement of the anticodon ends of the tRNAs in close proximity with parts of the 30S subunit (Dahlberg, 1989; Noller, 1991; Frank et al., 1995). In terms of the molecular constituents in the 30S decoding region, ribosomal proteins S4, S5, and S12 have been implicated in influencing the fidelity of decoding (see Karimi & Ehrenberg, 1994). However, there are a number of 16S rRNA nucleotides that are footprinted by tRNA or at which mutations affect the decoding and translocation

process (Noller, 1991). Photoaffinity labeling and site specific cleavage experiments by tRNA, tRNA derivatives, and mRNA analogs (see Green & Noller, 1997) also indicate important nucleotide positions. Several proposals for the three-dimensional arrangement for the 16S rRNA already place many of these sites in positions in the central part of the subunit where they could participate in the expected interactions (Malhotra & Harvey, 1994; Noller et al., 1995; Fink et al., 1996; Mueller & Brimacombe, 1997; Wang et al., 1999). However, the arrangements still are inconclusive about the proximity of some of the 16S rRNA sites with respect to the bound tRNA, and models are not available yet to describe the details of the binding pockets for the tRNAs.

The region in the 16S rRNA close to the 5' terminus plays a crucial role in the structure of the 30S subunit. Base-pairs (9–13)·(21–25) and (17–19)·(916–918) are the two interactions that define the central pseudoknot structure (Brink et al., 1993). The integrity of this structure is important for the structure, stability, and activity of the 30S subunit (Pinard et al., 1994, 1995; Poot et al., 1996). Further, the structural arrangement of the RNA within the interval 885–912 has been shown to control the fidelity of the binding of the ternary complex

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EFTu(aminoacyl-tRNA(GTP and is also important in the global structure of the 30S subunit (Lodmell & Dahlberg, 1997). The RNA element within the interval 885–912 must be a short distance from the 5' terminus in the three-dimensional structure, because of constraints determined by the secondary structure.

In an effort to find convenient methods for the analysis of the structure in the 5' terminal region, we have adopted part of the photocrosslinking approach of Nolan et al. (1993) and Harris et al. (1994, 1997). In this strategy, a guanosine monophosphorothioate (GMPS) is incorporated at the 5' terminus of the RNA during in vitro transcription and the GMPS subsequently is reacted with the reagent azidophenacyl bromide to yield the photoreactive azidophenacyl (APA) group attached to the RNA. Crosslinking from APA then can be done to obtain crosslinking sites that represent internal RNA–RNA distance measurements. A key feature of the Nolan et al. (1993) strategy is the ability to make circularly permuted molecules in which the RNA is continuous in the region that is normally its 5' and 3' termini; this allows an engineered 5' terminus to be positioned at locations throughout the RNA molecule. In 16S rRNA, the 5' and 3' ends are not joined by base pairing (as in the case for the RNase P RNA studied by the Pace group) (Nolan et al., 1993). Furthermore, the termini are not expected to be close to one another, based on the immunoelectron microscopic analysis of their locations in the 30S particle (Mochalova et al., 1982; Stoffler & Stoffler-Meilicke, 1984). Therefore we have not attempted the circular permutation of the whole 16S rRNA; instead we have concentrated on constructs with length differences in the normal 5' terminal region to obtain information about that neighborhood.

16S rRNAs were constructed with the 5' terminus extended by 5 nt or truncated by up to 20 nt. The structural integrity and functional activity of the reconstituted subunits that contain the modified 16S rRNA were examined first. For APA crosslinking, APA-derivitized RNA was reconstituted and 30S subunits were isolated. Irradiation with near-UV light resulted in crosslinking that reflects the RNA neighborhood in the vicinity of the 5' terminus in the 16S rRNA three-dimensional arrangement. From the pattern of sites, in the isolated 30S subunit, the 5' terminus must be in the

central front part of the subunit; from the data it is possible to estimate intramolecular distances from a reference nucleotide (guanosine at position 9).

RESULTS

Synthesis and reconstitution of 16S rRNA with 5' extensions and truncations

DNA for in vitro transcription was constructed by PCR reactions using a plasmid pKK3535 (Brosius et al., 1978) containing the 16S rRNA sequence. Left primers contained the promoter sequence for T7 RNA polymerase joined to sequences corresponding to the desired 5' RNA sequence. Right primers contained the 16S rRNA 3' terminal sequence joined to a *Bsu*36I restriction site; digestion of the *Bsu*36I site gives DNA templates that end at the 16S rRNA 3' terminus. Full-length PCR products were isolated and cloned into an appropriate DNA vector. RNA was prepared from plasmid DNA, after *Bsu*36I digestion, and the 5' terminal sizes and sequences were confirmed in primer extension experiments (data not shown). Table 1 contains a summary of the 5' sequences of the RNAs that were made from these DNA constructs. RNAs were synthesized in the presence of GMPS that is incorporated into the 5' terminal position. For site-specific crosslinking experiments, RNAs were reacted with azidophenacyl bromide, which specifically reacts with the sulfur of the GMPS.

Reconstitution reactions with the RNAs were done according to the procedures of Krzyzosiak et al. (1987) with total protein from 30S subunits (TP30). 30S subunits were isolated on sucrose gradients and showed profiles typical for the reconstitution of synthetic RNA (Krzyzosiak et al., 1987; Ericson et al., 1995) except for constructs +14 and +21, in which there was a much larger amount of partially reconstituted material. Typical yields for the recovery of 30S particles from reconstitution reactions were about 20–30% of the input RNA; this did not depend on whether the RNA contained APA. The yield of 30S particles was the same for the –5, +1, +5, and +8 constructs, but was reduced for the +14 and +21 constructs. The protein compositions of all constructs were determined by the reverse phase

TABLE 1. 5' terminal sequences.

