The environment of 5S rRNA in the ribosome: cross-links to 23S rRNA from sites within helices II and III of the 5S molecule

Monika Osswald and Richard Brimacombe*

Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 73, 14195 Berlin, Germany

Received February 24, 1999; Revised and Accepted April 10, 1999

ABSTRACT

Three contiguous fragments of Escherichia coli 5S rRNA were prepared by T7 transcription from synthetic DNA templates. The central fragment, comprising residues 33–71 of the molecule, was transcribed in the presence of 4-thiouridine triphosphate together with [32P]UTP. The three transcripts were ligated together, yielding a 5S rRNA analogue carrying 4-thiouridine residues at positions 40, 48, 55 and 65 in helices II and III. After ligation, the 4-thiouridine residues were derivatised with p-azidophenacyl bromide. The modified 5S rRNA was reconstituted into 50S subunits and these subunits were used to prepare 70S ribosomes in the presence or absence of tRNA and mRNA. The azidophenyl groups were then photoactivated by mild irradiation at 300 nm and the products of cross-linking analysed by our standard procedures. Multiple cross-links from 5S rRNA to two distinct regions of the 23S rRNA were observed. The first region was located in helix 38 in Domain II of the 23S molecule, with cross-links at sites between nucleotides 885 and 922. The second region covered helices 81–85 in Domain V, with sites between nucleotides 2272 and 2345. Taken together with previous data, these results serve to define the arrangement of the 5S rRNA molecule relative to the 23S rRNA within the 50S subunit.

INTRODUCTION

The small 5S rRNA is a well-studied component of the 50S ribosomal subunit and is known to lie within the central protuberance of the latter. This placement is based both on direct localisation by immunoelectron microscopy (IEM) of specific nucleotides in the 5S molecule (1–4) as well as on similar IEM localisations (5,6) of ribosomal proteins L5, L18 and L25, which have been shown by binding (7,8) and footprinting (9,10) studies to interact specifically with the 5S rRNA. The central protuberance of the 50S subunit lies opposite the head of the 30S subunit in the 70S ribosome. Chemical probing analyses of the 5S rRNA have indicated precise contacts with the 23S and 16S rRNAs (11,12). Our own interest in the 5S rRNA is more concerned with its interactions with the 23S rRNA. The relevance of this question has been enhanced by the recent dramatic advances that have been made in cryo-electron microscopic (cryo-EM) (13,14; H.Stark and M.van Heel, personal communication) and X-ray crystallographic (15) analyses of ribosomes and their subunits. These studies have led to the derivation of ribosomal structures with resolutions in the range 9–15 Å, a level at which it should in principle be possible to fit elements of the rRNA secondary structures directly into corresponding elements of electron density. However, in practice the complexity of the 16S and 23S rRNA molecules is such that a plausible fitting can only be accomplished by taking into account the large body of biochemical data relating to the 3-dimensional folding of these molecules that has been accumulated over the years (see for example 16 for a review). In this way we have recently published a structure for the 16S rRNA fitted to a cryo-EM reconstruction of the 30S subunit within the 70S ribosome (17–19). It is clear that, in order to derive a corresponding 3-dimensional structure for the 50S subunit, a detailed understanding of the interactions and contacts between the 5S and 23S rRNA molecules is of central importance.

In our previous studies on the environment of the 5S rRNA we have made use of a cross-linking approach, in which 5S rRNA analogues carrying randomly distributed photoreactive uridine derivatives were prepared by T7 transcription and reconstituted into 50S subunits. The uridine derivatives involved were either 4-thiouridine (20,21), 5-methylethamino uridine (22) or 2′-amino 2′-deoxyuridine (22); 4-thiouridine (thioU) is itself photoreactive, whereas in the latter two cases the photoreactive moiety was introduced after the T7 transcription by reaction with appropriate diazirine compounds. These experiments led to the identification of a number of precise contacts between the 5S rRNA and the 23S rRNA (20–22). However, despite the fact that the photoreactive residues were distributed throughout the 5S molecule, all of the observed cross-links were from the same residue (U89) of the 5S rRNA. This at first sight surprising finding is not unreasonable when considered in the context of the structure that has been derived by NMR (23) when the 50S and 30S subunits associate to form 70S ribosomes (11,12).

