

## Stem–loop IV of 5S rRNA lies close to the peptidyltransferase center

(ribosome structure/thiouridine crosslinking/5S–23S RNA contact)

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**ABSTRACT** A DNA fragment containing the *Escherichia coli* 5S rDNA sequence linked to a T7 promoter was prepared by PCR from an M13 clone carrying the 5S-complementary sequence. The DNA was transcribed with T7 polymerase using a mixture of [ $\alpha$ -<sup>32</sup>P]UTP and 4-thio-UTP, yielding a transcript in which  $\approx$ 18% of the uridine residues were randomly replaced by thiouridine. This modified 5S RNA could be reconstituted efficiently into 50S ribosomal subunits or 70S functional complexes. The reconstituted particles were irradiated at wavelengths above 300 nm, and the crosslinked ribosomal components were identified. A crosslink in high yield was reproducibly observed between the modified 5S RNA and 23S RNA, involving residue U-89 of the 5S RNA (at the loop end of helix IV) linked to nucleotide 2477 of the 23S RNA in the loop end of helix 89, immediately adjacent to the peptidyltransferase "ring." On the basis of this result, and in combination with earlier immunoelectron microscopic data, we propose a model for the orientation of the 5S RNA in the 50S subunit.

Interactions and neighborhoods between 5S rRNA and other components of the *Escherichia coli* 50S ribosomal subunit have been investigated in a number of laboratories by a variety of techniques. From protein binding studies (e.g., refs. 1 and 2) the 5S RNA has long been known to interact with proteins L5, L18, and L25, and the binding sites of these proteins on the 5S molecule have been studied in detail by footprinting methods (e.g., refs. 3 and 4). The 5' and 3' ends of the 5S RNA (5–7), as well as residues A-39 and U-40 at the loop end of helix III (8), have been localized by immunoelectron microscopy (IEM) on the central protuberance of the 50S subunit, in good agreement with the corresponding IEM locations of proteins L5, L18, and L25, all of which were also found to lie on the central protuberance (9, 10). On the other hand, very little information is available relating to possible interactions between 5S and 23S RNA (11).

Our laboratories have made extensive use of mRNA analogues containing 4-thiouridine (thio-U) residues, in order to study contacts between mRNA and 16S RNA by photocrosslinking (12–15), and the success of this approach has prompted us to apply the same methodology in the search for contacts between 5S and 23S RNA. Accordingly, a modified 5S RNA was constructed containing a random distribution of thio-U residues in place of normal uridine, and the modified molecule was reconstituted into 50S subunits or 70S ribosomes as a substrate for photocrosslinking. The principal result of the subsequent analysis, which we report here, was the characterization of a high-yield crosslink from the loop

end of helix IV of the 5S RNA to a site close to the peptidyltransferase ring of the 23S RNA.

### MATERIALS AND METHODS

**Construction of Modified 5S RNA.** Plasmid pKK223-3 (Pharmacia), which carries the *E. coli* 5S rDNA sequence, was cut with *Hind*III and *Ssp* I to yield a 500-bp fragment encompassing the 5S rDNA; this fragment was cloned by standard procedures (16) into phage M13mp18 (Boehringer Mannheim) that had been digested with *Hinc*II and *Hind*III restriction endonucleases. This phage was constructed for other purposes but was a convenient source of 5S rDNA, which was amplified by PCR (17) using *Taq* DNA polymerase (Amersham) together with the oligodeoxynucleotides 5'-GGCAAGCTTAATACGACTCACTATAGGCCTGGCGGCCGTAGCGCGGTG-3', carrying a *Hind*III site (underlined), the T7 promoter sequence (in italics), and the 5'-most 23 nt of the 5S sequence, and 5'-CTCCTGCAGCCGGCCTG-GCAGTTCCTACTC-3', carrying a *Pst* I and an *Nae* I site (respectively underlined and overlined) and the complement to the 3'-most 20 nt of the 5S sequence. After PCR, the DNA was cut with *Nae* I to generate the correct 3' end of the 5S RNA in the subsequent transcription (see Fig. 1). The DNA sequence was checked (18) after subcloning into pUC18.

