New features of 23S ribosomal RNA folding: The long helix 41–42 makes a “U-turn” inside the ribosome

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ABSTRACT

23S rRNA from *Escherichia coli* was cleaved at single internucleotide bonds using ribonuclease H in the presence of appropriate chimeric oligonucleotides; the individual cleavage sites were between residues 384 and 385, 867 and 868, 1045 and 1046, and 2510 and 2511, with an additional fortuitous cleavage at positions 1117 and 1118. In each case, the 3' terminus of the 5' fragment was ligated to radioactively labeled 4-thiouridine 5',3'-biphosphate (“psUp”), and the cleaved 23S rRNA carrying this label was reconstituted into 50S subunits. The 50S subunits were able to associate normally with 30S subunits to form 70S ribosomes. Intra-RNA crosslinks from the 4-thiouridine residues were induced by irradiation at 350 nm, and the crosslink sites within the 23S rRNA were analyzed. The rRNA molecules carrying psUp at positions 867 and 1117 showed crosslinks to nearby positions on the opposite strand of the same double helix where the cleavage was located, and no crosslinking was detected from position 2510. In contrast, the rRNA carrying psUp at position 384 showed crosslinking to nt 420 (and sometimes also to 416 and 425) in the neighboring helix in 23S rRNA, and the rRNA with psUp at position 1045 gave a crosslink to residue 993. The latter crosslink demonstrates that the long helix 41–42 of the 23S rRNA (which carries the region associated with GTPase activity) must double back on itself, forming a “U-turn” in the ribosome. This result is discussed in terms of the topography of the GTPase region in the 50S subunit, and its relation to the locations of the 5S rRNA and the peptidyl transferase center.

Keywords: ribosome structure; 23S rRNA; ribonuclease H cleavage; chimeric oligonucleotides; 4-thiouridine cross-linking; GTPase center

INTRODUCTION

A number of different biochemical methods have been applied to the study of the 3D folding of the large ribosomal RNA molecules, and the data obtained have been incorporated into crude models or “cartoons” representing the 3D arrangement of the double-helical elements of the *Escherichia coli* 16S (e.g., Brimacombe et al., 1988; Stern et al., 1988; Malhotra & Harvey, 1994) or 23S (Nagano et al., 1988; Mitchell et al., 1990) rRNA. More recently, rapid advances in the cryo-electron microscopy (EM) of ribosomal particles combined with computerized image processing techniques have led

to the derivation of 3D structures of the *E. coli* 70S ribosome at a resolution of ca. 20 Å (e.g., Stark et al., 1995, 1997a; Agrawal et al., 1996). At this resolution, ligands such as tRNA or elongation factor EF-Tu can be directly visualized (Stark et al., 1997a, 1997b), and many fine structural elements are discernible that have dimensions suggestive of RNA double helices. By combining the rRNA modeling studies with these new EM structures, it has been possible in the case of the 16S rRNA to fit the rRNA model to the EM contour (Mueller & Brimacombe, 1997a, 1997b; Mueller et al., 1997), thus giving the previous “cartoon” models for the first time a detailed physical basis.

Although the resolution of the EM structures is continuously improving, there are still a number of areas of uncertainty or ambiguity in the 16S rRNA structure, where additional biochemical information relating to the 3D arrangement of the rRNA helices would be very helpful. In the case of the 23S molecule, where the 3D

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models are considerably more primitive (Nagano et al., 1988; Mitchell et al., 1990) and have yet to be fitted to the EM contour, the need for this type of information is even greater. Paradoxically, whereas the older types of approach to the problem, such as in situ intra-RNA crosslinking using short bifunctional reagents (e.g., Döring et al., 1991) or direct UV irradiation (Stiege et al., 1986), have yielded relatively high-resolution data, some of the methods that have been applied more recently offer only a significantly lower level of resolution. For example, Fe(II)EDTA tethered to the ends of tRNA anticodon loop analogues has been used to generate hydroxyl radical cleavages in the rRNA (Joseph et al., 1997); here the length of the tether combined with the diffusion distance of the hydroxyl radical gives cleavages lying within a large (40–50 Å diameter) sphere encompassing the site of the probe (Joseph & Noller, 1996). In another approach, complementary oligodeoxynucleotides carrying a photolabel at one end have been hybridized to single-stranded hairpin loop-ends in the rRNA, and the resulting sites of attachment to the photolabel have been analyzed (e.g., Muralikrishna & Cooperman, 1995); here again, the probe length is more than 20 Å, without taking into account the considerable distortions introduced into the hairpin loop by the presence of the hybridized oligonucleotide.