*To whom correspondence should be addressed. Tel: +49 30 8413 1592; Fax: +49 30 8413 1690
for helices IV and V of the 5S rRNA; in the latter structure the uracil base of residue 89 is oriented conspicuously outwards from the bulk of the molecule.

The predominance of the cross-links from U89 has the consequence that cross-links in lower yield from other parts of the molecule might have been masked or overlooked in our previous studies (20–22). Accordingly, in order to investigate cross-linking from other regions of the 5S rRNA, we have instigated an approach in which the photoreactive residues are selectively introduced into a specific sequence of the molecule. For this purpose, a 5S rRNA analogue is prepared by T7 transcription of oligodeoxynucleotides (26) in 10 mM Tris–HCl pH 8.0, 250 mM MgCl₂, 100 mM dithiothreitol, 20 mM spermidine, 3 μl each of ATP, CTP, GTP and UTP (100 mM), 3 μl of RNasin (40 U/μl Promega) and 12 μl of T7 RNA polymerase (69 U/μl Pharmacia). For the 3′-terminal fragment (Fig. 1), 6 μl of GMP (300 mM) was added to the transcription mixture. For the central fragment, the transcription mixture also contained 6 μl of GMP (300 mM), together with 5 μl of thiouUTP (10 mM; Amersham) in place of UTP and 1 μl of [α-32P]UTP (Amersham). In each case the reaction volume was adjusted to 100 μl and incubated at 37°C overnight. The transcription products were separated by electrophoresis on 15% polyacrylamide gels containing 7 M urea. Bands corresponding to the 5S rRNA fragments were localised on the gels by UV shadowing or autoradiography as appropriate and the RNA was extracted in the presence of phenol and precipitated with ethanol.

**Ligation of 5S rRNA fragments**

The ligation was performed by the method of Moore and Sharp (27). For this purpose, the three transcribed fragments in roughly equimolar amounts (900–1400 pmol) were combined in a volume of 11 μl of water and mixed with 900 pmol of the two ‘bridging’ oligodeoxynucleotides (Fig. 1), each in 9 μl of water. After a brief incubation (5 min) at 37°C, this hybridised complex was added to the ligation mixture, which contained 15 μl of 10x concentrated buffer (660 mM Tris–HCl pH 7.5, 50 mM MgCl₂, 10 mM dithiothreitol, 10 mM ATP), 56 μl of polyethylene glycol (40%), 4 μl of RNAasin (as above) and 35 μl of T4 DNA ligase (5 U/μl; Boehringer) in a final volume of 150 μl. After incubation at 26°C overnight, the mixture was extracted with phenol and the ligation products were separated on a 10% polyacrylamide gel containing 7 M urea. The band corresponding to ligated 5S rRNA was localised by autoradiography and extracted from the gel as above. The sequence of the ligated molecule was checked by primer extension, using an oligodeoxynucleotide complementary to the 3′-terminal 20 nt as primer (data not shown).

**Derivationisation of 5S rRNA with APAB, and reconstitution into ribosomes**

The thioU residues in the central region of the ligated 5S rRNA were derivatised with APAB (Sigma), using the conditions of Bochkariov and Kogon (28) as previously described (25). Under these conditions, the derivationisation with APAB has been shown to be essentially quantitative (29). Reconstitution into 50S subunits was performed with unlabelled 23S rRNA and total 50S ribosomal proteins by the procedure of Lietzke and Nierhaus (30). 70S ribosomal complexes were then prepared in the presence or absence of P site-bound TRNA and mRNA as before (20, 21). For control experiments, ligated 5S rRNA samples not derivatised with APAB or ligated 5S rRNA with no thioU residues were used.

