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Neighborhood of 16S rRNA nucleotides U788/U789 in the 30S ribosomal subunit determined by site-directed crosslinking

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ABSTRACT

Site-specific photo crosslinking has been used to investigate the RNA neighborhood of 16S rRNA positions U788/U789 in *Escherichia coli* 30S subunits. For these studies, site-specific psoralen (SSP) which contains a sulfhydryl group on a 17 Å side chain was first added to nucleotides U788/U789 using a complementary guide DNA by annealing and phototransfer. Modified RNA was purified from the DNA and unmodified RNA. For some experiments, the SSP, which normally crosslinks at an 8 Å distance, was derivitized with azidophenacylbromide (APAB) resulting in the photoreactive azido moiety at a maximum of 25 Å from the 4′ position on psoralen (SSP25APA). 16S rRNA containing SSP, SSP25APA or control 16S rRNA were reconstituted and 30S particles were isolated. The reconstituted subunits containing SSP or SSP25APA had normal protein composition, were active in tRNA binding and had the usual pattern of chemical reactivity except for increased kethoxal reactivity at G791 and modest changes in four other regions. Irradiation of the derivatized 30S subunits in activation buffer produced several intramolecular RNA crosslinks that were visualized and separated by gel electrophoresis and characterized by primer extension. Four major crosslink sites made by the SSP reagent were identified at positions U561/U562, U920/U921, C866 and U723; a fifth major crosslink at G693 was identified when the SSP25APA reagent was used. A number of additional crosslinks of lower frequency were seen, particularly with the APA reagent. These data indicate a central location close to the decoding region and central pseudoknot for nucleotides U788/U789 in the activated 30S subunit.

Keywords: 16S rRNA three-dimensional structure; chemical probing; intramolecular RNA–RNA crosslinks; molecular modeling; reverse transcription arrest assay

INTRODUCTION

The first secondary structure for the complete 16S rRNA was proposed in 1979 as a result of comparison of the *Escherichia coli* and *Bacillus brevis* sequences (Noller, 1980; Woese et al., 1980). Since that time, the secondary structure has been confirmed and elaborated using the large number of sequences in the rRNA database and presently nearly all of the secondary structure base pairs and some tertiary structure base pairs are supported by sequence covariances (Gutell et al., 1994). However, the 16S rRNA sequence comparison does not indicate many covariances between nucleotides distant in its secondary structure that would indicate its three dimensional structure. Many different approaches have been used to obtain information pertaining to the rRNA higher order structure. Biochemical experiments that have been most successful are (1) photoaffinity experiments, cleavage experiments, and footprinting experiments utilizing mRNA and tRNA (see Green & Noller, 1997) and (2) structural experiments involving the ribosomal proteins either to obtain footprints for the proteins (Stern et al., 1989; Powers & Noller, 1995) or by using the proteins to carry cleavage reagents into the subunit (Heilek et al., 1995; Noller et al., 1995). There would be several advantages to studying the rRNA structure with reagents that are in the rRNA. The rRNA is a highly compact structure (Serdyuk et al., 1983; Frank, 1997; Stark et al., 1997), so there should be a high density of RNA–RNA contacts. Changes in these may be correlated to changes in the ribosome structure, even if there are no changes in RNA–ribosomal protein contacts. In addition, data from reporter reagents in the ribosome rather than in mRNA or tRNA substrates will be inherently simpler to use for molecular modeling, since the location and flexibility of the tRNA or mRNA does not have to be considered as part of the modeled structure.

UV light irradiation produces 15 long-range intramolecular crosslinks in the 16S rRNA (Wilms et al.,

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1997). These provide some new information about its internal three dimensional arrangement and the crosslinking technique provides an opportunity to monitor conformational changes (Noah & Wollenzien, 1998). However, the relatively small number of UV crosslinks puts some limits on that approach. In the present article we describe the use of site-directed crosslinking using a psoralen derivative as the photoreagent (Teare & Wollenzien, 1989, 1990). In this method, psoralen first is linked to an oligonucleotide, the psoralen–oligonucleotide is annealed to the rRNA and the psoralen moiety is phototransferred to the RNA target site. 16S rRNA containing the psoralen adduct is then purified and reconstituted into 30S subunits. The advantages of the method are that all the 30S subunits contain psoralen, so the effects of the psoralen on the structure and function of the subunit can be determined independently of the experiments involving crosslinking, and relatively large amounts of reconstituted 30S subunits can be prepared. In addition, the site-specific psoralen that is used in the experiment contains a reactive sulfhydryl group which can be further derivatized with other types of reporter reagents before reconstitution of the 30S subunits.

The site chosen in the present experiment is U788 in the 790 loop of the 16S rRNA. This region is present in all small subunit RNAs and is highly conserved in sequence. It has been implicated in several important 30S functions including subunit association (Chapman & Noller, 1977; Herr et al., 1979; Santer et al., 1990), IF3 binding (Muralikrishna & Wickstrom, 1989; Moazed et al., 1995; Tapprich & Hill, 1986; Tapprich et al., 1989), and tRNA binding (Moazed & Noller, 1990). The physical location for the region in the subunit has been investigated by DNA hybridization electron microscopy and was proposed to be in the end of the 30S subunit platform structure (Oakes & Lake, 1990); however, there is a significant distance between the decoding region and the proposed site, and it has been difficult to reconcile the rRNA segment location with its function. The crosslinks made by the site-specific psoralen (SSP) reagent in these experiments are with a number of RNA sites that must be in the central part of the 30S subunit and this indicates a location for the nucleotides U788/U789 in the central part of the subunit much closer to the decoding region than previously thought.

RESULTS

Reaction of psoralen at U788/U789 in 16S rRNA and reconstitution of the modified 16S rRNA

Site-specific psoralen was covalently placed at a specific location in the 16S rRNA by irradiation of a complex containing 16S rRNA and the SSP linked to a DNA oligonucleotide complementary to the region of the target RNA. For synthesis of the SSP–DNA, the DNA was reacted first with cystamine and purified, and then the cystamine–DNA was reduced so that the SSP could be added to it through a disulfide bond (Fig. 1). The sequence of the DNA was designed so that when it is annealed to the RNA, it would cross the RNA target site (which needs to be a uridine) to create a favorable place for psoralen intercalation. This arrangement was previously shown to be optimum for directing SSP reaction at its 4′–5′ double bond onto the uridine nucleotide next to the unpaired nucleotide in the hybrid structure (Teare & Wollenzien, 1989). The annealed complex was exposed to a $1\frac{1}{2}$ min irradiation with 320–380 nm light, resulting in preferential activa-