Construct	Nucleotides	5' sequence		
		+1	+10	+20
–5	–5–1542	5' GGGAAAAUUGAAGAGUUUGAUC AUGG...3'		
+1	+1–1542	5' GGAUUGAAGAGUUUGAUC AUGG...3'		
+5	+5–1542	5' GGAAGAGUUUGAUC AUGG...3'		
+8	+8–1542	5' GGAGUUUGAUC AUGG...3'		
+14	+14–1542	5' GGAUC AUGG...3'		
+21	+21–1542	5' GG...3'		

HPLC method of Kervalage et al. (1983). The protein compositions were found to be the same as for native reconstituted subunits or the initial TP30 except for differences in the S12 and S21 peaks. For both S12 and S21, in some of the reconstituted subunit samples, the expected peak size was reduced by 40–60% compared to that of the input TP30 protein composition and, in the samples in which this happened, usually three extra small peaks appeared in the chromatograms. This suggested that S12 and S21 are present in the subunits in these preparations, but are subject to specific and limited proteolysis. However, among different preparations of the samples, there was no correlation between the presence of this unusual protein profile and the RNA structural arrangement determined either by UV-induced or APA-induced crosslinking.

Structural and functional characterizations of the reconstituted 30S subunits

Experiments to compare the overall structure of the reconstituted subunits to native 30S subunits and to 30S subunits reconstituted with authentic 16S rRNA were done by UV crosslinking. UV crosslinking induces a number of long-distance crosslinks in native 30S subunits and these have been separated and identified by reverse transcription analysis (Wilms et al., 1997; Noah & Wollenzien, 1998); the frequency of these should be an indicator of the correct folding of the 16S rRNA in the subunit. The reconstituted 30S subunits for this experiment did not contain APA, so that only the intrinsic intramolecular RNA crosslinking pattern would be observed. Crosslinks characteristic for native 30S subunits are seen in 30S subunits reconstituted with authentic and synthetic 16S rRNA (Fig. 1). The frequencies of several of these were determined for each of the constructs. Four of the six crosslink-containing bands that were measured do not show significant changes even in the +14 and +21 constructs. However, two other bands (the first containing the crosslinks 967×1400 together with 793×1517 and the other containing 1093×1182) in the +21 construct decrease to 20–30% of their frequency in the native reconstituted sample, consistent with the appearance in the gel. One crosslink is seen in the reconstituted subunits (in the band third from the top of the pattern) including the reconstituted authentic 16S rRNA that does not appear in native subunits. RNA from this band was purified and analyzed by primer extension experiments and found to contain a crosslink between positions C979 and C1359 (data not shown). A crosslink at C976 $\times$ C1361 is seen in native ribosomes after irradiation with 348 nm laser light (T. Shapkina, unpubl. results); therefore there is probably some structural difference in reconstituted subunits in this region.

tRNA binding assays were done to estimate the functional status of the reconstituted subunits. Two types of

FIGURE 1. Analysis of internal RNA structure by UV crosslinking. 30S subunits containing each of the RNA constructs (but not containing APA) were irradiated with far-UV light and RNA was purified, ^{32}P -labeled, and electrophoresed on denaturing polyacrylamide gels. The identification of the lanes is for native 30S subunits (N), reconstituted 30S subunits containing authentic 16S rRNA (RA), and reconstituted 30S subunits containing the indicated RNA constructs (–5, +1, +5, +8, +14, +21). Identities of crosslinks made in the native and reconstituted authentic subunits are written in the left margin. The mobility of RNA containing the C54 $\times$ A353 crosslink is sensitive to the 5' termini lengths in the different constructs. A crosslink C1402 $\times$ C1501 is present at a very low frequency in 30S subunits and is identified in parentheses.

assays were used. In the first, the kethoxal reactivity of G926 in 70S ribosomes containing the reconstituted 30S subunits was quantified in empty ribosomes and in mixtures that include poly(U) and tRNA^{Phe}. Because G926 becomes unreactive when tRNA binds the P site (Moazed & Noller, 1990), a decrease in kethoxal modification indicates the extent of tRNA binding (Ericson et al., 1995). This assay shows a gradual decrease in tRNA binding to approximately 30% (in the +21 sample) compared to the highest amount (Table 2). In the second assay, 70S ribosomes containing [^{32}P]-labeled reconstituted 30S subunits were incubated with tRNA^{Val} and a synthetic mRNA analog containing a codon for

tRNA^{Val}, and the mixtures were irradiated with 280–320 nm UV light. Ribosome-bound tRNA^{Val} is crosslinked with this frequency light to C1400 in the 16S rRNA (results not shown). Polyacrylamide gel electrophoresis separates the tRNA-16S rRNA crosslinked molecules, and the extent of crosslinking indicates the extent of tRNA binding (Fig. 2). This assay also indicates partial tRNA binding activity in the constructs, as the 5' terminus is truncated, with the lowest amount of activity again occurring in the +21 sample (Table 2).

Analysis of crosslinks made by APA attached to 16S 5' terminus in different 16S rRNA constructs

Crosslinking was carried out on reconstituted subunits containing APA-reacted 16S rRNA. Reconsti-

tuted 30S subunits were incubated in activation buffer at 37 °C and then cooled and irradiated at 10 °C with 320–380 nm light. RNA was recovered by proteinase K digestion and phenol extraction. 3'-[³²P]-labeled crosslinked RNA was then separated by electrophoresis on denaturing polyacrylamide gels (Fig. 3). Cross-linked molecules with different sized loops caused by the long-range crosslinks have reduced mobility and appear as distinct bands in the gel. Because all of the loops involve the 5' terminus, the gel mobility predicts loop size in a different way than for internal UV crosslinks (Wilms et al., 1997; see Harris et al., 1997). In fact, for the present APA crosslinks, molecules with smaller loop sizes tend to run with the slowest mobility, the opposite behavior than was seen for loops that involved sites away from the termini. For all of the constructs, except the +21 construct, 10–15% of the sample had reduced mobility indicating crosslinking. At the wavelengths used for this irradiation, all crosslinking is dependent on the addition of APA to the RNA before reconstitution (results not shown). The patterns of crosslinking have many similar features for the constructs –5, +1, +5, +8, and +14, but there are frequency differences that are seen in the PAGE analysis and in the primer extension experiments. The +21 construct shows the absence of many of the bands seen in the other samples and one particular band in the top part of the pattern is much more frequent than in the other samples.

