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Nuclease protection analysis of ribonucleoprotein complexes: Use of the cytotoxic ribonuclease α-sarcin to determine the binding sites for *Escherichia coli* ribosomal proteins L5, L18, and L25 on 5S rRNA

(nuclease protection/RNA–protein interaction)

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ABSTRACT **A rapid and convenient method has been devised to determine the binding sites for proteins on RNA. The procedure is an adaptation of one used to map DNA–protein complexes by protection against nuclease digestion. The method uses the cytotoxic ribonuclease α-sarcin, which hydrolyzes purines in both single- and double-stranded regions of RNA. It has been authenticated by confirming the binding sites for the *Escherichia coli* ribosomal proteins L18 and L25 on 5S rRNA and its value has been established by identifying the attachment site for protein L5. The procedure should be useful for the analysis of other ribonucleoprotein complexes.**

α-Sarcin is a small cytotoxic protein that is produced by the mold *Aspergillus giganteus* (1, 2) and inhibits protein synthesis by inactivating ribosomes (3–5). This inactivation is the result of hydrolysis of a single phosphodiester bond in a highly conserved purine-rich sequence near the 3′ end of the large RNA in the large ribosomal subunit (6, 7). If rat liver ribosomes are the substrate, α-sarcin action yields an oligonucleotide of 393 bases derived from the 3′ end of 28S rRNA. However, if free rRNAs are the substrate, α-sarcin causes extensive progressive digestion and no specific fragments are formed (8). Experiments with homopolynucleotides and 5S rRNA have established that α-sarcin cleaves on the 3′ side of purines, irrespective of the secondary structure of the substrate (8). Thus, the toxin has quite remarkable nuclease specificities: if ribosomes are the substrate it hydrolyzes only one of approximately 7,000 phosphodiester bonds whereas, with free RNA, it cleaves after purines in both single- and double-stranded regions. These properties suggested to us that α-sarcin might be useful for analysis of the structure of ribonucleoprotein complexes.

Identification of the binding sites for proteins on RNAs by protection against nuclease digestion has been difficult because of the unavailability of appropriate ribonucleases. For analogous experiments with DNA, there are a variety of enzymes that are relatively indifferent to the identity of the base, to the sequence in which the nucleotides occur, and to the secondary and tertiary structure. For that reason, a suitable ladder can be formed on a sequence analysis gel and regions protected by the binding proteins can be identified (9). That has not been the case for ribonucleoprotein complexes. RNAs generally have a good deal of secondary structure and the few enzymes that attack double-stranded regions do so feebly and do not affect nucleotides in single-stranded sequences. Thus, binding sites for proteins on RNAs have had to be laboriously determined by a combination of nuclease digestion, protection against chemical modification of nucleotides, and binding to oligonucleotide fragments.

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To test whether α-sarcin is a suitable enzyme for the protection analysis, we chose as substrates the complexes of *Escherichia coli* 5S rRNA and ribosomal proteins. Three proteins, L5, L18, and L25, bind specifically to *E. coli* 5S rRNA (10–13). The binding sites for L18 and L25 have been tentatively identified (14), despite conflicting results (11, 15–17), so we could authenticate the technique by comparing our results with the earlier data. The binding site for L5 had not been determined, so an attempt to elucidate it provided an opportunity to assess the value of the procedure.

MATERIALS AND METHODS

Preparation and Labeling of 5S rRNA. 5S rRNA was extracted with phenol and $NaDodSO_4$ from ribosomes (18) prepared from *E. coli* MRE 600 cells. The nucleic acid was labeled either at the 5′ end with [γ-^{32}P]ATP by using T4 polynucleotide kinase (19) or at the 3′ end with [5′-^{32}P]cytidine 3′,5′-bisphosphate by using RNA ligase (20). The labeled 5S rRNA was purified by electrophoresis on 10% polyacrylamide gels containing 7 M urea (21). The purified radioactive 5S rRNA was diluted with an appropriate and known amount of unlabeled 5S rRNA and renatured by incubation for 10 min at 55°C in 50 mM Tris·HCl, pH 7.6/0.3 M KCl/10 mM $MgCl_2$ followed by slow cooling to 15°C (22).

Preparation of Ribosomal Protein–5S rRNA Complexes. *E. coli* ribosomal proteins L5, L18, and L25 were a gift from R. A. Zimmermann (University of Massachusetts). Since L5 is poorly soluble it was stored in buffer containing 6 M urea. Prior to use, it was diluted with a solution containing 6 M urea and then dialyzed against 50 mM Tris·HCl, pH 7.6/0.2 M KCl/5 mM $MgCl_2$/0.1 mM 2-mercaptoethanol in a microapparatus. The ribonuclease activity of α-sarcin is inhibited by potassium and magnesium (8). For that reason, complexes of ribosomal proteins and 5S rRNA were formed at concentrations of the cations optimal for binding (23) and then diluted to lower concentrations of the salts prior to digestion with α-sarcin.