We recently described a new method for studying the 3D topography of the rRNA, whereby a photoreactive 4-thiouridine ("sU") residue in the form of its 3', 5'-biphosphate ("psUp") is inserted with the help of RNA ligase at a specific cleavage site in the rRNA (Baranov et al., 1997); the cleavages (in 16S rRNA) were induced in isolated rRNA by RNase H in the presence of complementary chimeric oligonucleotides. A similar approach was developed simultaneously by Yu and Steitz (1997), using mRNA analogues. Ideally, one would want to religate the cleaved rRNA so as to obtain a continuous RNA chain [as was done by Yu & Steitz (1997) in their experiments], but religation of the large rRNA molecules (as opposed to mRNAs) in reasonable yield has proved to be extremely difficult (Bogdanova et al., 1995). Fortunately, however, it is possible in many cases to reconstitute cleaved 16S rRNA into 30S ribosomal subunits (Afonina et al., 1991a, 1991b), and these subunits are able to re-associate with 50S subunits to form 70S ribosomes in a normal manner (Baranov et al., 1997). After photoactivation of the psUp residue (which at the same time is radioactively labeled with ³²P), the sites of crosslinking from the sU can be determined within the rRNA. Although the method does not offer quite such a high level of resolution as the older intra-RNA crosslinking methods already mentioned (Stiege et al., 1986; Döring et al., 1991), it has, on the other hand, the distinct advantage that the site for the insertion of the psUp residue can be chosen by the investigator; thus, the environment of any desired area of the rRNA can be studied, provided, of course, that the cleavage site

does not inhibit the subsequent reconstitution of the rRNA into ribosomal subunits.

In this paper, we present the first application of our methodology (Baranov et al., 1997) to the *E. coli* 50S ribosomal subunit, and describe the analysis of crosslinks from psUp inserted at four different cleavage sites within the 23S rRNA. The sites for cleavage were chosen so as to lie relatively close to (but not directly within) areas of particular topographical or functional interest. The first is located in Domain I of the 23S rRNA, near to helix 22, which is known to be involved in a long-range interaction with the remote helix 88 (Leffers et al., 1987). The second is in Domain II, within the long helix 38, whose loop end has been crosslinked from several sites in the elbow region of A site-bound tRNA (Rinke-Appel et al., 1995). The third site is also in Domain II, in the area of the 50S subunit that is associated with GTPase activity, and the fourth is in Domain V, close to the peptidyl transferase center. Crosslinks were observed from the first three of these sites, but not from the fourth; in this last case, the chimeric oligonucleotide concerned caused cleavage at a second (fortuitously complementary) site, from which crosslinking did occur.

RESULTS

Reconstitution of 50S ribosomes containing fragmented 23S rRNA with 4-thiouridine at the cleavage site

In order to insert a photoreactive sU residue into the 23S rRNA, the RNA must first be cleaved at the desired position. This was achieved in each case, as in our previous study (Baranov et al., 1997), using RNase H in combination with a synthetic chimeric oligonucleotide complementary to the rRNA region just upstream of the site to be cleaved. Each oligonucleotide consisted of four deoxynucleotides at the 5' end connected to a stretch of seven 2'-OMe ribonucleotides, followed by a single "normal" ribonucleotide. The sites chosen for chain scission were the internucleotide bonds 383–384, 867–868, 1045–1046, and 2510–2511 in the 23S rRNA; the sequences of the chimeric oligonucleotides used in the respective RNase H digestions to generate these predicted cleavages are listed in Table 1. The RNase H digestions were performed as before (Baranov et al., 1997) and, in order to estimate the yields of the RNase H digestions, the fragments were separated by electrophoresis on 4% polyacrylamide gels and stained with methylene blue. In every case, the yield of hydrolysis was greater than 80% [data not shown, but cf. Lapham & Crothers (1996); Baranov et al. (1997); Yu & Steitz (1997)].

The positions of the cleavage sites in the 23S rRNA were checked by the primer extension method (Moazed et al., 1986). Examples of the gels obtained are shown

TABLE 1. Chimeric oligonucleotides used and their cleavage sites in 23S rRNA.

Oligonucleotide	Complementary region in 23S rRNA	Cleavage site in 23S rRNA
1. Deoxy(GTCC)-2'-OMe-(CGCCCUA)-riboC	372–383	384–385 ^a
2. Deoxy(GTGC)-2'-OMe-(UCUACCC)-riboC	856–867	867–868
3. Deoxy(GGGC)-2'-OMe-(CUUCCCA)-riboC	1034–1045	1045–1046
4. Deoxy(GCCG)-2'-OMe-(ACAUCGA)-riboG	2499–2510	2510–2511
Deoxy(GCCG)-2'-OMe-(ACAUCGA)-riboG	(1107–1117) ^b	(1117–1118)

^aThis cleavage site is one nucleotide further downstream than the predicted site (see Discussion).