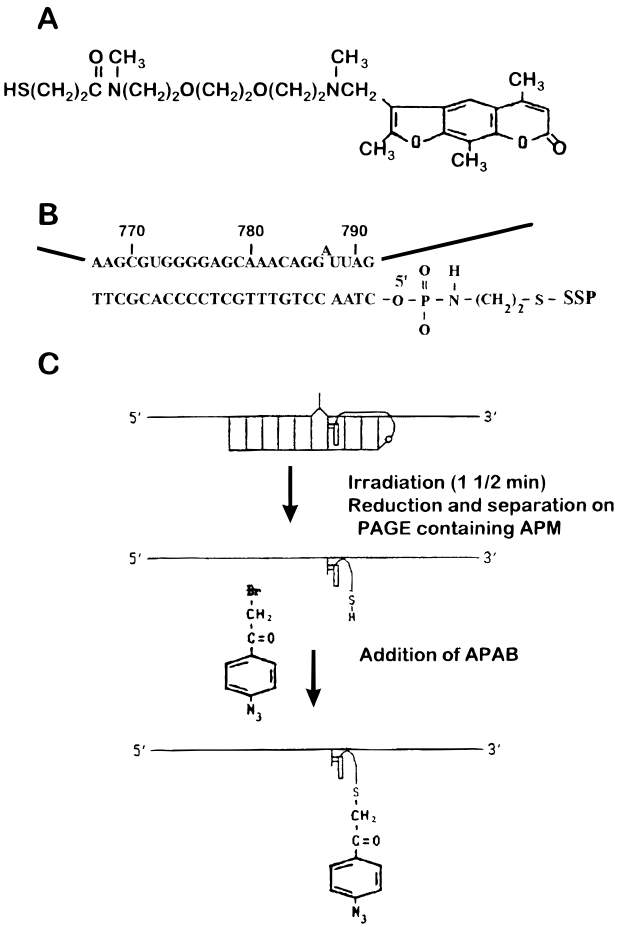

FIGURE 1. Scheme for targeting site-specific psoralen (SSP) to 16S rRNA positions U788/U789. **A**: SSP in its reduced form (Saffran et al., 1982). **B: S**equence of target region in 16S rRNA (top strand) and deoxyoligonucleotide with SSP attached to the 5′ phosphate through an aminoethanethiol (bottom strand). The sequence of the DNA was designed with 20 nt complementary to the RNA on the 5′side of the target site, 4 nt complementary on the 5′ side and a 1 nt bulge in the RNA on the 5′ side of the target site. **C**: Scheme for the placement of SSP into the 16S rRNA. After hybridization of the SSP–DNA, a $1\frac{1}{2}$ min irradiation was used to phototransfer SSP to the RNA. Reduction and electrophoresis through APM PAGE were then used to isolate the RNA that has the SSP group. For some experiments, the SSP 16S rRNA was reacted with APAB before reconstitution.

tion of the psoralen 4′5′ bond (Cimino et al., 1985). After the short irradiation, the sample was reduced with dithiothreitol (DTT) and electrophoresed on polyacrylamide gels containing *N*-acrylylaminophenyl mercuric chloride (APM, (Igloi, 1988)) to separate RNA containing the SSP from unreacted 16S rRNA and DNA–SSP. The derivitized RNA was identified by phosphorimaging, and the SSP–RNA was isolated from the gel material by sedimentation through cesium chloride cushions (Wilms & Wollenzien, 1994).

The efficiency of SSP addition was estimated to be 12–15% (not shown). It was also determined that the DNA oligonucleotide was completely removed from the RNA after reduction with DTT and electrophoresis under denaturing conditions (results not shown). The specificity of the monoaddition reaction was determined by primer extension analysis to be restricted to U788/U789 region. In primer extension experiments that used primers close to the monoaddition site, three stops of nearly equal intensity at A790, U789, and U788 were seen. This is consistent with monoaddition at the 5′ and 3′ side of U788, to the 5′ side of U789, and possibly also to the 3′ side of U789 (Teare & Wollenzien, 1989).

The electrophoretic purification on the APM-containing gels yields RNA that is nearly completely modified by the SSP at U788/U789. Part of the sample was treated with azidophenacylbromide (APAB) to add the azidophenacyl (APA) moiety to the SH of SSP. APA addition could be monitored on APM polyacrylamide gels (because APA addition reverses the retardation caused by free -SH groups) and was found to be >95%. The 16S rRNA was incubated in reconstitution buffer for 10 min at 37 °C and then was reconstituted with protein from 30S subunits (TP30) according to the conditions of Krzyzosiak et al. (1987). Reconstitution reactions were sedimented through sucrose gradients (Fig. 2A) and showed peaks centered at 30S compared to native 30S particles but showed somewhat greater peak widths similar to the behavior of reconstituted synthetic 16S

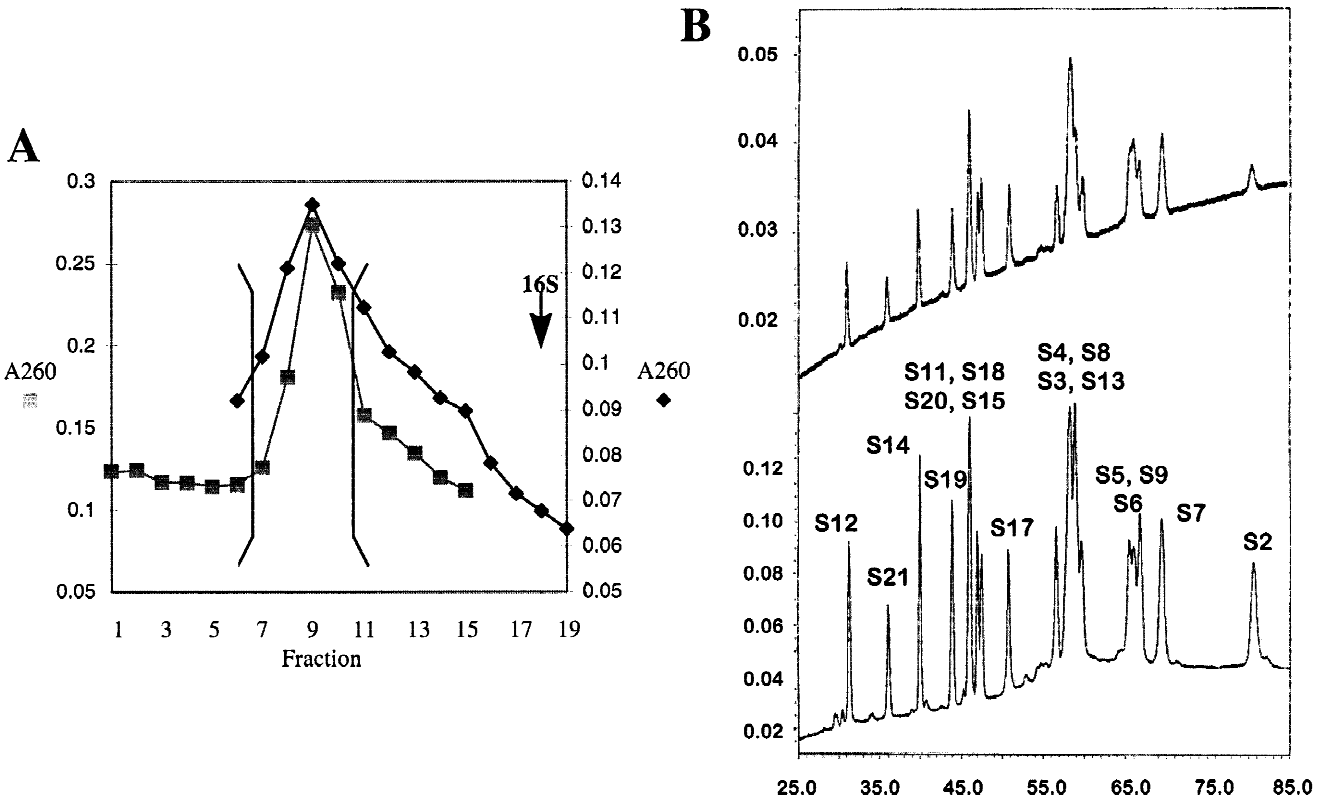

FIGURE 2. Purification and protein analysis of reconstituted subunits. Reconstitution reactions, usually consisting of 200 μg control or modified 16S rRNA and fourfold molar equivalents of TP30 (20 equivalents total), were cooled to ice temperature and sedimented on linear 10–30% sucrose gradients made with activation buffer for the purification of the completely reconstituted 30S subunits. **A** shows the gradient profile of reconstituted particles made with SSP25APA-modified 16S rRNA (diamonds) and from a parallel gradient the profile of native 30S subunits (squares). The position of 16S rRNA run in a parallel gradient is also shown. The first ten fractions of the gradients are not shown. The fractions within the brackets were combined to recover reconstituted particles. **B**: Reversed-phase HPLC analysis of ribosomal proteins. Top tracing: 20 μg of total protein from 30S containing SSP25APA in the U788/U789 positions. Bottom tracing: 80 μg of total protein from native 30S subunits. Each protein sample was injected in 20 μl of Rec 20 buffer onto SynchroPak RP-P column. Elution was with convex gradient 15–45% acetonitrile in 0.01% trifluoroacetic acid in 100 min. The conditions and assignments of peaks according to (Kervalage et al., 1983).

rRNA (Krzyzosiak et al., 1987). Fractions containing 30S particles were pooled and concentrated by sedimentation. Subunits were redissolved in activation buffer. The yield of 30S particles, for native 16S rRNA, 16S rRNA–SSP and 16S rRNA–SSP25APA averaged 30%, 25% and 25%, respectively compared to the amount of input RNA in the reconstitution reactions.