**Cross-linking and analysis of cross-linked products**

The ribosomal complexes were irradiated for 10 min using a UV lamp with an energy maximum of 254 nm, the samples being covered by a glass plate with a 280 nm cut-off as described previously (25). The 70S complexes were purified by sucrose gradient centrifugation at 10 mM magnesium and the 5S rRNA sequence of the ligated molecule was checked by primer complex was added to the transcription mixture, which contained 10 μl of 10x concentrated buffer (400 mM Tris–HCl pH 8.0, 250 mM MgCl₂, 100 mM dithiothreitol, 20 mM spermidine), 3 μl each of ATP, CTP, GTP and UTP (100 mM), 3 μl of RNasin (40 U/μl Promega) and 12 μl of T7 DNA polymerase (69 U/μl; Pharmacia). For the 3′-terminal fragment (Fig. 1), 6 μl of GMP (300 mM) was added to the transcription mixture. For the central fragment, the transcription mixture also contained 6 μl of GMP (300 mM), together with 5 μl of thioUTP (10 mM; Amersham) in place of UTP and 1 μl of [α-32P]UTP (Amersham). In each case the reaction volume was adjusted to 100 μl and incubated at 37°C overnight. The transcription products were separated by electrophoresis on 15% polyacrylamide gels containing 7 M urea. Bands corresponding to the 5S rRNA fragments were localised on the gels by UV shadowing or autoradiography as appropriate and the RNA was extracted in the presence of phenol and precipitated with ethanol.
cross-linked to 23S rRNA was isolated by a further sucrose gradient in the presence of SDS (refer to 20, 21). The cross-link sites on the 23S rRNA were then determined by a combination of ribonuclease H digestion and primer extension analysis using our standard procedures (20, 21). Individual thioU residues in the 5S rRNA involved in the cross-links were examined by ribonuclease A digestion and primer extension using our usual procedure (20, 21).

RESULTS

The sequences of the *Escherichia coli* 5S rRNA fragments used in this study are illustrated in Figure 1. In order to obtain efficient transcription with T7 RNA polymerase, the sequences for the central and 3′ fragments were chosen so as to begin with a G residue (positions 33 and 72, respectively). For the same reason, the U at position 1 of the 5′ fragment was changed to G and the corresponding base paired A residue (31) at position 119 in the 3′ fragment was changed to C. Our previous experiments (20, 21) had shown that changing the terminal base pair in this way does not impair the ability of the 5S rRNA to be reconstituted into 50S subunits or 70S ribosomes. Helices II and III of the 5S rRNA (31) cover positions 16–68 of the sequence, the central fragment (nt 33–71; Fig. 1) representing the hairpin loop end and the 3′ strand of the two helices (see Fig. 6 below).

The three fragments were transcribed from suitable DNA templates as described in Materials and Methods. In the case of the central and 3′ fragments, an excess of GMP was included in the transcription mixtures. GMP is only incorporated at the extreme 5′-terminus of the transcript and in this way transcripts carrying predominantly a 5′-monophosphate rather than a 5′-triphosphate are produced (J.Wower, personal communication). Such transcripts are direct substrates for the subsequent DNA ligase reaction. The central fragment contains four U residues (Fig. 1) and this fragment was transcribed in the presence of unlabelled thioUTP and [γ-32P]UTP. The specific activity of the latter was high, so that the incorporated thioU residues were not significantly ‘diluted’ with normal uridines. The 32P label was introduced for the dual purpose of following the cross-linking reactions and for potentially identifying the individual thioU residues involved in the various cross-links; digestion of the labelled 5S rRNA molecule with ribonuclease A should lead to the release of four distinguishable radioactive oligonucleotides carrying a 32P label at the positions 5′-adjacent to the thioU residues (namely AUp, GAACp, AGAAGUp and GUp; Fig. 1) and in the case of a cross-linked complex the corresponding oligonucleotide should be absent (refer to 20; see also below).

After T7 transcription, the three fragments were ligated together (27) using DNA ligase in the presence of the ‘bridging’ oligonucleotides indicated in Figure 1. The ligation products were separated by gel electrophoresis and a typical gel profile is illustrated in Figure 2. The band corresponding to the complete 5S rRNA molecule (Fig. 2, band 4) was extracted from the gel and the thioU residues (at positions 40, 48, 55 and 65) were then derivatised with APAB as described in Materials and Methods. In some control experiments this derivatisation step was omitted and for further controls ligated 5S rRNA was used in which the central fragment (Fig. 1) was transcribed in the presence of ‘normal’ UTP instead of thiout (see below).