^bThis 23S rRNA region is nearly complementary to oligonucleotide 4; the perfectly complementary sequence would be (GCCG)-(ACUCGAC).

in Figure 1 for oligonucleotides No. 1, 2, and 3 (Table 1), respectively. It can be seen that the corresponding reverse transcriptase stops are at 23S rRNA positions 385, 868, and 1046, and that the sites are, furthermore, unique, apart from small amounts of cleavage at positions one nucleotide upstream in Figure 1A and B. With oligonucleotide No. 4 (Table 1), two RNase H scissions were observed (not shown in Fig. 1), one at position 2510, as expected, and the other at position 1117; this second cut is due to a high level of fortuitous complementarity of oligonucleotide No. 4 to the 1107–1117 region of the 23S rRNA (see Table 1). Previously, we had observed (Baranov et al., 1997) that RNase H cleaves the rRNA between the extreme 3' nucleotide of the heteroduplex and the next (downstream) unpaired base of the rRNA, and, in the case of oligonucleotides No. 2–4 (Table 1), the cleavage sites were observed at the corresponding expected positions. However, with oligonucleotide No. 1, the cleavage site was one nucleotide further downstream (at internucleotide bond 384–385; see Discussion).

The ability of the fragmented 23S rRNA species to be reconstituted into 50S subunits or 70S ribosomes was tested using the procedure of Lietzke and Nierhaus (1988), with minor modifications as described in Materials and Methods. The products of the reconstitution reactions were analyzed by sucrose gradient centrifugation, and examples (in the case of 23S rRNA cleaved at position 1045–1046) are shown in Figure 2. All four of the cleaved rRNA samples showed similar profiles, in which the yield of reconstituted 50S subunits (Fig. 2A) varied between 60 and 80% for the rRNA samples carrying the different cleavages, and from one experiment to another. The structural integrity of the reconstituted 50S subunits was assessed by their ability to form 70S ribosomes. This was again similar for all four cleaved rRNA species (Fig. 2B), and the yields were almost identical to that obtained from reconstituted intact 23S rRNA. After each sucrose gradient, fractions were analyzed on 4% polyacrylamide gels in order to demonstrate the presence of the specific rRNA fragments (data not shown).

³²P-labeled psUp was ligated to the free 3' OH group of the nucleotide at the 5' side of the RNase H cleavage site in each of the fragmented 23S rRNA samples, using T4 RNA ligase, as outlined in Materials and Methods; the yield of the ligation reaction varied between 10 and 20%. In our previous study (Baranov et al., 1997), we had observed that the ligation of psUp to the 3' OH group at the cleavage site was much more efficient than that to the 3' OH group at the 3' terminus of the 16S rRNA. Here the same phenomenon was observed with the 23S rRNA (although, in contrast, the 3' end of 5S rRNA was a good substrate for the ligation of psUp). There is no obvious explanation for this anomalous behavior of the large rRNA molecules, and possible reasons for it are currently under investigation. In any event, low levels of incorporation of psUp at the extreme 3' terminus of the 23S rRNA do not interfere with the subsequent analysis of the crosslinking from the psUp residue inserted at the RNase H cleavage site. (It should furthermore be noted here that fortuitous degradation of the 23S rRNA also has no influence on the crosslink site analysis; fortuitous cleavages normally carry a 3' phosphate and are therefore not substrates for the ligation reaction.) The presence of the psUp residues ligated to the fragmented 23S rRNA samples did not affect their ability to be reconstituted into 50S subunits or 70S ribosomes (cf. Fig. 2, above); the peak fractions from the sucrose gradients of reconstituted 50S subunits were used for the crosslinking experiments described in the following sections.

Crosslinking and analysis of crosslink sites

The 50S subunits reconstituted from the four fragmented 23S rRNA species carrying ³²P-labeled psUp at the cleavage sites were separated by sucrose gradient centrifugation and subjected to mild UV irradiation at 350 nm (Rinke-Appel et al., 1991; Dontsova et al., 1992). The rRNA was then isolated from the irradiated subunits (or from nonirradiated control subunits) by phenol extraction, and the crosslinked regions within the rRNA were localized using the RNase H

method (Dontsova et al., 1991; Rinke-Appel et al., 1991). For this purpose, either two or three oligodeoxynucleotides (10–20 nt long) complementary to the 23S rRNA were necessary in the RNase H digestions. The first oligodeoxynucleotide is complementary to a region upstream of the psUp site, so as to release a short rRNA fragment carrying the ^{32}P -labeled psUp residue. This oligodeoxynucleotide is kept constant in the RNase H digestions, whereas the second one (and the third one, if present) is varied so as to excise different sections of the 23S rRNA encompassing the crosslinked target site (or sites). In contrast to the digestions with the chimeric oligonucleotides used to generate the initial specific cuts in the rRNA, these RNase H digestions are performed at 55° with an excess of enzyme (cf. Rinke-Appel et al., 1991). Candidates for the “constant” oligodeoxynucleotide were carefully selected so as to obtain a clean and quantitative digestion in every case. Thus, in the subsequent gel electrophoresis of the rRNA fragments released by the RNase H, the lanes from the control (nonirradiated) 50S subunits should only show the short fragment carrying the labeled psUp in each case, whereas the lanes from the irradiated subunits should, in addition, show products corresponding to this short fragment crosslinked to the fragments of variable length excised from the target region. Examples of these gels are illustrated in Figure 3, and will be described below for the individual crosslinks.