The gels shown in Figure 3 were cut apart to separate molecules with different mobilities and the RNA was recovered by ultracentrifugation (Wilms & Wolenzien, 1994). The gel for constructs –5 to +8 were separated into 10 fractions with the most prominent bands as the principal components of each fraction. The gel for +14 and +21 were separated into eight and six fractions. Primer extension experiments were done on the isolated crosslinked RNA to identify crosslinking sites. Reverse transcription stops attributed to crosslinking should occur in specific fractions. The presence and relative frequency of these stops were judged after consideration of the intensity of the same position in control lanes and the loading of the experimental lane. The whole 16S rRNA, except for the 3' terminal 20 nt, was searched in this way. Stops were visualized with at least two primers to rule out reverse transcription artifacts. In each case sites of crosslinking were taken as 1 nt on the 5' side of the reverse transcription stopping site (Wilms et al., 1997). Images of the gel electrophoresis patterns that show the locations of crosslinking sites are shown in Figure 4.

Intramolecular crosslinks for most of the fractions could be established by reverse transcription. These are listed in the legend to Figure 3 and are summarized on the secondary structures shown in Figure 5. Crosslinking sites could not be found for RNA from fractions

FIGURE 2. UV crosslinking with tRNA^{Val}. Control and reconstituted 30S subunits containing the indicated 16S rRNA (but without APA addition) were ³²P-labeled, mixed with 50S subunits, mRNA analogue and tRNA^{Val} before incubation and UV irradiation. The UV irradiation causes internal 16S rRNA crosslinks and also intermolecular crosslinks between tRNA^{Val} and 16S rRNA and the mRNA analogue and 16S rRNA (indicated in the left margin).

FIGURE 3. Gel electrophoresis of crosslinked RNAs. Denaturing polyacrylamide gel electrophoresis was used to separate RNA from each of the six constructs after crosslinking in 30S particles. Each of the lanes containing the samples was divided into ten to six fractions, depending on the number of bands in the lane and the fraction numbers are indicated along the left of each lane. The following crosslinks were subsequently determined by primer extension experiments. Sample (-5): F2: 556-560, 562, 571, 892, 901, 906, 912-919, 923-926; F3: 1071-1072, 1080-1082, 1092; F4: 313-314; F5: 300; F6: 1392-1398, 1400-1401, 1413; F7: 1492-1493. Sample (+1): F2: 556-560, 562-563, 572-573, 916-918, 892, 906; F3: 1071-1072, 1080-1082, 1092; F4: 313-314, 345-347; F5: 1330; F6: 1392-1398, 1400-1401; F7: 1492-1493. Sample (+5): F2: 556-560, 562-563, 572-573, 892, 906, 912-921; F3: 1071-1072, 1080-1082, 1092; F4: 313-314, 335-337, 345-347; F5: 1330; F6: 1400-1401, 1413-1414; F7: 1492-1493. Sample (+8): F2: 556-560, 563, 572, 892, 906, 913-914; F3: 1071-1072, 1080-1082, 1092; F4: 1149-1151; F5: 334-335, 343-345; F6: 298; F7: 1492-1493. Sample (+14): F2: 556-560, 563, 572-573, 575-577, 892, 913-915, 921-923; F3: 1080-1083, 1092; F4: 335-338; F5: 1492-1493. Sample (+21): F1: 555-563, 569-574, 884-886, 912-913; F2: 327-328; F3: 300. Crosslinking sites were not identified for Fractions 8 and 9 in the constructs (-5) to (+8), nor for fractions 6 and 7 in (+14) construct and fractions 4 and 5 in the (+21) construct.

eight and nine for constructs -5, +1, +5, and +8 and RNA from fractions five and six for construct +14 and for RNA from fraction four for construct +21. Fraction eight (in constructs -5, +1, +5, and +8) contains a frequent crosslink and if it is similar in size to that of the adjacent fraction 7, the crosslink should be at position 1500 or greater. However, neither an RNase H cleavage experiment (Rinke-Appel et al., 1991) with a DNA oligonucleotide complementary to 16S rRNA 1416-1436 nor a partial alkaline hydrolysis experiment (Juzumiene et al., 1995) were able to detect a cross-

linking site in the 3' terminal region. Fraction nine may contain dimerized 16S, because it has the mobility of 23S rRNA or twice the size of 16S, and supports reverse transcription with primers against 16S, but gives no specific stops that would indicate crosslinks. Except for these bands, there is an agreement between the amount of the RNA in a particular band and the strength of the corresponding reverse transcription stop attributed to it. These results have also been summarized in Table 3, in which the relative frequency of the band and the crosslinking sites are listed.