Aliquots of the DNA ( $\approx$ 2  $\mu$ g) were transcribed with phage T7 polymerase in the presence of [ $\alpha$ -<sup>32</sup>P]UTP and 4-thio-UTP as described (12, 15), except that unlabeled UTP was added to give a UTP/thio-UTP molar ratio of 1:4. A typical yield was  $\approx$ 700 pmol of 5S RNA with a specific activity of  $\approx$ 10<sup>5</sup> cpm/pmol. The U/thio-U ratio in the transcript was determined in a small-scale parallel transcription using [ $\alpha$ -<sup>32</sup>P]ATP in place of [ $\alpha$ -<sup>32</sup>P]UTP (but with the same molar ratio of UTP to thio-UTP); the isolated 5S RNA was then totally digested with ribonuclease T<sub>2</sub>, and the mononucleotides were separated by thin-layer chromatography (12). The relative radioactivities in the thio-Up and Up spots indicated a thio-U incorporation of  $\approx$ 18%.

**Reconstitution of Modified 5S RNA into 50S Subunits or 70S Ribosomes.** The <sup>32</sup>P-labeled T7 polymerase-transcribed 5S RNA was mixed with unlabeled 23S RNA and total 50S ribosomal protein and reconstituted on a 700-pmol scale as described (19). The reconstitution mixture was then divided into three aliquots. The first was applied directly to a 10–30% sucrose gradient in 10 mM Tris·HCl pH 7.8/15 mM magnesium acetate/100 mM NH<sub>4</sub>Cl/2 mM dithiothreitol. The second aliquot was diluted into a buffer with final composition 10 mM Hepes·KOH, pH 7.5/10 mM magnesium acetate/100 mM NH<sub>4</sub>Cl/3 mM 2-mercaptoethanol; an equimolar amount

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Abbreviations: IEM, immunoelectron microscopy; thio-U, 4-thiouridine.

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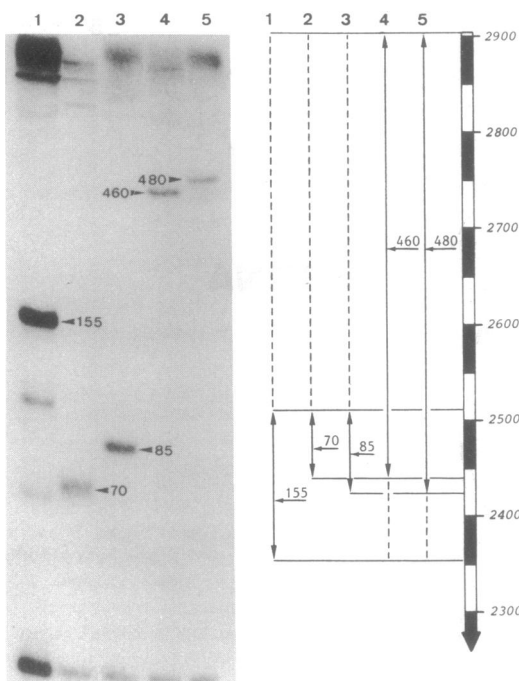


FIG. 3. Ribonuclease H digests of crosslinked  $^{32}\text{P}$ -labeled 5S RNA-23S RNA complexes (see Fig. 2B) after electrophoresis in 4% polyacrylamide gels (15). The digests were made in the presence of the following pairs of oligodeoxynucleotides (listed according to the position in 23S RNA complementary to the central base of the oligonucleotide): lane 1, positions 2358 and 2512; lane 2, positions 2442 and 2512; lane 3, positions 2426 and 2512; lane 4, positions 2358 and 2442; lane 5, positions 2358 and 2426. The approximate size (in nucleotides) of the 23S RNA fragment (linked to  $^{32}\text{P}$ -labeled 5S RNA) in each case is indicated, and the locations of these fragments within the 3' region of the 23S sequence are summarized in the diagram at right. [The amount of radioactivity in lane 1 is higher than the amounts in lanes 2-5, as this was a semipreparative digest used to obtain material for a fingerprint analysis (see Fig. 5).]

oligonucleotide 13 (UCUCCCAUG, positions 87-96, Fig. 1) is clearly absent from the fingerprint, and this same result was observed in the crosslinked samples derived from reconstituted 50S subunits and all types of 70S complex. When the "alternative" fingerprint system of ref. 24 was used (data not shown), the material remaining at the sample application point (Fig. 5B) could be resolved into two spots, one of which was not present in the parallel fingerprint from free 5S RNA. This spot represented the unlabeled 23S RNA oligonucleotide crosslinked to oligonucleotide 13 of the  $^{32}\text{P}$ -labeled 5S RNA. Secondary digestion of this complex with ribonuclease A (23, 25) showed the presence of radioactive AUp but the clear absence of Cp, whereas a similar digestion of non-crosslinked oligonucleotide 13 showed the presence of both AUp and Cp (see Fig. 1). This result demonstrates that residue U-89 of the 5S RNA is the crosslinked nucleotide.