Characterization of 30S subunits containing SSP at U788/U789

Reconstituted 30S particles containing 16S rRNA, 16S rRNA-SSP, or 16S rRNA-SSP25APA were tested for protein content by HPLC analysis (Kervalage et al., 1983) in which the 21 proteins of the small subunit are separated into 17 peaks. The profiles for the protein from 30S subunits reconstituted with SSP25APA-modified 16S rRNA (and SSP-modified 16S rRNA, results not shown) were found to be very similar in peak position and relative amounts compared to the protein obtained from native 30S subunits (Fig. 2B). Protein S1 (retention time 115 min, not included in Fig. 2B) also is present to about the same amount in subunits reconstituted with SSP or SSP25APA compared to unmodified 16S rRNA.

Chemical probing of the 16S rRNA was done to determine whether SSP or SSP25APA in the reconstituted subunits caused any structural changes. Subunits reconstituted with unmodified 16S rRNA and with SSP25APA-modified 16S rRNA (and SSP-modified 16S rRNA; results not shown) were reacted with dimethylsulfate and kethoxal and the pattern of reaction was determined by reverse transcription. The pattern of modification of A915, G926 and other strongly reactive positions indicated that the subunits overall have a global structure not greatly affected by the psoralen. On the other hand, there was an increase in reactivity of G791 and detectable changes in four other RNA regions. These included some nucleotides in the intervals 806–818, 675–714, 557–575 and 1408–1455 (Fig. 3, see Fig. 8). All of these were increases in reactivity at sites which were normally not reactive or marginally reactive, except for the positions G557 and A1408 at which there were modest decreases in chemical reactivity.

Control and reconstituted 30S subunits were associated with 50S subunits and were tested for tRNA binding using $tRNA^{Phe}$ in the presence of poly(U) under conditions in which the P-site is nearly completely filled in native 30S subunits. The fraction of active subunits able to bind tRNA was determined by kethoxal reactivity of position G926 (Fig. 4). This base is known to be reactive in functionally active subunits (Moazed et al., 1986b; Ericson et al., 1995) and is protected from keth-

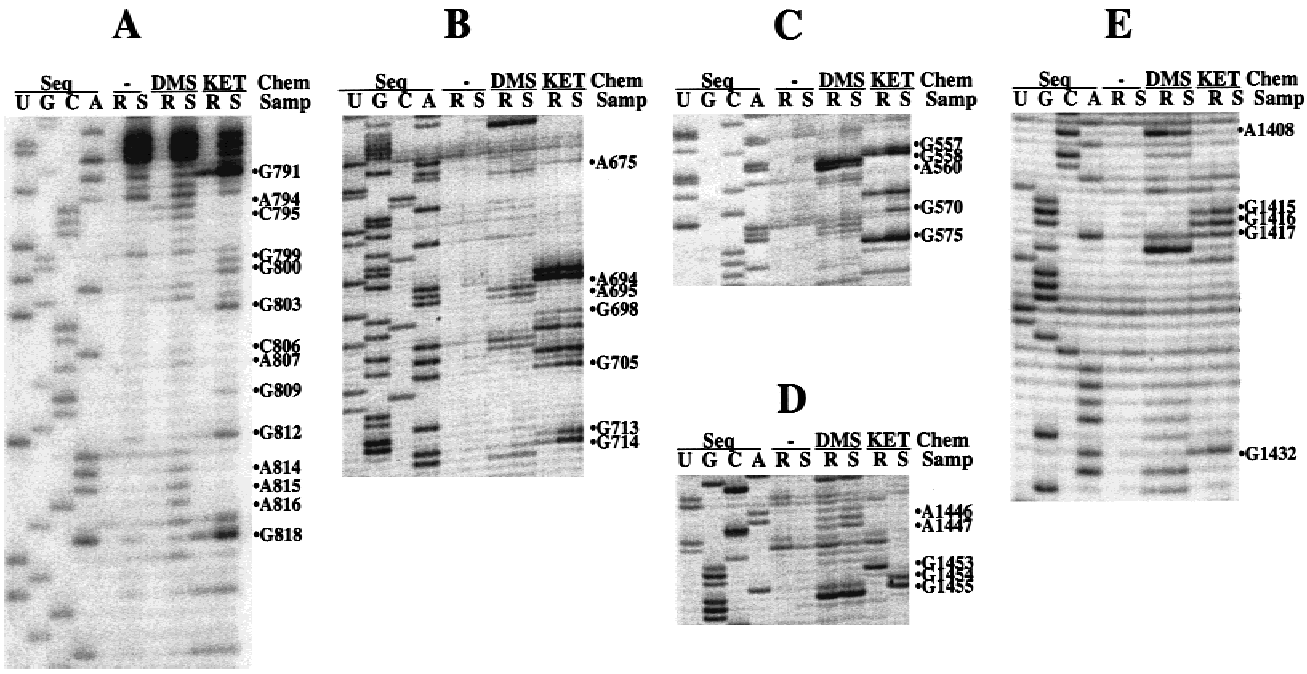

FIGURE 3. Chemical reactivity of 16S rRNA in reconstituted subunits. Kethoxal (reactivity with guanosines at N1, N2) and DMS (reactivity with adenines at N2 and with cytosines at N3) were used to determine reactivity. Reverse-transcription analysis of RNA in intervals in which there are structural distortions are shown for 30S subunits containing control unmodified 16S rRNA (lanes R), 16S rRNA with SSP25APA at position U788/U789 (lanes S). Sites where there are differences in reactivity are indicated. The intervals shown in the figure are **A**: 788–822; **B**: 665–716; **C**: 550–582; **D**: 1441–1459; **E**: 1407–1434. The sites of SSP modification at U788/U789 cause a complete block to reverse transcriptase so the reactivity comparison cannot be made past those sites.

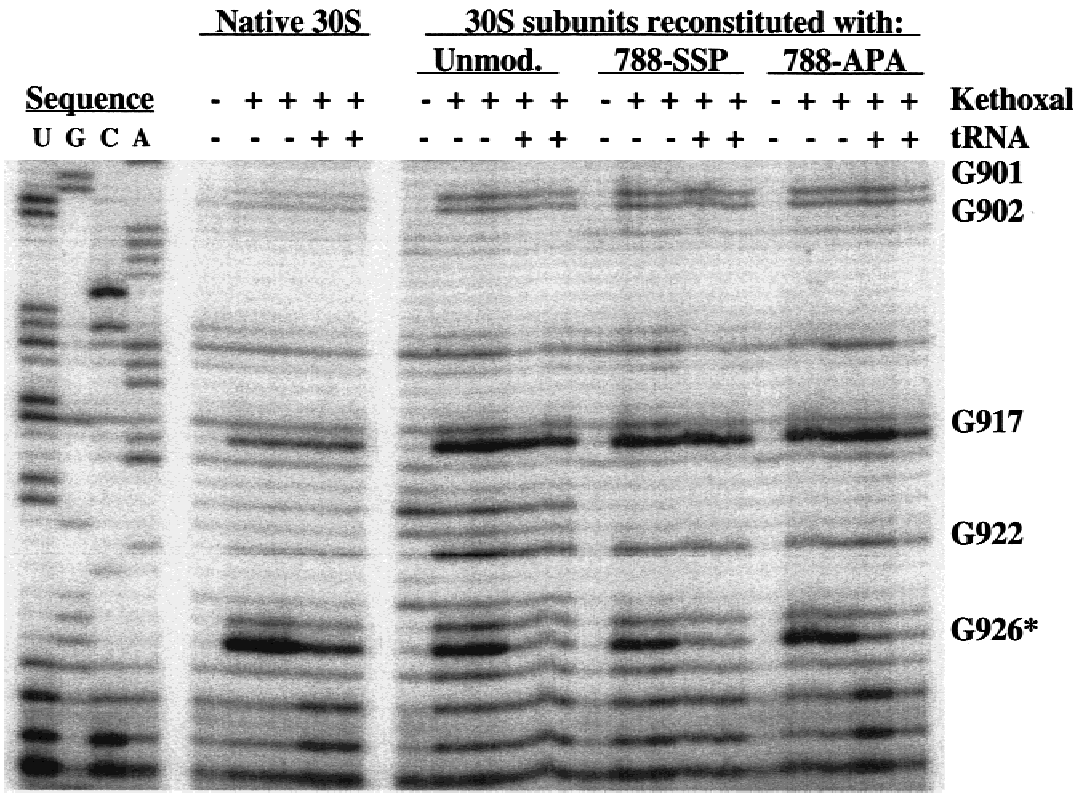

FIGURE 4. Determination of tRNA binding in 30S subunits containing SSP and SSP25APA. The extent of tRNA binding to native subunits and reconstituted subunits containing unmodified 16S rRNA or containing SSP or SSP25APA 16S rRNA was determined by observing the decrease in reactivity of G926 to kethoxal caused by the presence of bound $tRNA^{Phe}$. The degree of kethoxal reaction was determined by reverse-transcription analysis through the region around G926. Reactions without tRNA and with tRNA were done in duplicate for kethoxal reacted samples. Poly(U) was included in the tRNA samples and was necessary for the observed protections.