Differences in APA crosslinking in the 30S subunit, 70S ribosome and 16S rRNA

Experiments were done with three of the constructs to determine if there were similar patterns of crosslinking in 70S ribosomes and 30S subunits. In the first experiment, APA-derivitized (−5) 16S rRNA was reconstituted into 30S particles and the subunits were used in an association experiment. 50S subunits and the reconstituted APA-containing 30S subunits were mixed and incubated under conditions that favor 70S formation. The mixture was irradiated and then separated on sucrose gradients to isolate 70S ribosomes and 50S and 30S subunits that failed to associate. RNA was isolated from each of the fractions, [³²P]-end-labeled, and analyzed by PAGE. Controls for the experiment were APA-containing 16S rRNA that was incubated in activation buffer and irradiated and a sample of the reconstituted 30S subunits (APA-containing) that were irradiated. Before association, the isolated 30S subunits show the pattern of crosslinking (the second lane in Fig. 6A, labeled 30S) described before. The 16S rRNA sample without reconstitution shows a pattern of crosslinking similar to that seen in the 30S sample (Fig. 6A, lane labeled 16S). (Reverse transcription has not been done on these crosslinked molecules, so we do not know if the locations of the crosslinks are the same as seen in the 30S subunit.) The mixture of 30S plus 50S subunits that was incubated, irradiated, and extracted for analysis before separation (Fig. 6A, lane Mix 30S + 50S—Tot.), showed normal 16S rRNA crosslinking. When the 70S fraction from the sucrose gradient was analyzed, linear 23S rRNA and 16S rRNA were seen, but there was very low intensity of bands that indicate crosslinking within the 16S rRNA (Fig. 6A, lane Mix 30S + 50S—70S). At long exposures of the gel light crosslinking can be seen in the 70S fraction. This is estimated to be about 5–10 times lower than the

amount of crosslinking seen in the 30S sample. When the 30S fraction from the sucrose gradient was analyzed (Fig. 6A, lane Mix 30S + 50S—30S), 16S rRNA showed the normal degree of crosslinking.

The association experiment has been repeated for the −5, +5, and +8 16S rRNA constructs (Fig. 6B). In all three instances after incubating the samples for association, irradiation, and separation on sucrose gradients, 16S rRNA from the 30S fractions shows the normal pattern of crosslinking expected for each of the constructs, but 16S rRNA from the 70S fraction showed very much reduced levels of crosslinking (Fig. 6B). Because the incorporation of GMPS during *in vitro* transcription is not complete, some reconstituted 30S subunits do not contain APA and it was possible that these were the only subunits capable of associating to form 70S ribosomes. An experiment was done to determine whether APA was present in the 30S of associated 70S ribosomes; this took advantage of the observation that APA was active in crosslinking in protein-free 16S rRNA (see Fig. 6A, lane 16S). 30S subunits containing APA were incubated with 50S subunits, and the mixtures were separated on sucrose gradients. RNA from the 70S fraction was extracted and irradiated. The lanes marked 16S from 70S in Figure 6B show that the samples from the +8, +5, and −5 constructs show crosslinking in the 16S rRNA. This demonstrates that APA was present in the 16S rRNA in the reassociated 70S ribosomes. Another explanation for the low levels of RNA crosslinking in 70S ribosomes could be an increase in reactivity with proteins. The level of crosslinking of APA-containing [³²P] 16S rRNA to proteins was determined in the 30S versus 70S ribosome by examination of labeled proteins on SDS PAGE gels. No significant qualitative differences were seen (results not shown). These experiments show that 30S subunits containing APA are capable of subunit association, but that in the 70S ribosomes, the APA must be in a con-

TABLE 2. tRNA-binding and UV-crosslinking frequency for different 16S rRNA constructs.

tRNA binding assay	Relative activity (%) of reconstituted 30S subunits						
	Rec. Auth. ^a	−5	+1	+5	+8	+14	+21
G926 reactivity ± tRNA	100	65	51	55	53	39	30
tRNA ^{Val} crosslink to C1400	100	79	39	40	34	70	20
UV crosslink	Relative crosslink frequency (%)						
	Rec. Auth.	−5	+1	+5	+8	+14	+21
C54 × A353	100	105	87	92	76	86	86
C582 × G760	100	142	110	67	138	123	119
C967 C967 × C1400							
+ U793 × G1517	100	99	94	55	61	73	34
U991 × U1212	100	82	94	70	91	76	88
U1126 × C1281	100	112	125	92	125	114	90
A1093 × G1182	100	41	85	50	49	85	30

^aRec. Auth. column contains results from 30S subunits reconstituted with authentic 16S rRNA and was taken to represent 100% activity in tRNA binding and in the frequency of each UV-induced crosslink.

figuration that does not lead to the same degree of internal 16S rRNA crosslinking.

DISCUSSION

The extension and truncation of the 16S rRNA 5' terminus, and the subsequent analysis of the reconstituted 30S particles, was done to determine the effects of the length of the 5' terminal single-stranded region on the function and structure of the 30S subunit. For constructs that show sufficient similarity to native sub-

units, the 5' end can then be used to estimate distances to other rRNA sites in its neighborhood. In fact, the decrease in tRNA binding activity in the constructs with termini positions of -5 to +21 is gradual. The fact that there is reduction in tRNA binding activity for the +5, +8 constructs that only removes nucleotides in the 5' terminal single-stranded region without altering the secondary structure indicates some role for that part of the 16S rRNA in the status of the P tRNA binding site. Interestingly, there does not seem to be an equal gradual loss in the structural integrity of the subunits in the same truncation series, judged by the pat-

FIGURE 4. (Continues on facing page.)

tern of UV-light-induced crosslink in the 16S rRNA that monitors the general global 16S rRNA structure. Only the +21 construct was significantly altered at a number of sites, reflecting a partial unfolding of the 30S subunit. This is consistent with reports of the importance of the central pseudoknot structure in the folding and function of the 16S rRNA in the subunit (Pinard et al., 1995; Poot et al., 1996).