When the locations of the crosslink sites had been narrowed down in this way to rRNA regions of less than ca. 50 nt, the sites of crosslinking within the regions concerned were analyzed by primer extension (Moazed

et al., 1986). The substrates for the primer extension reactions were isolated from RNase H digestions on a preparative scale. For this purpose, the digestions were chosen so as to release the crosslinked material as a relatively large rRNA complex of 100–200 nt (cf. Fig. 3A, lane 5, or Fig. 3B, lane 1), and a control fragment was extracted from the same gel lane in each case. These control fragments represent the non-crosslinked rRNA from the target region and do not contain the region with the radioactive psUp residue; they could accordingly be located by UV shadowing as bands moving just below the corresponding cross-linked complexes in the gels. Examples of the primer extension gels obtained from the crosslinked and control fragments in the various cases are shown in Figure 4, and are described below for the individual crosslinks. As a result of the artifacts that are often observed in primer extension analyses of crosslink sites (see Brimacombe, 1995, for discussion), we only consider a stop signal in the reverse transcriptase gels as being indicative of a crosslink site if it is reproducibly observed within the short rRNA region already defined by the RNase H digestions (cf. Fig. 3).

Crosslinks from psUp at position C-867

Reconstituted 50S subunits carrying psUp at position C-867 showed the highest level of crosslinking (ca. 30%), and examples of the RNase H digestions for the localization of the corresponding crosslink site are shown in Figure 3A. Each digest was made in the presence of an oligodeoxynucleotide just upstream of the psUp site,

FIGURE 1. Reverse transcriptase analysis of the sites of scission by RNase H in the 23S rRNA. In each panel, the dideoxy sequencing lanes are indicated by A, C, G, U, and the slots marked S or K are from the cleaved and intact (control) 23S rRNA, respectively. **A:** Cleavage site produced by RNase H in the presence of chimeric oligonucleotide No. 1 (Table 1). Primer extension was performed using a primer complementary to nt 490–510 of the 23S rRNA. **B:** Cleavage site from chimeric oligonucleotide No. 2; primer complementary to nt 935–952. **C:** Cleavage site from chimeric oligonucleotide No. 3; primer complementary to nt 1123–1140. Positions corresponding to the sites of scission (385, 868, and 1046 in A, B, and C, respectively) are marked by arrows.

FIGURE 2. Sucrose gradient profiles of ribosomal particles containing cleaved 23S rRNA. **A:** Reconstitution of 50S subunits. Dotted line, control optical density (A_{260}) profile of native 50S subunits; light solid line, control profile of 50S subunits reconstituted from un-cleaved 23S rRNA; heavy solid line, profile of 50S subunits reconstituted from 23S rRNA cleaved at position 1045–1046. Direction of sedimentation is from right to left. **B:** Ability of reconstituted 50S subunits to form 70S ribosomes. Samples correspond to those shown in A.

releasing a short rRNA fragment (ca. 35-nt long) carrying the psUp label as outlined in the previous section. Preliminary experiments indicated that the site of crosslinking was in the downstream region, between positions 868 and ca. 990. The different digests in Figure 3A demonstrate how this region was progressively narrowed down. Slot 1 (Fig. 3A) shows that the crosslink site lies in the 110-nt stretch between positions ca. 880 and 990, whereas slot 3 localizes it to between positions 868 and ca. 940, from which it can be deduced that the site must lie in the 880–940 area. Slot 2, using an oligodeoxynucleotide complementary to positions 900–920, shows only a faint band (ca. 40-nt long) in the gel, which suggests that this oligonucleotide may have encompassed the actual crosslink site, and that, as a result, the RNase H digestion was inhibited. This suggestion was borne out by the primer extension analysis (Fig. 4B), which shows a strong series

of stop signals in the crosslinked sample between nt 909 and 921. The signals divide into two groups, comprising nt 909–914 and 917–921. We cannot distinguish whether the stop signals within these groups represent genuine multiple crosslink sites, or whether this is a case of the “stuttering” that is often seen in reverse transcriptase analyses (see Denman et al., 1988; Brimacombe, 1995, for discussion). Nevertheless, the gel of Figure 4B does indicate that there are at least two distinct crosslink sites, separated by 8–10 nt, and this result was observed reproducibly.