oxal reaction by P-site bound tRNA (Moazed & Noller, 1990). On a per-subunit basis, G926 in subunits containing SSP and SSP25APA was about two thirds as reactive as in 30S subunits reconstituted with unmodified 16S rRNA and G926 was decreased by nearly the same extent in the presence of excess $tRNA^{Phe}$ compared to control reconstituted subunits.

Analysis of the crosslinking sites

The occurrence of crosslinking within the 16S rRNA from the SSP or SSP25APA to other positions was determined by a gel electrophoresis assay. In this experiment, 30S subunits reconstituted with modified 16S rRNA were irradiated to activate the psoralen or APA moiety and then deproteinized, 5′-dephosphorylated, and then 5′ labeled before electrophoresis on denaturing polyacrylamide gels. Control samples for this experiment were 30S samples that did not contain SSP and were irradiated and subunits that contained SSP but were not irradiated. Polyacrylamide gel electrophoresis of 16S rRNA from control and crosslinked reactions are shown in Fig. 5. Under the gel conditions used, crosslinked 16S rRNA molecules are retarded approximately according to the loop sizes, so they can be seen as distinct bands in the gel (Wilms et al., 1997). The formation of crosslinks is dependent on the presence of SSP and SSP25APA in the subunits and on irradiation (Fig. 5). The pattern seen on the polyacrylamide gel was the same for 5′ and 3′ labeling, indicating there was no preferential labeling of molecules after crosslinking. The amount of crosslinking did not increase with the removal of DTT or oxygen from the buffer during the irradiation. Crosslinking done with 23S particles (incompletely reconstituted subunits that appear as a shoulder between 16S and 30S in the sucrose gradients) showed a much smaller frequency of bands in electrophoresis, indicating that the crosslinking seen here occurs in completely reconstituted 30S particles.

Figure 6 shows preparative gel electrophoresis of 16S rRNA from SSP and SSP25APA containing 30S subunits after crosslinking and indicates the separation of each gel into 17 fractions. The overall pattern of crosslinking changed in a few ways when APA had been added to the RNA: There is greatly increased frequency of a band (this band will be shown to contain a crosslink to position G693) with mobility of about 0.7 of that of linear 16S rRNA. In addition, with APA, there

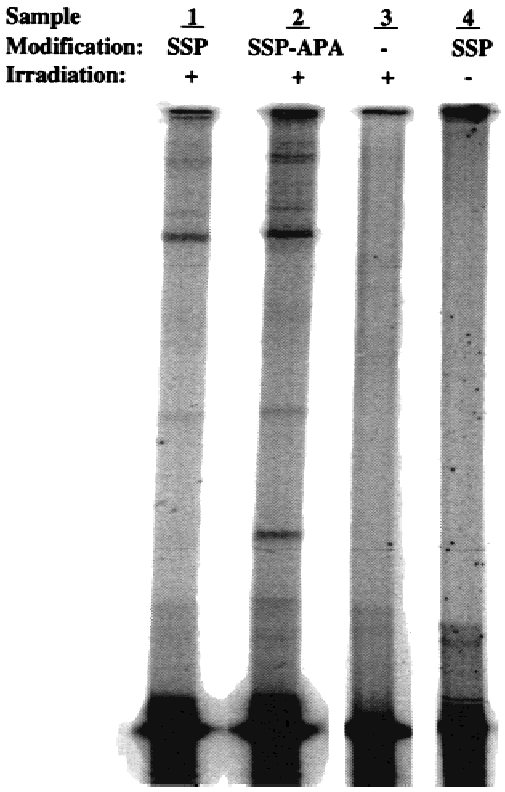

FIGURE 5. Analysis of crosslink formation by SSP and SSP25APA with irradiation in the 16S rRNA in 30S subunits. Reconstituted samples containing SSP- and SSP25APA-modified 16S rRNA or unmodified 16S rRNA were irradiated to demonstrate the action of the SSP and SSP25APA (samples 1–3) or SSP-containing subunits were not irradiated to demonstrate the necessity of irradiation. Crosslinking was detected by electrophoresis of the RNA samples on polyacrylamide gels under denaturing conditions in which crosslinked RNA has reduced mobility (Wilms et al., 1997).

are a number of new low frequency crosslinks in the middle of the gel and in the top part of the gel. RNA from each fraction from the preparative gel electrophoresis was recovered by centrifugation through a cesium chloride cushion. After phenol extraction and reprecipitation, the RNA samples were subjected to primer extension analysis using Avian myeloblastosis virus (AMV) reverse transcriptase and usually seven primers. Psoralen is a complete stop for reverse transcriptase so it is not possible to read 22 nt on the 5′ side of 788 and it is not possible to read the 3′ terminal 38 nt because of the dimethylation of bases at A1518/A1519. The presence of covalent crosslinks in the RNA determines their position in the electrophoresis pattern, so if novel reverse transcription stops are seen in the RNA from specific bands, these stops can be attributed to crosslinking sites. All of the crosslinking sites were detected with multiple primers so they must not be from mispriming or other reverse transcription artifacts.

Fraction-specific reverse transcription stops are seen reproducibly at a number of positions in the 16S rRNA (Fig. 7). The most prominent crosslinks seen with SSP are: U561/U562 (Fraction 4), U920/U921 (Fraction 7), C866 (Fraction 13), and U723 (Fraction 15). The frequency of these are between 0.2% (C866) and 0.8% (U561/U562). Crosslinks lesser in frequency are G693 (Fraction 11), U564 (Fraction 11), U434 (Fraction 1), and U916 (Fraction 8). The frequency of the minor crosslinks is at best, about 0.03% (for G693, U564, and U434) and difficult to measure above background for many of the others, even though distinctive and reproducible bands can be seen in the gel images. For the G693 site, crosslinking by the SSP by itself is surprising since psoralens are not known to be active in crosslinking to purines (Cimino et al., 1985). The stop here might be due to some type of unusual psoralen photochemistry at G693.

Additional crosslinks are made by the SSP25APA reagent and these are attributable to crosslinking by the APA moiety. The crosslink to G693 increases to about 0.36% as determined by the appearance in the PAGE gel (Fig. 6) consistent with the primer extension experiment (Fig. 7) and there is some crosslinking at the adjacent U692 and A694. There are a number of sites in the interval 916–943 that are new crosslinking sites (Fig. 7). These include G926 (Fraction 6), C936 (Fraction 3) and U943 (Fraction 2).

One other major crosslink to position A239 occurred in experiments in which the 30S subunits were UV-irradiated immediately after collection from the sucrose gradients, without purification out of the sucrose and reactivation in activation buffer. This crosslink has a very low frequency in the present experiments in which the 30S subunits are purified and reactivated. The A239 crosslink may occur because of some conformation difference in the subunit due to the high sucrose concentration and pressure experienced during ultracentrifugation. It did not occur if sucrose was added back to activated 30S subunits. This crosslink is not included in our summary of the crosslinks made in the 30S subunit in activation buffer.

The summary of the data obtained is represented on Figure 8, in which crosslinking sites and the sites in which there are differences in chemical modification in 30S subunits containing SSP25APA are indicated in the 16S rRNA secondary structure.