We conclude that 30S subunits containing the -5 to +8 constructs are structurally intact and possess a significant, but not full, activity in tRNA binding. We choose G9, the nucleotide in the first secondary structure element as a reference point and we will use the cross-

linking data to estimate distances from the crosslinking sites to G9. Phosphate-phosphate distances are 5.9 Å and 7.0 Å in the C3' endo and C2' endo conformation of the nucleotide unit (Saenger, 1984). If a value of 6 Å is taken as an approximate average distance of a nucleotide unit in the unstructured 5' terminal region, the reactive nitrene of GMPS-APA in the +8 construct is a maximum physical distance of about 16 Å from the phosphate group of nucleotide G9. Using these same values, distances of crosslinking in the +5, +1, and -5 constructs are about 34, 58 and 88 Å from the phosphate of nucleotide G9. It is difficult to assign distances of crosslinking for the +14 and +21 constructs, be-

FIGURE 4. Analysis of crosslinking sites by primer extension on crosslinked fractionated 16S rRNA. RNA from the fractions indicated in Figure 3 was isolated and subjected to primer extension analysis using reverse transcriptase and a series of DNA oligonucleotide primers. Six regions of the 16S rRNA contained crosslinking sites. Nucleotide positions determined by sequencing reactions (lanes UGCA in each panel) are shown in the left margins. The nucleotide numbers of crosslinking sites or intervals are listed on the right margins. Small dots have been placed in the figures on the right of each lane (except the lane in **B**, sample 14, fraction 2, where dots were placed on the left) to indicate bands that are significantly darker than control lanes and indicate crosslinking sites. Sample numbers and fraction numbers are listed at the top of the lanes. The nucleotide involved in the crosslink in all cases is 1 nt in the 5' direction from the reverse transcription.

FIGURE 5. Summary of crosslinking sites for the six RNA constructs. The diagram summarizes patterns of APA crosslinking in each of the six constructs in reconstituted 30S subunit. All sites crosslinked are indicated without regard to the relative intensity. The structure of each of the RNAs at their 5' termini is shown. For the +14 and +21 constructs we assume that the truncations result in the elimination of first and second base-paired regions without another rearrangement.

cause the secondary structure in the 5' terminal region must be altered and the global subunit structure is altered (at least in the +21 construct).

Given the patterns of crosslinking and estimated crosslinking distances, the distances between the crosslinking sites and G9 were estimated (Table 3). Several assumptions were made to create this table. First, it may not be a complete list of the neighborhood, as it is unlikely the crosslinking agent can reach or react with all sites within the specified radius. In fact, there are a few sites that are crosslinked by the short- or intermediate-length constructs but not by longer length constructs (see Table 3); this means that the crosslinker may be somewhat inflexible and overshoot some sites. Second, the distances listed are the length of the extended reagent based on its chemical structure—crosslinking may occur at distances greater than these estimates due to molecular motions. Last, there may be structural heterogeneity in the 30S subunit so that a number of different conformations are being sampled simultaneously during the irradiation—this could lead to an underestimate of some distances.

The crosslinking sites can be separated into six groups according to their locations in the secondary structure: I: sites in the interval 298–347; II: sites around 556–577; III: 892, 901–906, and 912–926; IV: sites at 1071–1072, 1080–1082, 1092, 1149–1151; V: sites within the decoding region (1392–1413 and 1492/1493); VI: nucleotide 1330. There is one other interval containing crosslinks at 884–886; these occur only in the +21 construct and we make no distance estimate for these to nucleotide G9 in the native structure.

Existing structural information helps locate the 5' terminal region in the 30S subunit. Proteins S4, S5, S12, and S16 (Stern et al., 1988; Powers & Noller, 1995) have footprints close to the 5' terminus and these proteins are located in the middle left to middle low part of the subunit (Capel et al., 1987) when it is viewed from the 50S interface side. An immunoelectron microscopy experiment was done to locate the 5' end of the 16S rRNA in the 70S ribosome, and a position in the lower left side of the subunit was described (Mochalova et al., 1982). Positions for several of the nucleotides crosslinked to G9 in this study (906, 1392–1398, 1400–

FIGURE 6. Comparison of the conformation of the 5' terminal region in 30S versus 70S ribosomes. **A:** APA-derivitized 16S rRNA (the +5 construct) was irradiated as protein-free RNA (lane 16S) and after reconstitution and isolation of 30S subunits (lane 30S). To test for crosslinking in 30S versus 70S ribosomes, 30S and 50S subunits were incubated, irradiated, and then separated on sucrose gradients. RNA was isolated from 30S, 50S, and 70S particles, labeled and examined by PAGE. Tot is RNA from the 30S + 50S mixture before sucrose gradient separation, 70S, 50S, and 30 are RNA samples from the corresponding parts of the sucrose gradient. **B:** Comparison of crosslinking in 30S versus 70S for the +8, +5, and -5 constructs. 30S subunits containing APA-modified 16S rRNA were prepared, mixed with 50S subunits, irradiated, and then separated on sucrose gradients. Lanes marked 30S and 70S contain RNA from corresponding parts of sucrose gradients for each construct. The lanes labeled 16S from 70S contain RNA that was isolated from 70S ribosomes, deproteinated, and then irradiated before PAGE analysis.

TABLE 3. Summary of the crosslinks and distance estimates from nucleotide G9.

Site	Crosslinking in constructs						Distance estimated (Å) to nucleotide G9	Comments
	-5	+1	+5	+8	+14	+21		
298–300	+			++		++	~16	Not seen in +1, +5
313–314	+	+	+				16–34	
327–328						+	—	Not in native structure
334–338			+	++	+++		~16	Not seen in -5, +1
343–347		+	+	++			~16	Not seen in -5
556–563	+	+	+	++	++	+++	≤16	
570–577	+	+	+	+	++	+++	≤16	
892, 906	+	+	+	+	+		≤16	
901	+						58–88	See Discussion for comment
912–920	++	+	++	+	+	+	≤16	
921–926	++				+		58–88	
1071–1072	+	+	+	+			≤16	
1080–1083	+	+	+	+	+		≤16	
1092	+	+	+	+	+		≤16	
1149–1151				+			≤16	Not seen in -5, +1, +5
1392–1398	++	+					34–58	
1400–1401	++	++	+				16–34	
1413	+		+				16–34	Not seen in +1
1492–1493	+	+	+	+	++		~16	
1330		+	+				16–34	Not seen in -5
884–886						++		Not in native structure

Crosslink frequency is scored as (+) moderate, (++) strong, (+++) very strong. Distance estimates are based on distances of the crosslinking group from the phosphate of G9: for constructs -5, +1, +5, +8 these are 88 Å, 58 Å, 34 Å, and 16 Å. The constructs +14 and +21 have defective function or structure and crosslinks that occur only in them are not used for distances estimates. The list shows the sites in groups of nucleotide positions that are close in the secondary structure.