Crosslinks from psUp at position C-1045

In contrast to the situation with the psUp residue at position C-867, the crosslinking from C-1045 proved to be upstream from the psUp site, and examples of the corresponding RNase H digestions are shown in Figure 3B. The psUp-labeled region was excised in each digest as a ca. 50-nt fragment with the help of an oligodeoxynucleotide complementary to positions 985–995, a second oligodeoxynucleotide being added that was complementary to different upstream positions of the rRNA in each gel lane. The gel lanes 1, 3, and 5 (Fig. 3B) illustrate how the crosslink site was narrowed down from a 210-nt region to a 110- and a ca. 20-nt region, respectively. The appearance of the latter band in lane 5 of the gel indicates that the crosslink site should lie between ca. nt 970 and 990. Lanes 2 and 4 show a number of spurious cuts by the RNase H (probably arising from fortuitous complementarities to sequences in the 23S rRNA other than the targeted site), but here also bands of the expected lengths (160 and 40 nt) can be seen.

In the primer extension analysis (Fig. 4C), a single strong and reproducible stop signal was observed at nt 994, which—according to the usual convention—is indicative of a crosslink site at the adjacent nt, G-993. Position 993 lies within the binding site of the oligodeoxynucleotide (complementary to positions 985–995) used to excise the 50-nt fragment carrying the psUp label (Fig. 3B) and, therefore, at first sight, it might appear that the RNase H and primer extension results are contradictory. This is, however, not the case. The presence of the crosslink at position 993 would prevent hybridization of this oligodeoxynucleotide and thus inhibit the RNase H digestion at the 985–995 site in the crosslinked rRNA molecules, whereas, in the non-crosslinked molecules, the digestion would proceed normally. In other words, the supposed RNase H fragments upstream and downstream of the 985–995 cutting site are “held together” in the crosslinked molecules both by the crosslink from 1045 to 993 itself, and by the absence of an RNase cut at this position. The net result does not affect the interpretation of the RNase H digestions of Figure 3B, and we conclude that the cross-

FIGURE 3. Analysis by RNase H digestion of crosslinked 23S rRNA on 4% polyacrylamide denaturing gels. In each pair of lanes, the left sample is rRNA from 50S subunits containing cleaved 23S rRNA without UV irradiation, and the right sample is with irradiation. Diagrams beneath the gel autoradiograms show a scale of nucleotides of the appropriate region of the 23S rRNA, together with the positions of the RNase H cuts for each gel lane. Asterisks indicate the positions of the ^{32}P -labeled psUp residues and the numbers give the approximate lengths of the 23S rRNA fragments excised by the RNase H. **A:** Digests of crosslinked 23S rRNA carrying psUp at position C-867. Each digest was made in the presence of two or three oligodeoxynucleotides. One of these (complementary to positions 822–842) was present in all five gel slots; the released non-crosslinked fragment (ca. 35 nt long) runs in a lower part of the gel (not visible). The other oligodeoxynucleotides were complementary to the following 23S rRNA sequences: Slot 1, positions 870–886 and 985–995 (releasing a fragment ca. 100-nt long); Slot 2, 900–920 (ca. 40 nt from the psUp site); Slot 3, 936–952 (ca. 75 nt from the psUp site); Slot 4, 962–978 (ca. 100 nt); Slot 5, 985–995 (ca. 120 nt). **B:** Digests of crosslinked 23S rRNA carrying psUp at position C-1045. Each digest was made in the presence of two oligodeoxynucleotides, one of which (complementary to positions 985–995) was present in all five gel slots; as in A, the short non-crosslinked fragment released (ca. 50-nt long) is not visible in the gel region shown. The other oligodeoxynucleotides were complementary to: Slot 1, positions 774–788 (releasing a fragment ca. 210-nt long); Slot 2, 822–842 (ca. 160 nt); Slot 3, 870–886 (ca. 110 nt); Slot 4, 936–952 (ca. 40 nt); Slot 5, 962–978 (ca. 20 nt). **C:** Digests of crosslinked 23S rRNA carrying psUp at position C-1117. Each digest was made using two oligonucleotides, one of which was present in all five gel slots, namely the chimeric oligonucleotide 3 (Table 1), which cuts at position 1045–1046. Again the non-crosslinked fragment (ca. 75-nt long) is not visible in the gel region shown. The other oligodeoxynucleotides used were complementary to: Slot 1, positions 962–978 (releasing a fragment of ca. 75 nt); Slot 2, 1004–1016 (ca. 35 nt); Slot 3, 1021–1035 (ca. 15 nt). Fragment lengths indicated on the gel panels do not include the length of the common excised fragment in each case (35, 50, or 70 nt in panels A, B and C, respectively). See text for further explanation.

link site is indeed at position 993, as indicated by the primer extension analysis.