The ligated and APAB-derivatised 5S rRNA was reconstituted into 50S subunits and from these subunits 70S ribosomal complexes with or without tRNA and mRNA were prepared, under the same conditions as those used in our previous experiments (20, 21). The reconstitution was typically made on a 150 pmol scale, with a level of 5S rRNA radioactivity incorporated into 50S subunits of the order of 8–10 × 10^6 c.p.m. Cross-links from the APAB-derivatised residues were induced by mild UV irradiation at wavelengths above 280 nm, again using our previously established conditions (25). The cross-linked 70S complexes were purified on sucrose gradients in the presence of 10 mM magnesium, followed by a second sucrose gradient in the presence of SDS. In the latter gradients (data not shown, but cf. 20), 5S rRNA cross-linked to 23S rRNA is separated from free 5S rRNA, the amount of 5S radioactivity remaining associated with the 23S rRNA being typically of the order of 15–20 × 10^6 c.p.m.; this represents a cross-linking yield of ~2% (cf. 22). Small amounts of 5S rRNA cross-linked to 16S rRNA were also detected in these gradients.
The cross-link sites on the 23S (or 16S) rRNA were analysed by our usual procedure (20,21), involving ribonuclease H digestion of the cross-linked 5S–23S rRNA complex in the presence of oligodeoxynucleotides complementary to selected sequences of the 23S rRNA, followed by primer extension analysis. Cross-linking of the 5S rRNA to ribosomal proteins (either from the 50S or the 30S subunit) was also investigated, by an immunological method (32); cross-linked 5S rRNA–protein complexes should run together with the peak of free 5S rRNA in SDS-containing sucrose gradients.

A preliminary scan of the cross-link sites on 23S rRNA using ribonuclease H revealed that two regions of the latter were involved, the first covering nt 800–1150 of the 23S sequence (in Domain II of the secondary structure) and the second nt 1900–2600 (in Domain V). Typical examples of the more detailed ribonuclease H analyses of these two sequence regions are given in Figure 3. Figure 3A compares the digestion patterns in the Domain II area obtained from cross-linked complexes in the presence or absence of RNA and mRNA and shows, in fact, that there are no differences. Lanes 1 and 2 indicate that there is a cross-link site between the approximate positions 865 and 933 (releasing a fragment of ~70 nt), but that there is no site in the short contiguous region from nt 933 to 950. Lanes 3 and 4 indicate the presence of two cross-links, one between nt 865 and 950 (the 85 nt fragment) and one between nt 950 and 970 (the 20 nt fragment); here the additional band on the gel of 105 nt arises from incomplete scission by ribonuclease H at the intermediate site (position 950). Lanes 5 and 6 show a cross-link between nt 865 and 929 and lanes 7 and 8 an apparent cross-link between nt 990 and 1130 (see below).

Figure 3B compares the results of Figure 3A with those from a control sample in which the derivatisation with APAB was omitted. It can be seen that the cross-linked gel bands with 70, 85, 65 and 140 nt (in lanes 2, 4, 6 and 8, respectively) are entirely dependent on the presence of APAB. On the other hand, the 105 and 20 nt bands in lane 4 are also seen in the absence of APAB (lane 3), indicating that the cross-link site between nt 950 and 970 is APAB-independent. Furthermore, this site was also observed in another control experiment (not shown) in which ligated 5S rRNA carrying normal uridine in place of the thioU residues in the central fragment (Fig. 1) was used for the 50S reconstitution. The site between nt 950 and 970 is thus a fortuitous UV-induced cross-link, most probably corresponding to the very high yield cross-link previously observed (21) from U89 of the 5S rRNA (either from the 50S or the 70S ribosomes with or without tRNA and mRNA and the same type of local variability in the distribution of the primer extension stop signals observed in each case.