1401, 1413, 1492–1493, 1330) have been determined as being tRNA footprinted sites (Moazed & Noller, 1986, 1990) and are expected therefore to be located in the front (interface) middle of the 30S subunit.

Three of the crosslinking sites in the first group, 298–300 [in helix H11 (helix numbering is according to Malhotra & Harvey (1994)], 334–338 (in helix H13), and 343–347 (in helix H14) based on their crosslinking in the +8 construct are within 16 Å of the G9 nucleotide. The 313–314 crosslinking site (in helix H6) is 16–34 Å from G9. A UV crosslink 244 × 894 (Stiege et al., 1986; Wilms et al., 1997) and a chemical crosslink 31 × 306 (Atmadja et al., 1986) place strong constraints on the locations of H6 and H12, which must be close to the middle front of the subunit. Chemical crosslinking done in the protein-free 16S rRNA included crosslinks that were between nucleotides at about 340 and nucleotides close to the 5' and 3' termini (Expert-Bezancon & Wollenzien, 1985; Wollenzien et al., 1985). In addition, site-directed cleavage experiments in which the BABE reagent (Rana & Meares, 1991) was added to Cys C31 of protein S4 (Heilik et al., 1995) or was added to Cys99 of protein S5 (Noller et al., 1995) produced cleavages in the 16S rRNA in the interval 297–301. S4 and S5 have been positioned in the middle left and middle of the subunit based on neutron scattering experiments (Capel et al., 1987). Thus the APA crosslinking results confirm the locations of helices H6 and H11 and indi-

cate new distances for helices H13 and H14 to G9 in the center of the subunit.

Sites in the intervals 556–563 and 912–920 are crosslinked in all of the constructs indicating a distance to G9 of less than 16 Å. Crosslinking of 892 and 906 in helix H27 also indicates a distance of less than 16 Å to G9. The crosslink involving 901 occurs only in the -5 construct; this suggests a much longer distance of 58–88 Å. However, this would be physically unfeasible and there must be other reasons for the absence of crosslinking to 901 in the other constructs. The sites 892 and 906 in helix H27 are expected to be in the front middle of the subunit based on a UV-induced crosslink between 894 and 1468 (Wilms et al., 1997). In addition, interactions between H27 and the decoding region are suspected based on the known importance of H27 in the decoding process (Lodmell & Dahlberg, 1997) and on streptomycin binding studies (Spickler et al., 1997). Nucleotides in the interval 923–926 are located at a longer distance from G9 as 923–926 is crosslinked only by the -5 construct. Some abrupt bend or turn in the RNA strand at the central pseudoknot (17–19)((916–918) may be responsible for this apparently much longer distance to 923–926 compared to 912–920.

A set of sites at 1071–1072, 1080–1082, and 1092 in helices H35, H36, and H37 in domain 3 of the 16S rRNA are APA crosslinking sites in the -5, +1, +5, and

+8 constructs. There is one additional site 1149–1151 that is crosslinked with only the +8 construct (Table 3). There has been only circumstantial evidence for the location and arrangement of these sites. Helix H34, adjacent to H35–H37, is positioned close to the decoding region in the front middle of the subunit based on results that involve crosslinks from position +5 mRNA to nucleotides 1052 (Rinke-Appel et al., 1993), from +11 in the mRNA to position 1196 (Juzumiene et al., 1995), from +2 in the mRNA to nucleotide 1080 in 30S subunits reconstituted with synthetic 16S rRNA (D. Juzumiene, unpubl. results), and from +2 in the mRNA to 1167–1168 in native 30S subunits (D. Juzumiene, unpubl. results). Other mRNA crosslinking sites in the region occur at 1156 (Bhangu & Wollenzien, 1992) and 1131–1132 (Wollenzien et al., 1991), but the location of these sites with respect to the decoding site was not known. A site-directed cleavage experiment was done in which the BABE reagent was tethered to position 922; it cleaved nucleotides in the interval 1192–1198 (Samaha et al., 1999). This places the 1192–1198 region not more than 22 Å from 922. In another site-directed cleavage experiment, in which Cys 21 of protein S5 was modified with the BABE reagent, cleavage in the 16S rRNA was observed in the intervals 1062–1066 and 1090–1096 as well as at sites around the central pseudoknot at 920, 1400, 535, and 420 regions. This indicates that part of domain 3 is close to the position of S5 in center of the subunit (Noller et al., 1995). Nucleotides in the 1060–1110 interval have also been positioned in the upper left of the subunit above the constriction that separates the body from the head subunit based on their association with S2 and S3 (Stern et al., 1989; Powers & Noller, 1995) and the positions of those proteins (Capel et al., 1987). The surprising part of the APA crosslinking result is the very small distance between the sites 1071–1072, 1080–1082, 1092, and 1149–1151 in this region and G9. This indicates the H35, H36, and H37 helices have to be very close to the middle part of the subunit. There has been speculation about the flexibility of the head of the subunit around helix 28 (see Wilson & Noller, 1998). A rotational motion of the subunit head would allow the H35 top H37 helices to be close to the front middle of the subunit and this may account for the APA crosslinking to these sites.