Crosslinks from psUp at positions C2510 and C1117

Preliminary analyses showed that there were no detectable crosslinks from the psUp residue at position C-2510, and only the psUp at the concomitant fortuitous cleavage site at C-1117 (Table 1) was involved in crosslink formation. The situation here was similar to the case of the psUp residue at C-1045, in that the crosslink was upstream of the psUp site, and Figure 3C shows examples of the RNase H digestions concerned, which served to localize the crosslink site to positions ca. 1030–1045. The corresponding primer extension

analysis (not shown in Fig. 4) indicated that the crosslink site was in the region of nt 1037, but, as with the crosslinks from the psUp residue at position 867 (Fig. 4B), there were two or three adjacent reverse transcriptase stop signals in this area of the gel. We conclude that the crosslink site (or sites) lay between nt 1036 and 1038.

Crosslinks from psUp at position A-384

Crosslinking from the psUp residue at A-384 was only observed in low yield (1–2%), but this was nonetheless reproducible. RNase H digestions (not shown in Fig. 3) showed that the crosslink site was downstream from the psUp site, and the crosslinked region was localized to the sequence between positions 384 and ca. 440. An

FIGURE 4. Primer extension analysis of the crosslink sites in the 23S rRNA. In each panel, the dideoxy sequencing lanes are indicated by A, C, G, U and the slots marked X or K are the crosslinked and control samples, respectively. (K1 and K2 are two independent control lanes in each case.) Arrows denote the positions of reverse transcriptase stops specific for the crosslinked samples. **A:** Crosslinks from psUp at position A-384; reverse transcription was performed using a primer complementary to 23S rRNA nt 490–510. **B:** Crosslinks from psUp at position C-867; primer complementary to nt 962–978. **C:** Crosslinks from psUp at position C-1045; primer complementary to nt 1022–1034.

example of the corresponding primer extension analysis is given in Figure 4A, where a reverse transcriptase stop can be seen at position 421, indicative of a cross-link site at the adjacent nt, C-420. In other experiments, additional stop signals were sometimes seen at nt 417 and 426, indicating possible crosslinks at U-416 and G-425, but the signal at position 421 was the most reproducible.

DISCUSSION

We have described here the second application of our new approach (Baranov et al., 1997) for the site-directed photo-crosslinking of the long 16S and 23S rRNA molecules. Growing interest in RNA structures in general has led to the appearance of a number of new techniques in “RNA engineering,” and several methods have been described that allow the insertion of modified nucleotides into relatively short RNA molecules (Moore & Sharp, 1992; Strobel & Cech, 1993; Yu & Steitz, 1997). All these approaches are based on the restoration of a continuous RNA chain, using T4 DNA ligase. However, in the case of the long rRNA molecules, this type of religation has proved to be extremely difficult (Bogdanova et al., 1995; see Baranov et al., 1997, for discussion), and our method is accordingly based on the observation that fragmented rRNA can, in many cases, be successfully reconstituted back into ribosomal subunits or 70S ribosomes. Many lines of evidence indicate that ribosomes containing fragmented rRNA can retain their correct structure and competence in the

basic ribosomal functions (Afonina et al., 1991a, 1991b; Green & Noller, 1996; Winter et al., 1997). Indeed, the insertion of the photoreactive nucleotide (sU) into the rRNA chain without religation can be advantageous for the generation of crosslinks, because this allows the photoreactive moiety more flexibility and hence a better chance of contacting potential crosslinking targets in its vicinity.

The specific chain scissions within the rRNA are induced by RNase H in the presence of complementary chimeric oligonucleotides, and there are some contradictions in the literature as to the precise sites of cleavage that are generated by this procedure. Our previous experience (Baranov et al., 1997) has been that, in the presence of such modified oligonucleotides, the RNase H cleaves the rRNA exactly between the extreme 3' nucleotide of the heteroduplex and the first downstream nonpaired nucleotide of the rRNA. Lapham and Crothers (1996) also found cleavages at this position. On the other hand, Yu and Steitz (1997) reported that the cleavage occurs between the two last 3' RNA nucleotides within the duplex, and—in the other direction—Lima and Crooke (1997) described cleavage between the first two nonpaired RNA nucleotides on the 3' side of the duplex. In a further report, Lapham et al. (1997) demonstrated that the location of the cleavage site depends on the method of isolation and the storage conditions of the RNase H. Here we have used RNase H prepared by the method of Brimacombe et al. (1990), and the sites of cleavage were at the same position as we had observed previously (Baranov et al., 1997),

with the exception of the cleavage at internucleotide bond 384–385, which corresponds to the situation reported by Lima and Crooke (1997) (see Table 1). Thus, it seems likely that structural features of the rRNA itself, or of the individual heteroduplexes, may influence the exact site of RNase H cleavage. Much more important is the fact that the use of the chimeric oligonucleotides does indeed lead to a clean scission, which is almost exclusively at a single internucleotide bond, although it is obviously necessary to check the precise site of cleavage in each case, as we have done here (Fig. 1).