The primer extension method is not appropriate for a corresponding analysis of the cross-linked residues in the 5S rRNA, because the APAB-modified thioU residues themselves would be expected to cause reverse transcriptase stops. Accordingly, the fingerprint method was used, as in our previous experiments (20–22). As already noted above, digestion of the ligated 5S rRNA with ribonuclease A should lead to the release of four distinct 32P-labelled oligonucleotides, and such a digest is illustrated in Figure 5A. In the cross-linked complexes, the 5S rRNA oligonucleotide involved in the particular cross-link should be absent, as we have previously demonstrated (20–22). However, in this series of experiments the multiple cross-link sites observed (Fig. 4) are too close together for the individual cross-links to be segregated into single complexes. This difficulty was compounded by the local variability in the distribution of the primer extension stop signals just mentioned, as well as by the fact that the excised ribonuclease H fragments that are to be used for reverse transcription have to be sufficiently long to allow hybridisation of a deoxyoligonucleotide primer. As a result, any
Figure 3. Autoradiograms of ribonuclease H digests on 6% polyacrylamide gels of 23S rRNA cross-linked to 32P-labelled, APAB-modified 5S rRNA. In each gel lane the ribonuclease H digest was performed in the presence of two or more oligodeoxynucleotides (10–20 nt long) complementary to selected regions of the 23S rRNA. The central complementary positions of each of these oligodeoxynucleotides within the 23S rRNA sequence, together with the approximate lengths of the 23S rRNA fragments that would be released in each case, are indicated in the diagrams at the bottom of the figure. In (A) the odd numbered lanes are digests derived from 70S complexes carrying tRNA and mRNA, whereas the even numbered lanes are from 70S complexes without tRNA or mRNA. Lanes 1 and 2, oligodeoxynucleotides centred on 23S positions 865, 933 and 950; lanes 3 and 4, positions 865, 950 and 970; lanes 5 and 6, positions 865 and 929; lanes 7 and 8, positions 990 and 1130. In (B) the even numbered lanes are from 70S complexes with tRNA and mRNA and the odd numbered lanes are from similar control complexes but where the APAB derivatisation of the 5S rRNA was omitted. The oligodeoxynucleotide positions are exactly the same as those in (A). In (C) the digests are again from 70S complexes with tRNA and mRNA. Lane 1, oligodeoxynucleotides centred on 23S positions 1904 and 2170; lane 2, positions 2170 and 2360; lane 3, positions 2360 and 2573; lane 4, positions 2235 and 2309; lane 5, positions 2235 and 2292; lane 6, positions 2292 and 2360. Fragment lengths shown in parentheses in the lower diagrams are those that were not observed on the gels.

Isolated 23S rRNA fragment cross-linked to 5S rRNA usually contained several cross-link sites involving two or more of the four modified 5S nucleotides. Thus, although reductions in the intensities of one or more of the four oligonucleotide spots from
the digested cross-linked complexes were often observed, reliable identification of the individual cross-linked nucleotides among the four modified residues was in general not possible. A rare example (showing the absence of the AUp sequence at positions 39–40 of the 5S sequence) is included in Figure 5B, to demonstrate that this fingerprint method is indeed viable, as in our previous studies (20–22), when only a single cross-linked residue is present in the sample. The particular result shown in Figure 5B was, however, not seen frequently enough to be regarded as proven and, accordingly, we limit our conclusion to the statement that the observed cross-link sites within the 23S rRNA lie in the neighbourhood of residues 40–65 in helices II and III of the 5S rRNA (see also Discussion below).

It was mentioned above that weak cross-linking from the modified 5S rRNA to the 16S rRNA in 70S complexes was sometimes observed, but the yield of this cross-link proved to be too low for the cross-link site(s) to be localised. Similarly, the immunological investigation of possible cross-links from the 5S rRNA to ribosomal proteins from either subunit did not show any significant levels of reaction.