Several sites in the decoding region are subject to crosslinking in a way that suggests their locations and orientations. Nucleotides 1492–1493 are crosslinked in constructs –5 to +8, but there is somewhat of a reduction in the frequency for the +8 crosslink (see Fig. 2), so we conclude the distance for 1492–1493 to G9 is about 16 Å. 1400–1401 and 1413 are not crosslinked at the same frequency as 1492–1493 in the +8 construct, so they both may be somewhat greater distance (16–34 Å). The sites 1400–1401 and 1413 are each about one half turn away from 1492–1493 and if the

region were helical, all three sites would be approximately facing the same direction. Nucleotides in the interval 1392–1398 are crosslinked in constructs –5, +1, and +5, but not in +8, suggesting distances of 34–58 Å to these sites.

One other crosslinked site is 1330. This nucleotide is the only one in this part of the domain III RNA that is crosslinked. It is associated with the S7 binding, which is known to be in the head of the subunit on the surface facing the 50S subunit (Capel et al., 1987). The 1330 site is crosslinked in the +1 and +5 constructs (but not in the –5 construct) suggesting a distance of 16–34 Å from G9 to 1330.

Altogether, the crosslinks listed here provide new distance constraints for the RNA folding in the central part of the subunit. Given the large number of crosslinking sites in both domains I and III and the relatively short crosslinking distances, the 5' terminal region (specifically nucleotide G9), must be centrally located in the subunit and accessible to both the front of the body and head of the subunit.

The frequency of APA crosslinking in all of the constructs tested is very greatly reduced in the 70S ribosomes compared to 30S subunits. The spatial arrangement of the APA crosslinking sites with respect to G9 therefore might only apply to the 30S subunit. However, UV-induced crosslinking experiments in 30S versus 70S ribosomes do not indicate a rearrangement of the 16S rRNA during 70S formation except for crosslinking frequency differences in the decoding region (Noah & Wollenzien, 1998). Cryoelectron microscopy also has been used to investigate structural changes during association (Lata et al. 1996; Agrawal et al., 1999). There are measurable shape differences in the 30S subunit upon association. These are located in shoulder, neck, and platform regions and are approximately 5-Å changes in the surfaces and approximately a 13° change in the tilt of the head toward the shoulder (Agrawal et al., 1999). Thus it is unlikely that the changes are large enough to account for the nearly complete disappearance of APA crosslinking. We confirmed that APA is present in the 70S ribosomes. This suggests that the APA moiety on the 5' terminus changes its location during the association process from its position in the front middle of the subunit to a position a significant distance away. Immunoelectron microscopy determined a location for the 5' terminus on the surface of the 30S subunit in the lower left side of the subunit body (Mochalova et al., 1982). This is likely to be a location in which APA would not be able to reach sites in the decoding region or in the front of the head and body of the subunit and could account for the decreases in APA crosslinking. Therefore, the 5' APA probe may not be suitable for monitoring the 16S conformation in the 70S ribosome. However, it may be of use for investigating the structure of the 30S subunit during its function in the translation initiation reaction.

Newcomb and Noller (1999) described directed hydroxyl radical cleavage from three positions in the 16S rRNA in 70S ribosomes. In one of those experiments, the BABE probe was tethered to the 5' terminus; results from that experiment indicated cleavages at a series of sites in the interval 600–640 as well as at positions close to the probe tether site. The strength of cleavage was described as strong at some sites, so this indicates a distance of 0–22 Å from the point of attachment of the probe (Joseph et al., 1997). We have not seen any crosslinking in the interval 600–640 in the 30S subunit or in 70S ribosomes, so it is possible that these sites are outside of the reach of the APA crosslinking probe in the 30S subunit, even in the –5 construct, the longest construct we have tested. Alternatively, the 600–640 region may be within reach of APA but is simply not reactive for crosslinking.

As a control to verify the presence and reactivity of APA in the 16S rRNA, experiments were done in which the synthetic protein-free RNA was incubated in activation buffer, cooled, and irradiated. Analysis of that sample by PAGE showed a pattern of bands similar to those seen for crosslinking in the subunit. Reverse transcription analysis has not been done on these crosslinked molecules, so the extent to which the interactions are the same before and after reconstitution is not known. An experiment relevant to this has been done by Montpetit et al., (1998) in which domain III of 16S rRNA was prepared by *in vitro* transcription and then APA was added to the 5' GMPS residue incorporated at its 5' terminus. The protein-free RNA was crosslinked and examined by gel electrophoresis and reverse transcription analysis. Specific crosslinks were found in that experiment and were consistent with other structural information determined in the 30S subunit (Montpetit et al., 1998). This indicates the protein-free rRNA of domain III has a specific three-dimensional structure similar to its fold in the 30S subunit.

MATERIALS AND METHODS

DNA templates for 16S rRNA constructs

Six 16S rRNA constructs carrying different 5' ends were used in this work: extended by 5 nt, normal length (with nucleotides A1 and A2 changed to Gs), or truncated by 4, 7, 13, and 20 nt. DNA templates for these RNAs were obtained by PCR from plasmid pKK3535 containing the *rrnB* operon (Brosius et al., 1978) using overhanging left primer with T7 RNA polymerase promoter and right primer with a *Bsu*36I restriction site. PCR was done in 100 µL reaction mix containing 50 ng pKK3535, 10 mM Tris HCl, pH 8.4, 50 mM KCl, 0.001% Triton X-100, 1.5 mM MgCl₂, 0.2 mM each dNTPs, 40 pmol each kinased primer and 5 U Taq DNA polymerase. Synthesis was carried out for 25 cycles [denaturation (95° for 30 s), annealing (40° for 30 s), and extension (72° for 30 s)] and PCR

products were agarose-gel purified and isolated using a GeneCleanII kit (BIO 101). DNA fragments were ligated into the pTZ19U phagemid (for the –5 16S rRNA construct) or pUC18 plasmid (for the other 16S rRNA constructs). The plasmids were previously cut with *Sma*I to produce blunt ends and treated with CIAP to have 5' OH in order to prevent self-ligation. White clones were screened for restriction sites; size and the edges of the inserts were sequenced.