### DISCUSSION

Thio-U has several advantages as a photocrosslinking agent. It is effectively a "zero-length" reagent, and the high activation wavelength (above 300 nm) leads to no unwanted side reactions. Furthermore, its similarity to uridine is such that relatively high levels of substitution can be tolerated without loss of biological specificity (26). In this case the level of thio-U incorporation ( $\approx 18\%$ ) did not impair the specificity of the interaction between the modified 5S RNA and the ribosome, as evidenced by its ability to be reconstituted with reasonable efficiency into 50S subunits or 70S ribosomes and functional complexes. Furthermore, we observed (data not

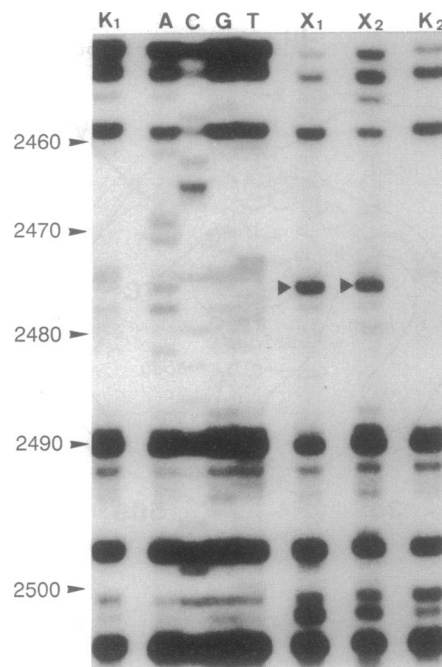


FIG. 4. Reverse transcriptase analysis (13, 22) of the crosslink site in 23S RNA. The dideoxy sequencing lanes are marked A, C, G, and T, and the lanes marked X or K contained crosslinked or control fragments, respectively, from independent experiments. Both the crosslinked and the control fragments were derived from 70S complexes in this example; the crosslinked fragments were isolated from preparative ribonuclease H gels (see Fig. 3), and the control fragments (from noncrosslinked 23S RNA) were isolated from parallel lanes in the same gel. The transcription stops corresponding to the crosslink site are marked with filled triangles.

shown) that the 5S RNA became crosslinked to L18, a well-known 5S RNA-binding protein (1, 2), which provides additional evidence for the specificity of the reconstitution. About 14% of the reconstituted 5S RNA was reproducibly crosslinked to 23S RNA (Fig. 2B), and this predominantly involved a single product, the crosslink from U-89 of the 5S RNA to U-2477 of the 23S RNA; if the 18% incorporation of thio-U was indeed random, then this implies an extremely high yield of crosslinking (up to 75%) from the 18% of the 5S RNA molecules carrying a thio-U at position 89.

The significance of the U-89/U-2477 crosslink in relation to other crosslinking and topographical data is summarized in Fig. 6. Residue 2477 is at the loop end of helix 89, which is directly connected to the peptidyltransferase ring of the 23S RNA (24, 28, 32); this ring contains the target sites of

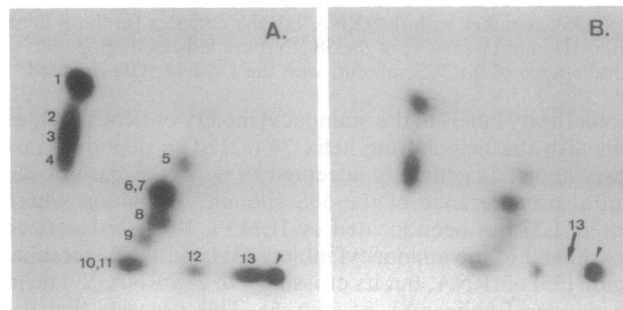


FIG. 5. Ribonuclease  $T_1$  fingerprints (14, 23) of free  $^{32}\text{P}$ -labeled 5S RNA (A), and of  $^{32}\text{P}$ -labeled 5S RNA crosslinked to 23S RNA (B). Direction of the first dimension is from right to left, and that of the second from bottom to top; the sample application point is marked by an arrowhead in each case. The spots in A are numbered as in Fig. 1, and in B the missing spot 13 is indicated.