DISCUSSION

The strategy used here for the placement and use of the SSP reagent is an extension of the procedures that were used earlier to investigate pre-mRNA structure (Teare & Wollenzien, 1990), with changes to take into account the larger size of the 16S rRNA, its more stable structure (which makes efficient and specific annealing more difficult), and the need to obtain large amounts of the derivatized RNA. For the U788 site targeted here, the phototransfer reaction resulted in placement of the SSP at U788 and U789, to nearly

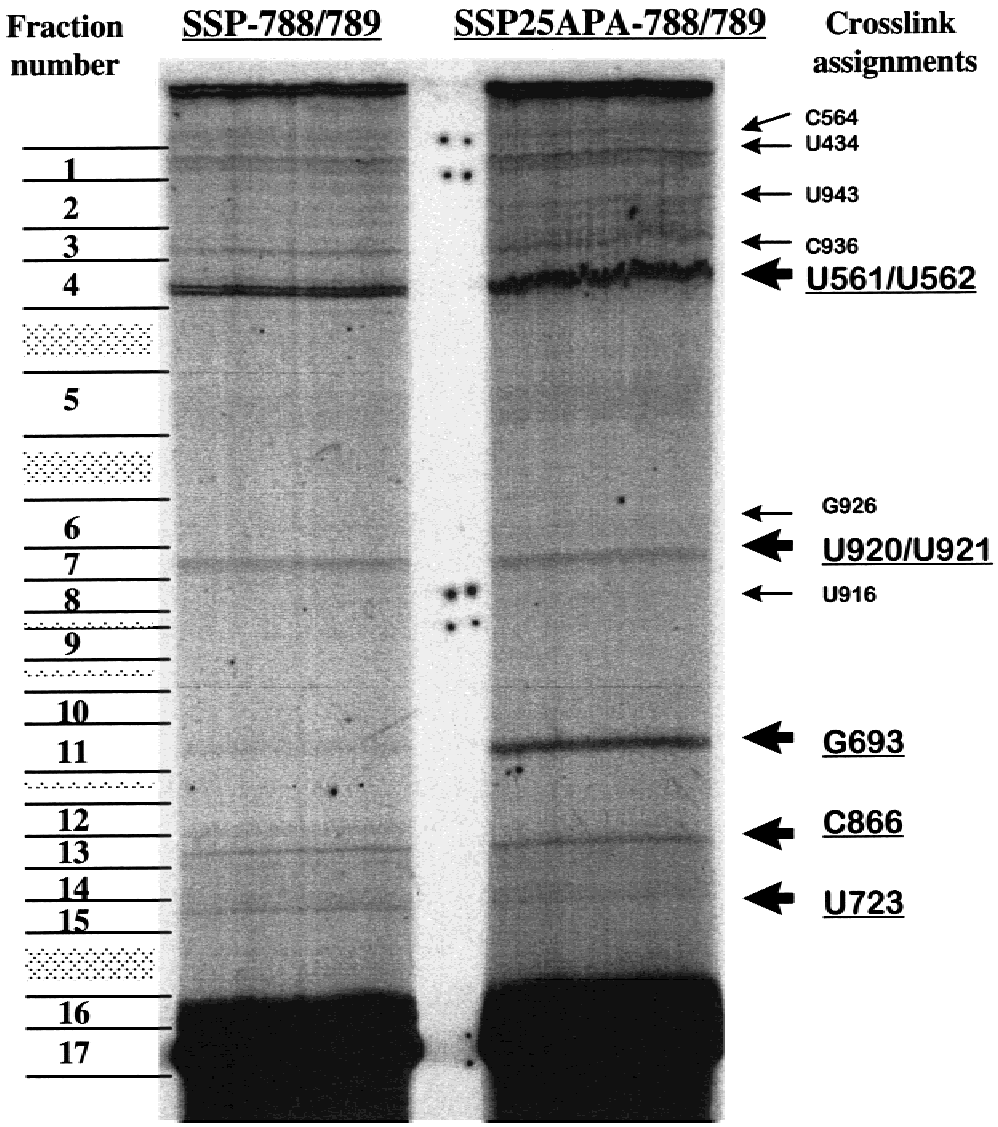

FIGURE 6. Preparative PAGE showing separation of the crosslinked 16S rRNA. Left lane: crosslinked RNA from irradiated 30S containing SSP at position U788/U789. Right lane: crosslinked RNA from irradiated 30S containing SSP–APA containing 30S. Numbering on the left indicates separation of the gel material into fractions from which RNA was purified by centrifugation. The same selection of fractions was made for the left and right gels. Crosslinks are listed on the right of the gel, from the results of reverse transcription experiments.

equal amounts. These are apparently equally reactive in crosslinking because there is no preference in the location of the SSP on either U788 or U789 determined by the primer extension experiments on the fractionated crosslinked RNA molecules. The sequence of the 790 end loop is highly conserved, with U788 being highly conserved among naturally occurring sequences (Gutell et al., 1985; Santer et al., 1990) and U789 being the preferred nucleotide in selection experiments (Lee et al., 1997). The presence of the SSP has a local structural effect since G791 is significantly more reactive with kethoxal and A794 and C795 are somewhat more reactive with DMS. However, there was no modification in this region that inhibited P-site tRNA binding (von Ahsen & Noller, 1995) and, from the analysis of tRNA binding, the majority of the SSP and SSP25APA monoadducts apparently do not interfere with tRNA binding. Because of the frequency of the crosslinking it is not possible to make a straightforward determination whether the presence of crosslinks affects tRNA binding.

16S rRNA with SSP at positions U788/U789 is readily reconstituted into subunits, judging by sedimentation of reconstituted subunits and HPLC analysis of the protein composition of the reconstituted particles. Chemical probing done on control unmodified and modified 30S subunits indicates that the psoralen alters the 16S rRNA reactivity in a limited number of sites including G791 within the end loop and to a much lesser extent in some other regions. Two of these regions (the intervals 675–715 and 557–575) are associated with cross-