The locations of the crosslinks that we have identified from the psUp residues at the RNase H cleavage sites are shown within the appropriate regions of the secondary structure of 23S rRNA in Figure 5. Crosslink 1, from the psUp at position A-384, is predominantly to C-420, with two other possible sites at U-416 and G-425. This result (although the crosslink was only formed in low yield) indicates that the loop-end of helix 21 lies close to helix 22, and simple model building (not shown) demonstrates that the A-384/C-420 crosslink is furthermore in good agreement with the base pair between

C-385 and G-411 proposed by Larsen (1992) on the basis of phylogenetic analysis. In view of the well-established tertiary interaction (Leffers et al., 1987) already mentioned in the Introduction between the loop ends of helix 22 (in Domain I of the 23S rRNA) and helix 88 (in Domain V), these data also imply that helices 21 and 88 are in close proximity.

Crosslink 2, from the psUp residue at C-867 (Fig. 5), clearly lies within the secondary structure of the 23S rRNA in helix 38. As noted in the Results, at least two crosslink sites are involved here, separated by several nucleotides, but the sites cannot be specified precisely as a result of the "stuttering" of the reverse transcriptase in the primer extension analysis (Fig. 4B). The cleavage at C-867 generates a potentially flexible single-stranded "arm" comprising residues 863–867 (although it should be noted that additional base pairs could be formed within the internal loop of helix 38, such as 863–864/912–913), and this flexibility would enable the psUp residue to reach a number of sites on the opposite strand of the helix. The distance between the two principal reverse transcriptase stops in Figure 4B represents almost one full turn of an A-form RNA helix, suggesting that the crosslink sites are concentrated on one side of the helix. Crosslink 2 is very similar to the psoralen crosslink observed by Turner and Noller (1983), which they localized to positions C867 and U-913. Crosslink 4, from the psUp residue at C-1117, is—like crosslink 2—also a "secondary structural" crosslink, within helix 42 of the 23S molecule.

Crosslink 3, on the other hand, from the psUp residue at C-1045, defines an important new tertiary structural feature of the 23S rRNA. This crosslink, from C-1045 to G-993, demonstrates that the internal loop of helix 42 (Fig. 5) is folded back so as to contact the base of helix 41, thus generating a "U-turn" within the helix 41–42 region. The established pseudoknot in this area between nt 1137–1138 and 1005–1006 is likely to be important for the formation of this U-turn; it has been demonstrated (Rosendahl et al., 1995) that mutations that disrupt the pseudoknot base pairing are detrimental to ribosomal activity, but that compensatory mutations cause the activity to be restored. The U-turn has the effect of bringing the well-known GTPase-associated area of the ribosome (defined by the conserved region between nt 1050 and 1110 in helices 42–44) close to the "ring" in Domain II enclosed by helices 36–41 and 45 (Fig. 5). It is noteworthy that such a U-turn is implicit from sequence comparison studies on the large subunit rRNA (e.g., Gutell & Fox, 1988); large deletions occur within helices 41–42 in certain mitochondrial rRNAs and, if the 41–42 stem did not fold back, these deletions would result in the "misplacing" of the GTPase area in the mitochondria ribosomes. The 1050–1110 region is the binding site for protein L11 (Egebjerg et al., 1990), and is the target for the antibiotics thiostrepton and micrococin, both of

which affect GTP hydrolysis (Egebjerg et al., 1989; Rosendahl & Douthwaite, 1994). The internal loop in helix 42 is part of the binding site of the pentameric complex L10(L7/L12)₂ (Egebjerg et al., 1990; Rosendahl & Douthwaite, 1993), and L7/L12 is known to be involved in binding of the elongation factors. Recently, a direct visualization of the EF-Tu/tRNA complex (Nissen et al., 1995) within the ribosome was presented, based on an electron cryomicroscopic reconstruction of kirromycin-stalled 70S ribosomes (Stark et al., 1997b); the reconstruction showed an apparent bridge connecting domain 1 of the EF-Tu with the L7/L12 region of the 50S subunit, thus implicating this region as being an important part of the "gate" for admitting the incoming EF-Tu/tRNA ternary complex or EF-G to the ribosome.