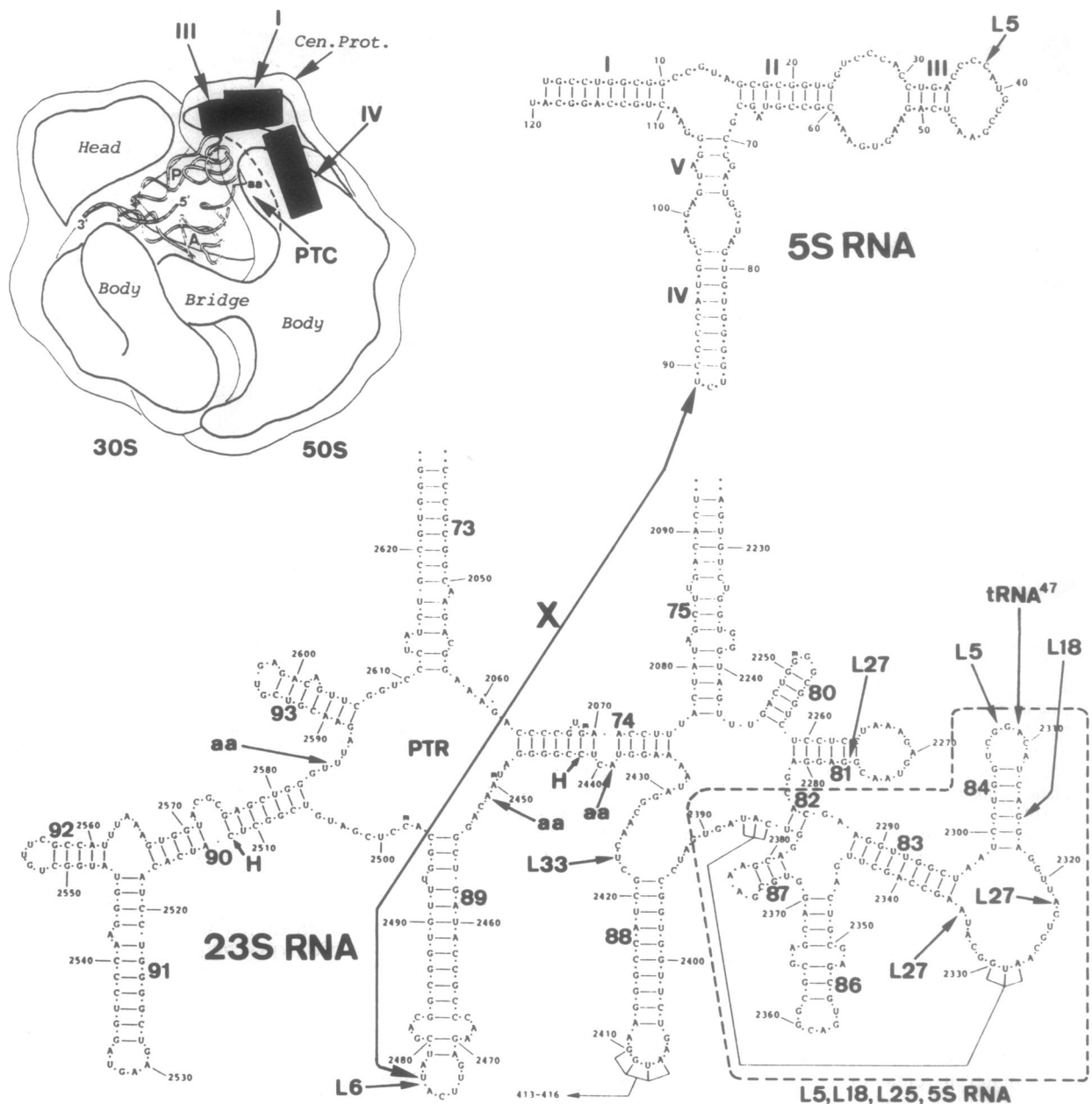


FIG. 6. Secondary structures of 5S RNA (4, 27) and part of 23S RNA (24, 28), showing the location (X) of the 5S–23S crosslink. The symbols H indicate the ends of the shortest ribonuclease H fragment encompassing the crosslink site (Fig. 3). Relevant RNA–protein crosslink sites (29) are marked, aa denotes the positions of crosslinks (30, 31) to the aminoacyl moiety of tRNA, and tRNA<sup>47</sup> shows the site of a crosslink from position 47 of P-site-bound tRNA (31). PTR is the peptidyltransferase ring (32). The 23S RNA region found in a ribonucleoprotein fragment with 5S RNA and proteins L5, L18, and L25 (11) is boxed in by the broken line. At upper left is a sketch of the 70S ribosome as described by Frank *et al.* (33), together with the tRNA–mRNA complex [in the R configuration (34)]; the suggested orientations of the three principal regions (I, II plus III, and IV plus V) of the 5S RNA are indicated roughly to scale by the boxes marked I, III and IV, respectively. Cen. Prot., the central protuberance of the 50S subunit, with the L7/L12 stalk oriented “toward” the reader. PTC, peptidyltransferase center.

photoaffinity labels in the aminoacyl moiety of tRNA (30), as does also the neighboring helix 74 (31). The peptidyltransferase center is generally accepted to lie at the base of the central protuberance of the 50S subunit, a position where protein L27 has been located by IEM (9, 10). L27 has been crosslinked to the aminoacyl moiety (31) and the 3'-terminal region (35) of tRNA, but its crosslinks to 23S RNA (29) lie in the region of helices 81–84 (Fig. 6). This suggests that the latter helices must fold back toward the central protuberance, a contention which is supported by the crosslinks to proteins L5 and L18 in helix 84 (29). A ribonucleoprotein fragment (11) found to contain 5S RNA, together with L5, L18, and L25 and a segment of 23S RNA, also involves this area of the 23S RNA (Fig. 6). A further connection is protein L33, which has

been crosslinked to helix 88 (29) as well as to the 3' terminus of E-site-bound tRNA (36), and to positions 20a (37) and 47 (31) of P-site-bound tRNA, the latter position in turn having a crosslink site (31) in helix 84. The majority of the footprint sites for P- or A-site-bound tRNA (38) also lie within this general area of the 23S RNA.