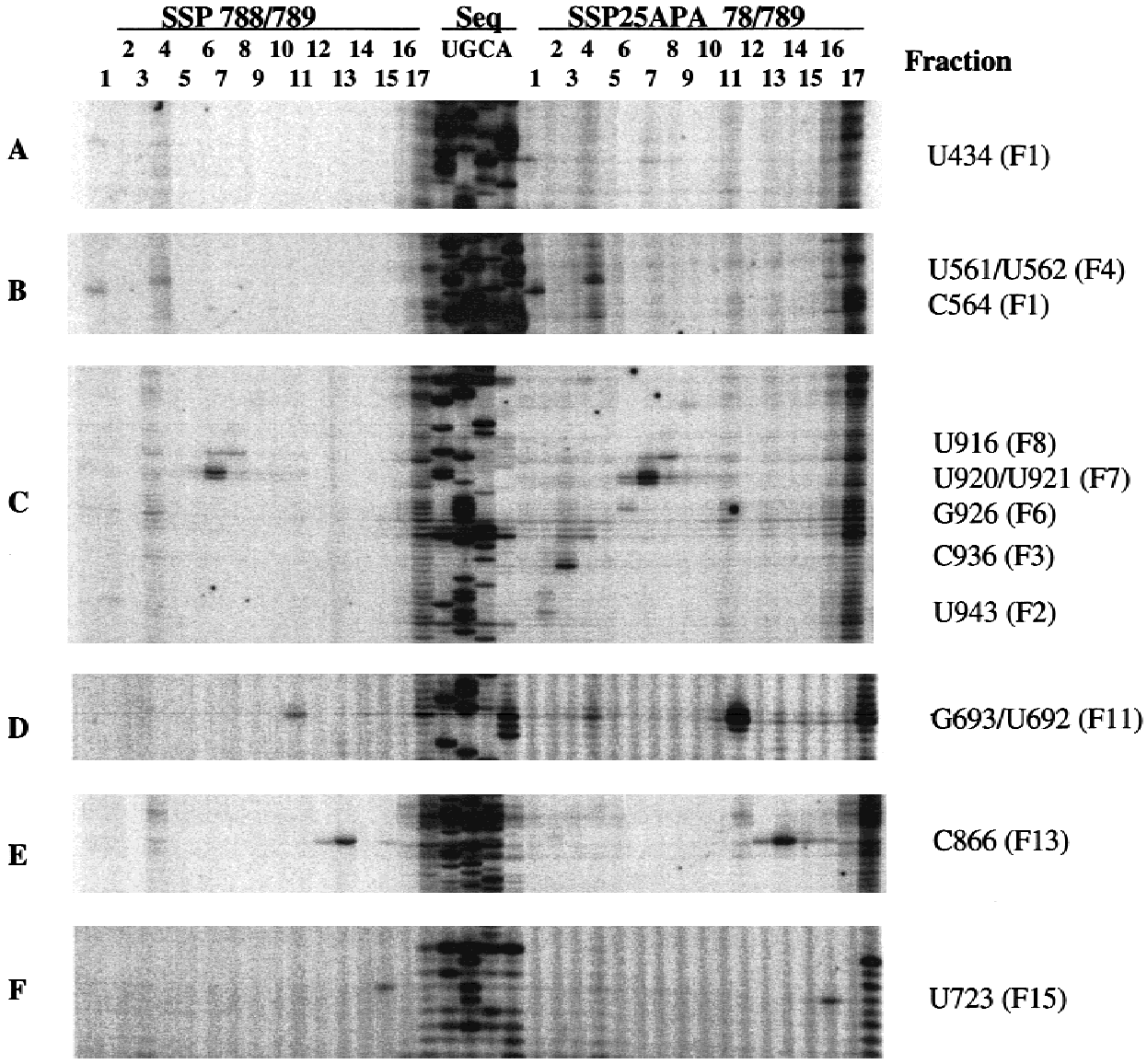

FIGURE 7. Reverse-transcription analysis of crosslinked 16S rRNA. RNA from the indicated fractions in each experiment was used for reverse transcription in a series of reactions with different primers. The regions were selected to show the major and minor sites attributable to crosslinking. Sites of crosslinking (1 nt on the 5′ side of the reverse transcription stop) and the fraction in which they occur are indicated in the right margin. An approximately tenfold greater amount of RNA was used in fraction 17 in each experiment to identify the sites of nonspecific photochemical reactions.

linking. In the two other intervals (794–818 and 1408–1455) there are a number of nucleotides that are slightly changed in their reactivity by the presence of the SSP and SSP25APA but are not crosslinking sites. SSP- and SSP25APA-derivatized 30S subunits were about two thirds as active in tRNA binding capacity as control reconstituted 30S subunits containing unmodified 16S rRNA indicating that whatever perturbations are made by the SSP, these are not important enough to grossly alter the subunit. However, measurements to determine tRNA association constants have not been made, so we cannot rule out more subtle differences induced by the SSP and SSP25APA.

Four crosslinks to positions U561/U562, U920/U921, C866, U723 are made from the U788/U789 site with the SSP reagent. These crosslinks must occur upon the activation of the 3,4 double bond of the psoralen, so the 2 nt involved in the crosslink are separated by an 8 Å distance. Minor crosslinks are also made at positions C564, U434, U916 and G693. With SSP25APA reagent, additional crosslinks are seen—the frequency of G693/U692/A694 increases tenfold, the frequency of U916 and U926 increase and new minor crosslinks at C936 and G926 are seen. The frequencies of the five major bands (0.2–0.9%) in this study are at the lower end of frequencies for crosslinks made by APA (0.5–12.5%) in the circularly permuted RNase P experiments of Harris et al. (1997) and are also in the lower range of the frequency range (0.3–6%) for crosslinks made by 4-thiouridine incorporated into a fragment of

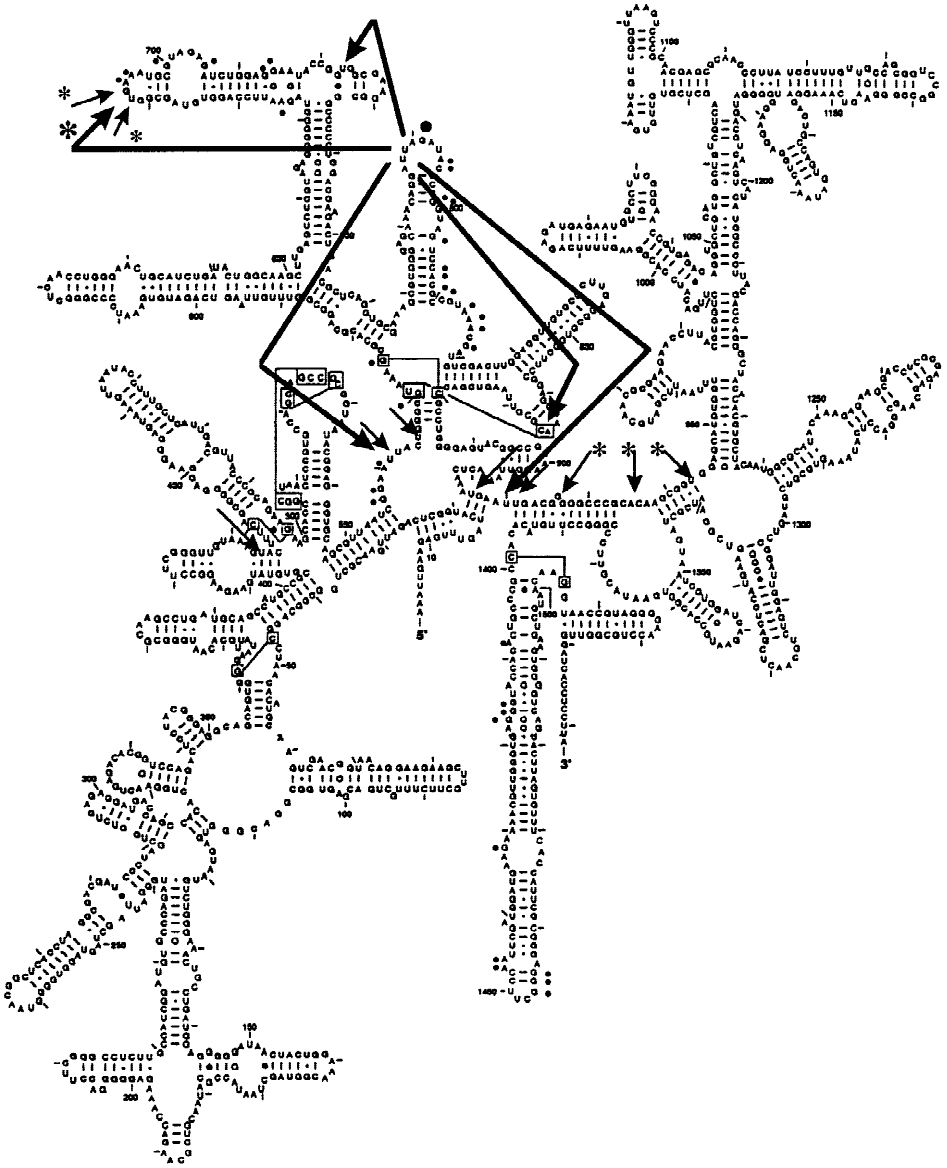

FIGURE 8. Summary of the SSP and SSP25APA crosslinking sites in the secondary structure (Gutell et al., 1994) of the 16S rRNA. The large filled circle at G791 and small filled circles indicate positions of medium or marginal change in chemical reactivity in subunits containing SSP25APA compared to subunits reconstituted with unmodified 16S rRNA. The five major sites of SSP crosslinking are indicated by heavy arrows and minor sites are indicated by light arrows. Asterisks are used at those crosslinking sites dependent on APA or increased more than twofold by APA. Based on the pattern of crosslinking and the specificity of psoralen the experiment suggests the following approximate distances to U788/U789—8 Å: U561/U562, C866, U723, U920/U921, (U343, U564, U916); >25 Å: G693 (G926); between 8 and 25 Å: (C936, U943).