Preparation of 16S rRNA constructs

16S rRNA constructs were synthesized by *in vitro* run-off transcription reactions using the Ribomax protocol from plasmid DNA cut with *Bsu*36I. The six 16S rRNA constructs were synthesized with “normal” phosphorus on the 5' end or with 5' guanosine monophosphorothioate (Burgin & Pace, 1990). After DNase I digestion, RNA transcripts were phenol and ether extracted, concentrated with Centricon-100 columns (Amicon), and ethanol precipitated. The usual yield from a 500-µL reaction was 300–500 µg RNA. 16S rRNA constructs with 5' GMPS were used for azidophenacyl bromide (APAB) addition according to the procedure of Stade et al. (1989).

Reconstitution of 30S ribosomes

Two hundred micrograms of each 16S rRNA construct were reconstituted with TP30 ribosomal proteins according Krzyzosiak et al. (1987). Reconstitution mixes were sedimented on 10–30% linear sucrose gradients and fractions containing 30S particles were pooled, concentrated by ultracentrifugation, and stored in the activation buffer (20 mM Tris HCl, pH 7.5, 200 mM NH₄Cl, 20 mM MgCl₂, 2 mM DTT) at –80°C. Approximately 75 pmol (1 A₂₆₀ unit) reconstituted 30S subunits were obtained from the sucrose gradients. For most experiments reconstituted 30S were 3' end labeled with [³²P]-pCp and T4 RNA ligase (Krzyzosiak et al., 1987). Unligated [³²P]-pCp was removed by centrifugation and washing with activation buffer on Microcon-10 columns (Amicon).

UV crosslinking

Ten picomoles reconstituted 30S subunits containing the different 16S rRNA constructs (without 5' APA modification and 3' [³²P]-labeled with pCp) were heated 20 min at 37°C to activate them and were irradiated with 280–320 nm UV light at 4°C (Wilms et al., 1997). In experiments to study tRNA binding, 10 pmol of 3' end-labeled 30S in 50 µL activation buffer first was heated 20 min at 37°C and then were incubated with 10 pmol of 50S. tRNA^{Val} crosslinking to 16S rRNA was done with the associated 70S ribosomes, 50 pmol mRNA analog (54 nt long with codon for tRNA^{Val}) and 50 pmol tRNA^{Val}. Complexes were formed for 30 min at 37°C and irradiated with the same far-UV light. RNA from irradiated ribosomes was incubated for 30 min at 37°C with 2% SDS, 20 mM EDTA and 1 mg/mL proteinase K, extracted with phenol/ether and was then ethanol precipitated. 16S rRNA was purified on 1% agarose gel to remove fragmented 16S rRNA and 23S rRNA. Crosslinked 16S rRNA was separated by gel electrophoresis on gels made with 3.6% acrylamide: bis-acrylamide (70:1), 8.3 M urea in BTBE buffer (30 mM bis-Tris, pH 6.8, 30 mM boric acid, 2.5 mM EDTA). The gel was

run for 18 h in BTBE buffer at 10 W (Hoeffer Scientific apparatus) or at 6 W (Owl Scientific) keeping the temperature at 45 °C (Wilms et al. 1997).

30S ribosomes carrying APA on the 5' end of 16S rRNA were irradiated with the CoNO₃-filtered mercury light source that provides light in the range 320–385 nm (Isaacs et al. 1977). Seventy-five picomoles of reconstituted 30S in 375 µL activation buffer were activated for 20 min at 37 °C, cooled on the ice, and irradiated for 10 min at 4 °C. 16S rRNA was extracted from irradiated samples as described above and part of it was 3' end labeled with [³²P]-pCp (England & Uhlenbeck, 1978). Crosslinked 16S rRNA species were separated on the same kind of gel, except 4% acrylamide:bis-acrylamide (40:1) was used. After the electrophoresis (18 h at 10 W and 45 °C using Hoeffer Scientific thermostatted apparatus), cross-linked and uncrosslinked 16S rRNA was detected with a phosphorimager system and extracted from the gel by ultracentrifugation through 2 M CsCl₂, 0.2 M EDTA, pH 7.4, cushions for 12 h at 40,000 rpm (Wilms & Wollenzien, 1994). RNA pellets were dissolved in H₂O, phenol extracted, ethanol precipitated, and used for reverse transcription analysis.

Reverse transcription analysis

16S rRNA crosslinked nucleotides were determined by primer extension using AMV reverse transcriptase as described previously (Bhangu & Wollenzien, 1992). Using different DNA primers, it is possible to read the whole 16S rRNA except for the terminal 20 nt, as the synthetic RNAs do not have the dimethylated A1518 and A1519 nucleotides that arrest reverse transcriptase. cDNA usually was electrophoresed on 8% acrylamide:bis-acrylamide (19:1), 8.3 M urea in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3) gels.

30S association with 50S and separation on sucrose gradients

Fifty picomoles of reconstituted 30S were mixed with 50 pmol 50S subunits in 200 µL activation buffer (20 mM Tris HCl, pH 7.5; 200 mM NH₄Cl; 20 mM MgCl₂; 3 mM β-mercaptoethanol). After 30 min incubation at 37 °C, the reaction mix was irradiated at 320–380 nm UV light and loaded on the linear 10–40% sucrose gradients in activation buffer. Ultracentrifugation was done in an SW40 rotor for 18 h at 25K rpm. Fractions containing associated 70S and not associated 50S and 30S subunits were collected and ethanol precipitated. Total RNA from those ribosomes was extracted by proteinase K/SDS/EDTA treatment and 3' end labeled with [³²P]-pCp and T4 RNA ligase.

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