Proteins L5 and L18 have both been located by IEM on the central protuberance of the 50S subunit (9, 10), and within the 5S RNA these proteins have footprint sites involving helix I (3) and helices II and III (3, 4), respectively; L5 has a crosslink (29) at the loop end of helix III (Fig. 6). This suggests that helix III is folded back toward helix I (4), in agreement with the IEM locations of the 3' terminus (5–7) and of residues A-39 and U-40 (8) relatively close together on the

upper side of the central protuberance. In combination with these results, the U-89/U-2477 crosslink would thus imply that helix IV is drawn down toward the base of the central protuberance, so that helix 89 bridges the gap between the loop end of helix IV and the peptidyltransferase center. This would place the crosslink to protein L6 [also at the loop end of helix 89 (29)] at a position latitudinally in agreement with its IEM location at the base of the L7/L12 stalk (9), albeit more toward the solvent side of the 50S subunit (24); this can readily be accommodated, since L6 has recently been shown to have an extended conformation (39). The overall result (Fig. 6) is that the 5S RNA appears to be constrained into a bent Y-shape in which helix III is directed toward the 30S subunit [to accommodate the IEM location of residues A-39 and U-40 (8)], helix I is angled toward the upper side of the central protuberance [to accommodate the IEM location of the 3' terminus (5-7)], and helix IV is directed downward toward the peptidyltransferase center.

The direct neighborhood between 5S RNA and the peptidyltransferase region of 23S RNA demonstrated here is in agreement with the observations of Dohme and Nierhaus (40), who found that reconstituted 50S subunits lacking 5S RNA showed a drastic reduction in peptidyltransferase activity. However, the same authors also noted a drastic reduction in the elongation factor Tu-dependent binding of tRNA to the A site. Since the topographical data just discussed (Fig. 6; see also ref. 24) imply that the 5S RNA is located more toward the solvent side of the 50S subunit, with no direct contacts to the A-site tRNA, then it seems likely that the effect of 5S RNA on the A-site binding is mediated via the neighboring elements of 23S RNA at the peptidyltransferase center. In this context it is interesting that base-substitution mutations at positions 2460, 2492, and 2493 within helix 89 (Fig. 6) affect translational accuracy, as measured by suppression of frameshift and nonsense mutations (M. O'Connor and A. Dahlberg, personal communication). Thus, taken together, the data support the notion that the 5S RNA and the peptidyltransferase region of the 23S RNA play a complex multifunctional role in protein biosynthesis.

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