16S rRNA (Lemaigre-Dubreuil et al., 1991). In an experiment by Osswald et al. (1995), intermolecular crosslinks made from diazirine-modified tRNA to the ribosomal RNAs occurred at frequencies of 0.25–2%. Factors that limit RNA–RNA crosslinking within the ribosome with these types of reagents may be the relative inflexibility of the RNA in the ribosome at some locations, reactions of the photoreagent with ribosomal proteins, and for APA, chemical reaction or quenching by reducing agents present in the reconstitution and activation buffers (Bayley, 1983).

All of the crosslinking results pertain to the structure of the reconstituted 30S subunit after it has been incubated under conditions which should activate it. This state has been chosen since a number of experiments have already been done under this condition, i.e. determination of the three dimensional arrangement of the ribosomal proteins (Capel et al., 1987) and RNA–protein interactions (Stern et al., 1989; Powers & Noller, 1995), so the structural information obtained here should be compatible and appropriate for use with data from those experiments. On the other hand, from other experiments, it is known that there are structural changes in the 30S subunit upon association with the 50S subunit (Noah & Wollenzien, 1998), and there may be additional changes upon mRNA and tRNA associ-

ation. We cannot rule out completely that the crosslinks in the present experiment come from a nonnative arrangement of the 790 end loop region. However, the chemical probing experiments to determine sites of altered chemical accessibility due to SSP and SSP25APA should sample the whole population of the subunits, and that also indicates that the SSP and SSP25APA must be in a crowded region of the subunit, consistent with the major sites that are crosslinked.

The 790 loop is known to be of functional importance. Initiation factor IF-3 footprints are at positions G791 and U793 as well as at positions G700, U71 and G703 (Muralikrishna & Wickstrom, 1989; Moazed et al., 1995). IF3 also was crosslinked to two sites in the vicinity of the 790 end loop at position A829 and G859 (Ehresmann et al., 1986). A mutation G791C inhibits initiation complex formation by decreasing the association rate constant for formation of the IF3·30S subunit complex (Tapprich et al., 1989). In addition, the mutation A792G or C affects subunit association implicating the 790 end loop in contacts with the 50S subunit (Santer et al., 1990). tRNA footprints for tRNA situated in the P site are at positions A794 and C795 in the 790 loop and at G693 in the 700 region (Moazed & Noller, 1990). The proximity of the 700 and 790 end loops was shown before by chemical crosslinking between fragments 693–696 and 794 (or 799) using nitrogen mustard as the crosslinking reagent (Atmadja et al., 1986). Position G693 was also crosslinked by a diazirine reagent that was attached to position 32 of the anticodon loop of $tRNA^{Arg}I$ when that tRNA was in the P-site and E-site (Doring et al., 1994). These data indicate that the 790 loop must be closely involved in determining the functional status of the decoding region and that the region G693–G703 also participates in this.

The rRNA region containing the U788/U789 sites has been proposed to be in the platform structure of the 30S subunit at a significant distance from the decoding region in several recent models for the 16S rRNA three dimensional arrangement (Stern et al., 1988; Malhotra & Harvey, 1994; Fink et al., 1996; Mueller & Brimacombe, 1997). In these models the helices containing the 690 and 790 end loops originate in the central part of the subunit and point outward so that the end loops are pointing toward the end of the platform structure. This placement is consistent with data obtained by electron microscopy of complexes in which sites in the rRNA were tagged by hybridization with DNA complementary to rRNA sequences at 787–803 (Oakes & Lake, 1990). In addition, DNAs complementary to rRNA sequences at 686–703, 714–733, (Oakes & Lake, 1990) and at 685–696 and 694–705 (McWilliams & Glitz, 1991) were also used. Placement of the 790 end loop in the platform structure is also not inconsistent with information for the RNA contacts of protein S11, located the furthest out in the platform (Capel et al., 1987), which has Fe(II)–EDTA footprints at nucleotide positions 794, 796, 803, 804, and 820 and chemical footprints at 777, 778 and for protein S21, also located in the platform, which has an incomplete chemical footprint at 800. However, the placement of the 790 end loop in the end of the platform structure is at odds with its known function and data pertaining to its proximity to the decoding site. Suggestions have been made that there may be significant structural flexibility in the 690 and 790 end loop to account for this discrepancy (von Ahsen & Noller, 1995; Mueller et al., 1997) and that there also may be some ambiguity in the exact location of the P site in the 30S subunit (Mueller et al., 1997).

Three of the major SSP crosslinks (U561/U562, U920/U921 and C866) are key in helping to determine the location for the 788/789 region in the activated 30S subunit. Nucleotides U561/U562 must be in a central location in the interior part of the subunit because they are between RNA elements known to be located in the left and right parts of the body of the subunit. Sites that are relevant to this placement are the Fe(II) EDTA footprints (Powers & Noller, 1995) for the proteins S4 (at a number of nucleotide positions in the intervals 400–440, 490–510 and 540–550) and S12 (at nucleotides positions in the intervals 33–38 and 508–536) in the left part of the body and for the proteins S6 + S18 (at nucleotides in the intervals 670–738 and 844,845), S15 (at nucleotides 655–658 and 749–751) and S8 (at nucleotides 598, 643,644,653, 821–827, and 873–878), in the right part of the body (Capel et al., 1987). Nucleotide C866 must also be close to U561/U562, by virtue of the constraints imposed by a sequence covariance between C866 and G570 (Gutell et al., 1994). The position of U561/U562 and U920/U921 must be close to the central pseudoknot (the base-pairing interaction (17–19)·(916–918)) because of constraints imposed by the secondary structure. The location of nucleotides U920/U921 are closely associated with the decoding region since U921 is base-paired to nucleotide G1396, which is 2 nt away from the beginning of the base-paired interaction (1399–1413)·(1487–1504) containing the strong footprints for the tRNA P site and A site. Many of the nucleotides in the interval 1389–1398 are accessible to the mRNA, but the nucleotides U920/U921 are not, judging from the pattern of mRNA crosslinking in the region (Rinke-Appel et al., 1991; Juzumiene et al., 1995). The arrangement of the U561/U562, C866, and U920/U921 sites thus suggests that the loop containing U788/U789 is located in the central part of the subunit, in the vicinity to the central pseudoknot and that this placement is not accessible to the decoding site. The minor crosslink sites at C556, U916 are also consistent with the location. This would require the 788/789 to be somewhat behind the decoding region, so that in the 30S subunit the 790 end loop is buried and not on the subunit surface.

Minor crosslinks from U788/U789 to several other crosslinking sites at G926, C936, U943 and U434 would

be difficult to meet in an arrangement with the 790 end loop in the subunit interior in back of the decoding region. G926 is known to be facing the P site, since tRNA binding to the P site makes this position inaccessible to kethoxal reaction (Moazed & Noller, 1990) and kethoxal modification interferes with tRNA binding (von Ahsen & Noller, 1995). G926 is photoaffinity-labeled by s^4U at position +2 in mRNA (Sergiev et al., 1997) and U936 is photoaffinity-labeled by tRNA in the P and E site by a diazirine reagent attached to position 32 in tRNA (Doring et al., 1994). The positions C936 and U943 are footprints for protein S7 (Powers & Noller, 1995) and are part of the S7 minimum binding site (Dragon et al., 1994). S7 is known to be in the subunit surface facing the 50S subunit (Capel et al., 1987). Thus, the 926, 936, and 943 sites are likely to be accessible on the 30S surface in the vicinity of the P site. To account for these crosslinks it is necessary to propose an alternate location for the 790 end loop in which it is in a position on the subunit interface surface next to the decoding region. We are presently determining the crosslinking pattern in complexes with IF3 and the 50S subunit to see if the distribution of crosslinks from SSP at U788/U789 is significantly changed.