On the other hand, there is also evidence for involvement of the L7/L12 region in the regulation of peptidyl transferase activity. L7/L12 has been crosslinked to L2 (Traut et al., 1995), and L2 is known to be associated with the peptidyl transferase activity (reviewed by Wower et al., 1995). Furthermore, it has been shown that the mutants A1159G and A1187G (cf. Fig. 5), which show resistance to thiostrepton, are at the same time hypersensitive to other antibiotics (amicetin, anisomycin, and chloramphenicol) that act at the peptidyl transferase center (Mankin et al., 1994). 5S rRNA provides yet another link between these regions, because crosslinks in high yield have been reported (Dokudovskaya et al., 1996) between the loop end of helix IV of the 5S molecule to the loop ends of both helix 89 (in Domain V of the 23S rRNA, close to the peptidyl transferase center) and helix 39 (in Domain II; Fig. 5). More recently, further crosslinks from helix IV of the 5S rRNA have been found to the single strands joining helices 41 and 42 of the 23S rRNA (Sergiev et al., 1997), and from helices II and III to the loop end of helix 38 (M. Osswald & R. Brimacombe, unpubl. results). Taken together with the crosslinking from the latter loop end to the elbow region of A-site-bound rRNA (Rinke-Appel et al., 1995), all of these data define a complex network of interactions (cf. Brimacombe, 1995) between tRNA, 5S rRNA, and the areas of the 23S rRNA comprising the GTPase and peptidyl transferase centers. As the folding of the rRNA in these areas becomes clearer, it becomes progressively easier for us to predict sites where the insertion of a photoreactive psUp residue is likely to be informative, and we anticipate that the further application of this site-directed crosslinking method will help us gain a deeper insight into the structure and function of the large 16S and 23S rRNA molecules.

MATERIALS AND METHODS

Materials

T4 polynucleotide kinase, T4 RNA ligase, and AMV reverse transcriptase were purchased from Boehringer Mannheim.

RNase H from *E. coli* was isolated as described by Brimacombe et al. (1990). 70S ribosomes from *E. coli* were prepared according to the procedure of Rheinberger et al. (1988), and 23S and 5S rRNA were isolated as described by Nowotny et al. (1988). Chimeric oligonucleotides (Table 1) were synthesized by the procedure described by Baranov et al. (1997).

Cleavage of 23S rRNA and ligation of psUp

23S rRNA was cleaved at a single internucleotide bond in the presence of RNase H and one of the chimeric oligonucleotides (Table 1) as described by Baranov et al. (1997) for the case of 16S rRNA. ³²P-labeled psUp was also prepared by the procedure of Baranov et al. (1997), with the exception that the ratio of ³²P-ATP:sUp in the reaction with T4 polynucleotide kinase was increased from 1:10 to 1:1, because we had observed that sUp can inhibit the subsequent ligation reaction (data not shown). Ligation to the 3' termini of the 23S rRNA fragments was performed as before (Baranov et al., 1997), using T4 RNA ligase.

Reconstitution of fragmented 23S rRNA into 50S subunits or 70S ribosomes

A mixture of fragmented 23S rRNA (3 pmol/ μ L) and 5S rRNA (9 pmol/ μ L) at a molar ratio of 1:3 was pre-incubated for 30 min at 37° in a buffer consisting of 10 mM Tris-HCl, pH 7.5, 4 mM magnesium acetate, 400 mM NH₄Cl, and 4 mM 2-mercaptoethanol. Next, a solution of a 1.2-fold molar excess of total 50S ribosomal proteins [10 pmol/ μ L, prepared by the procedure of Lietzke and Nierhaus (1988)] in the same buffer was added, and the mixture was incubated for 30 min at 44°. The magnesium concentration was then raised to 20 mM and incubation continued for 2 h at 50°, following which the mixture was chilled on ice. The samples were used either for the isolation of 50S subunits by centrifugation through a sucrose gradient (10–40%, 23,000 rpm, 18 h, in 20 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 100 mM NH₄Cl, 4 mM 2-mercaptoethanol), or for the preparation of 70S ribosomes. In the latter case, the reaction mixture containing the reconstituted 50S subunits was diluted so as to give a composition of 10 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate, 200 mM NH₄Cl, and 4 mM 2-mercaptoethanol. A twofold molar excess of 30S subunits (10 pmol/ μ L) in the same buffer was pre-incubated for 15 min at 42° and added to the solution of 50S subunits. After incubating for 30 min at 37°, the mixture was chilled on ice and analyzed by sucrose gradient centrifugation as above (cf. Dontsova et al., 1994).

Crosslinking and identification of crosslink sites in 23S rRNA

Reconstituted 50S subunits containing fragmented 23S rRNA isolated by sucrose gradient centrifugation (cf. Fig. 2A) were irradiated at 350 nm (Dontsova et al., 1992) to activate the sU residues. The crosslinked 23S rRNA was then isolated by phenol extraction, and the crosslink sites on the RNA were identified by digestion with RNase H in the presence of oligodeoxynucleotides complementary to selected sequences

in the 23S rRNA, combined with primer extension analysis according to our usual procedures (Dontsova et al., 1992, 1994), exactly as described by Baranov et al. (1997). Yields of the individual crosslinks were estimated by comparing radioactivities in gel bands corresponding to undigested cross-linked complexes (cf. Baranov et al., 1997) with the total radioactivity in the gel lane concerned.

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