**DISCUSSION**

The results described in the foregoing show that helices II and III of the *E.coli* 5S rRNA become cross-linked to two specific regions of the 23S rRNA, in Domains II and V of the secondary structure, respectively. The multiplicity of the cross-link sites observed within these specific regions is, however, suggestive of a certain degree of flexibility in the 23S rRNA regions concerned or in the helix II/III region of the 5S molecule. The latter type of flexibility could either be inherent in the 5S rRNA structure (helices II and III have a somewhat ‘open’ secondary structure) or could result from the four modified nucleotides that were introduced (Fig. 1). It is noteworthy that these APAB–thioU modifications (at positions 40, 48, 55 and 65) did not cause a significant reduction in the ability of the reconstituted 50S subunits to associate with 30S subunits to form 70S ribosomes (either with or without tRNA and mRNA). This contrasts with our previous data, where randomly incorporated 2′-amino 2′-deoxy-uridine residues modified with a diazirine derivative were used (22); in those experiments formation of 70S complexes was shown to select against 5S molecules carrying the modified nucleotides at positions 32, 40, 48 and 55. As noted in the Introduction, nucleotide 55 of the 5S rRNA is strongly protected in 70S ribosomes as opposed to 50S subunits (12) and the precise nature of a modification at this site or neighbouring sites may well be critical in allowing or preventing the formation of 70S particles. Although we were unable to localise the cross-link site(s) from helix II/III of 5S rRNA to 16S rRNA (as a result of the low levels of cross-linking to the latter), the observation of cross-linking to 16S rRNA is consistent with an involvement of this part of the 5S molecule in a direct contact between the two subunits.

The cross-linking data from helices II and III, together with our previous cross-links (20–22) from residue U89 of the 5S molecule, are summarised in Figure 6. The cross-links from helices II and III in the Domain V region are located in helices 81–86, which is in good agreement with other results. A 23S rRNA region comprising helices 82–87 was identified many years ago (33) in a ribonucleoprotein fragment consisting of 5S rRNA, proteins L5, L18 and L25 (the 5S-associated proteins; 7–10) and a part of the 23S molecule. The same components clearly form the central protuberance of the 50S subunit (34). RNA–protein cross-links to proteins L5 and L18 have been observed in helix 84, as well as cross-links to L27 in helices 81 and 85 (35); L27 is known from IEM studies (5) to lie at the base of the central protuberance. Furthermore, site-specific cross-links from the elbow region of P site tRNA have been identified both at the loop end of helix 84 and around helix 85 (25).
The cross-links from helices II/III of the 5S rRNA to Domain II of the 23S rRNA are concentrated in the upper part of helix 38 (Fig. 6). This part of the 23S molecule was for a long time devoid of any topographical information, but recently we identified site-specific cross-links from three different positions in the elbow region of A site tRNA to the loop end of helix 38 (25). Helix 38 is thus a strong candidate for comprising the 'A site finger' observed in cryo-EM reconstructions of the 70S ribosome (13). Again, these data are consistent with a location of helices II and III of the 5S rRNA on the interface side of the central protuberance of the 50S subunit.

The experiments reported here represent our first attempt to study cross-linking from a specifically modified sequence region artificially ligated into the 5S rRNA. It is thus of interest to compare the quality of the results with those from our previous studies (20–22), where the modified nucleotides were randomly distributed throughout the whole 5S molecule. The cross-links identified in these latter studies exclusively involved residue U89, which, as already noted in the Introduction, protrudes conspicuously outwards from the 5S rRNA structure (23). By good fortune, this residue also happens to lie within a readily identifiable ribonuclease T1 oligonucleotide in the 5S sequence,
so that we had no difficulty in recognising it in our fingerprint analyses (refer to Fig. 5). In the present study, although we had deliberately chosen the modified 5S sequence region in such a way that the four cross-linkable modified uridine residues should in principle have been distinguishable from one another (Fig. 5), this turned out not to be possible as a result of the closely neighbouring multiple cross-links that were formed. There is no reason to suppose that this would be a general problem and an obvious development of the method in order to obviate it in this particular case would be to prepare individual 5S rRNA constructs each carrying only one of the four modified uridine residues. However, this would necessitate a different ligation strategy and for the moment the results described here are adequate for our purposes, namely to clarify the orientation of the 5S molecule relative to the 23S rRNA. Taken together, the data in principle have been distinguishable from one another (Fig. 5), in a similar manner to that already published for the 16S rRNA at 20 Å resolution (17–19). This structure is in the final stages of refinement and will be published in the near future.

REFERENCES