MATERIALS AND METHODS

Preparation of ribosomes, subunits and ribosomal proteins

Frozen *E. coli* MRE 600 ($\frac{1}{2}$ log) cells were obtained from the Cell Culture Fermentation Facility, University of Alabama, Birmingham. 70S ribosomes were prepared according to Makhno et al. (1988), except that washed 70S ribosomes were used for the isolation of ribosomal subunits. 70S ribosomes were dissolved in 20 mM Tris, pH 7.5, 200 mM NH_4OAc, 3 mM $Mg(OAc)_2$, 2 mM DTT and were incubated at 37 °C for 15 min to accomplish subunit dissociation (Makhno et al., 1988) and were separated in sucrose gradients in the same buffer. Subunits were dissolved in activation buffer (200 mM NH_4Cl, 20 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, 2 mM DTT, Zamir et al., 1973) and activated before freezing for storage. Total protein from 30S ribosomal subunits was obtained by the method described by Nierhaus and Dohme (1979). It was dialyzed into reconstitution buffer (20 mM HEPES, pH 7.5, 400 mM NH_4Cl, 20 mM $Mg(OAc)_2$, 4 mM mercaptoethanol, Krzyzosiak et al., 1987) and stored frozen in aliquots.

Preparation of SSP–modified RNA and reconstitution into 30S subunits

SSP (Saffran et al., 1982, obtained from Cerus Corp., Concord, California) was attached to the 5′-end of deoxyoligonucleotide pCTAACCTGTTTGCTCCCCACGCTT through a cystamine moiety through a reversible disulfide bond as previously described (Teare & Wollenzien, 1989). The purification of the cystamine oligonucleotide and of the final product (SSP–oligonucleotide) were carried out by reversed phase HPLC using a PRP-1 column (Hamilton, 0.41 × 15 cm) and, with 50 mM triethylamine acetate, pH 7.5 in both phases, a gradient in acetonitrile concentration from 8 to 32% run from 5 to 35 min. After purification, the SSP–oligonucleotide was lyophilized and redissolved in water. The hybridization of SSP–oligonucleotide and 16S rRNA was performed at 1:1 molar ratio in a buffer containing 1 × TE, 0.1 M NaCl (TE is 0.01 M Tris-HCl, pH7.5, 1 mM EDTA) for 10 min at 45 °C, followed by incubation on ice for 10 min. Usually 4 mg of 16S rRNA with 8 nmol SSP–oligonucleotide were hybridized in 600 μl total volume. Irradiation was at 320–360 nm for 1.5 min at 4 °C using a device that uses a mercury lamp and a circulating $Co(NO_3)_2$ solution to remove far-UV light below 320 nm (Isaacs et al., 1977). This device has an estimated intensity of 200 mW/cm^2. The short irradiation preferentially activates the 4′5′ furan-side reactive bond (Cimino et al., 1985). After this irradiation, the sample was ethanol precipitated, redissolved in 800 μl of a buffer containing 1 × TE, 7 M urea and then was reduced by adding DTT to a final concentration of 10 mM and incubating for 10 min at 37 °C. The samples were then subjected to electrophoresis on a 4% polyacrylamide gel containing APM(Igloi, 1988). Electrophoresis was at 200 V (12.5 V/cm) at 45 °C for 5 h in a thermostatted apparatus. SSP-containing 16S rRNA is greatly retarded on such a gel because of the sulfhydryl group it contains and migrates only few millimeters away from the well under this conditions. The gel containing the RNA–SSP was cut out and the RNA–SSP was recovered by ultracentrifugation through cushions containing 2 M CsCl, 0.2 M EDTA, pH 7.4, 5 mM DTT for 20 h at 40K rpm (Wilms & Wollenzien, 1994), resuspended in 5 mM DTT, and ethanol precipitated. The procedures of Stade et al. (1989) were followed for the addition of APAB. RNA was redissolved in 50 μM DTT, then 500 mM Tris-HCl, pH 8.2 and 20 mM APAB in methanol were added to a final concentration of 50 mM and 2 mM. After incubation for 1.5 h at room temperature, 16S rRNA was ethanol precipitated, redissolved in 1 × TE, extracted with phenol, and precipitated again. The RNA was redissolved in reconstitution buffer and incubated for 10 min at 37 °C prior to reconstitution.

Control and SSP- and SSP25APA-modified 16S rRNA were reconstituted with the total 30S protein according to the method of Krzyzosiak et al. (1987). 30S subunits were isolated by sedimentation on sucrose gradients in reconstitution buffer. They were concentrated by ultracentrifugation (20 h at 40K rpm) and were redissolved in activation buffer.

Characterization of the reconstituted 30S subunits

Protein composition of control or reconstituted subunits was determined by the method of Kervalage et al. (1983) that utilizes HPLC reverse-phase separation of the proteins into 16 peaks (plus protein S1 which elutes at higher acetonitrile concentrations than shown). The structure of the RNA in the reconstituted 30S subunits containing control 16S rRNA and SSP-or SSP25APA-containing 16S rRNA was determined by chemical probing of the 30S subunits at 37 °C in CMN buffer (80 mM NH_4 cacodylate, 20 mM $MgCl_2$, 100 mM NH_4Cl, pH 7.2) with DMS and kethoxal as described by Moazed et al. (1986a).

The ability of the reconstituted 30S subunits to bind tRNA was estimated by determining the degree of kethoxal reac-

tivity decrease at position G926 in the presence of an excess of $tRNA^{Phe}$ compared to the reactivity at this position without tRNA being present. For this, 2 pmol of control or reconstituted 30S subunits were first associated with 2 pmol of 50S subunits, and then were incubated 15 min at 37 °C in 20 μl reactions in CMN buffer with 10 μg poly(U), and 200 pmol yeast $tRNA^{Phe}$ and then were reacted 10 min at 37 °C with the addition of 1 μl of 20 × dilution of kethoxal in 20% ethanol. Control reactions were done under the same conditions without the addition of poly(U) and $tRNA^{Phe}$. The reactivity was determined by primer extension and analysis of the band intensity due to kethoxal reaction at G926.

Crosslinking and purification of specific crosslinked molecules

30S ribosomal subunits were activated in activation buffer and irradiated in the same buffer at 4 °C at a concentration of 1.5 μg/μl in an Eppendorf tube. Irradiation was with near-UV light, 320–360 nm, for 15 min in the device described previously (Isaacs et al., 1977). The 16S rRNA was recovered from 30S ribosomal subunits by proteinase K digestion with 1% SDS and 20 mM EDTA, followed by phenol extraction and ethanol precipitation. Part of the sample (5 μg) was dephosphorylated with calf intestinal phosphatase for 15 min at 37 °C, and purified by proteinase K digestion, phenol extraction, and ethanol precipitation. The 16S rRNA was then 5′ end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and mixed back with the unlabeled crosslinked sample.

The crosslinked 16S rRNA was separated by PAGE using 3.6% acrylamide:bis-acrylamide (40:1) with 8M urea and BTBE buffer (30 mM Bis-Tris base, 30 mM boric acid, 2 mM EDTA, pH 6.8, Wilms et al., 1997). For analytical separation, up to 2 μg RNA were loaded in each well (8 mm with 0.38 mm spacer); for preparative separation, up to 40 μg RNA were loaded in a well 6.75 cm wide on a gel with the same spacer size. Electrophoresis was at 800 V (20 V/cm) at 45 °C for 17 h in BTBE buffer in a thermostatted gel electrophoresis apparatus (Hoeffer Scientific). The location of bands containing uncrosslinked and crosslinked 16S rRNA were detected by autoradiography or phosphorimaging; they were cut out of the gel and eluted by ultracentrifugation through cushions containing 2 M CsCl, 0.2 M EDTA, pH 7.4, for 20 h at 40K rpm (Wilms & Wollenzien, 1994). RNA pellets were redissolved in 100 μl H_2O, phenol extracted and reprecipitated before further analysis.

Determination of crosslinked sites by reverse transcription primer arrest assay

Primer extension analysis was performed on all fractions using AMV reverse transcriptase (Wollenzien, 1988). Bases involved in photochemical crosslinking are identified by the presence of an increased stop in the reverse transcription pattern; the stop occurs 1 nt in the 3′ direction of the crosslinked base (Ericson & Wollenzien, 1988). Ten DNA primers complementary to regions throughout the 16S rRNA were used to allow reading of the 16S rRNA except for the 3′ terminal 40 nt and since the SSP monoadduct at U788/U789 is a complete stop for reverse transcriptase, an additional primer which begin reverse transcription at 768 was used. Reverse-transcription reactions were usually electrophoresed on 8% acrylamide:bis-acrylamide (19:1), 8 M urea in TBE buffer.

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