Digestion of Ribosomal Protein–5S rRNA Complexes with α-Sarcin. Immediately after dilution of the ribosomal protein–5S rRNA complexes to reduce the potassium and magnesium concentrations, α-sarcin was added and digestion was allowed to proceed for 15 min at 30°C. Digestion of 5S rRNA in the absence of ribosomal protein was carried out under the same conditions. The reaction was stopped by the addition of an equal volume of 10 M urea and then 0.1 vol of 0.1 M EDTA was added. The EDTA eliminated streaking on the sequence analysis gels, which apparently was due to oligonucleotide fragments bound to α-sarcin. The α-sarcin digests were analyzed by electrophoresis on 10% or 20% polyacrylamide gels (24).

RESULTS

Ribonuclease Activity of α-Sarcin. The ribonuclease activity of α-sarcin has been characterized (8). The toxin is specif-

ic for purines and can cleave single- and double-stranded RNA. The characterization had revealed a potential difficulty in the use of α-sarcin to map ribonucleoprotein complexes. The enzyme does not require cations for activity and is inhibited moderately by concentrations of potassium in excess of 100 mM and somewhat more severely by concentrations of magnesium exceeding 2 mM (8). However, this inhibition can be overcome by increasing the concentration of α-sarcin. With higher concentrations of α-sarcin, uniform digestion of 5S rRNA can be obtained at moderate concentrations of KCl and $MgCl_2$ (cf. the legends to the figures). The pattern obtained for the digestion of 5S rRNA under the conditions we used does not differ significantly from that obtained with lower concentrations of α-sarcin in the absence of cations (Fig. 1).

There are several purines in rat 5S rRNA that are relatively resistant to α-sarcin (8). This phenomenon is also observed with *E. coli* 5S rRNA and yeast tRNA (unpublished observation). The basis of the decreased sensitivity is not known; it does not appear to be related to the primary or secondary structure of the molecules. It may be the result of steric hinderance or of tertiary interactions that block access of the toxin to phosphodiester bonds. There are two regions of *E. coli* 5S rRNA that are less susceptible to cleavage by α-sarcin: positions 29–41 and 108–117.

Identification of the Binding Sites for Ribosomal Proteins on 5S rRNA. The concentration of the ribosomal protein had to exceed that of the 5S rRNA to ensure efficient formation of the complexes and that no appreciable free radioactive nucleic acid was present. Free radioactive 5S rRNA would interfere with determination of the protein binding site. The concentration of α-sarcin needed to generate a broad distribution of fragment lengths under the conditions used was determined in preliminary trials for each ribosomal protein–5S rRNA complex. This concentration range was quite narrow.

Ribosomal protein L18 protected residues adenosine-15 through guanosine-24 and guanosine-44 through adenosine-66 from digestion by α-sarcin (Fig. 1, lanes 3 and 5). It should be noted that the pattern of digestion of 5S rRNA with 0.8 μM α-sarcin in the absence of potassium and magnesium (Fig. 1, lane 7) was similar to that with 8 μM toxin in the presence of cations (Fig. 1, lane 4).

Ribosomal protein L25 protected residues guanosine-72 through adenosine-109 from digestion by α-sarcin (Fig. 2, lanes 3 and 5).

E. coli ribosomal protein L5 does not bind well to 5S rRNA because the association constant is low and the protein is poorly soluble in reconstitution buffer (25, 26). Protein L18 increases the solubility of L5 and enhances its binding to 5S rRNA (25). For that reason, the experiments designed to determine the L5 binding site on 5S rRNA were done in the presence of L18 (Fig. 3). The L18 binding site could be identified and subtracted on the basis of the results of concurrent (Fig. 3, lane 3) and of earlier experiments (Fig. 1). Thus, the

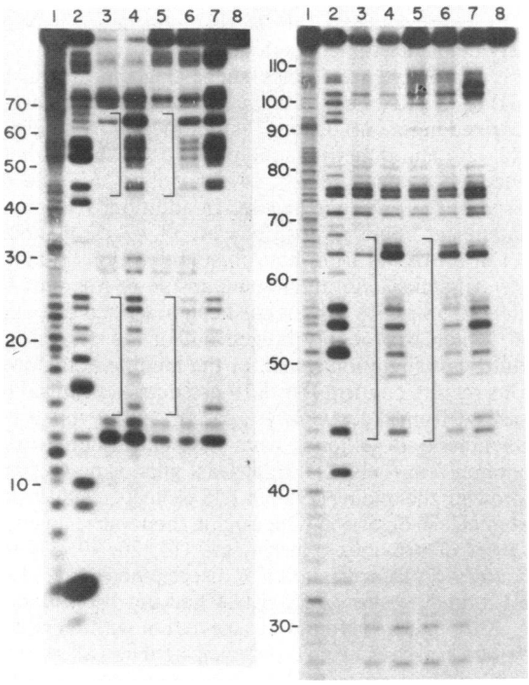

FIG. 1. Protection of 5S rRNA from digestion with α-sarcin by ribosomal protein L18. *E. coli* ribosomal protein L18 (10 μM) was incubated with renatured radioactive 5S rRNA (0.4 μM) in 50 mM Tris·HCl, pH 7.6/285 mM KCl/6 mM $MgCl_2$ for 30–45 min at 33°C and then the mixture was diluted to 50 mM Tris·HCl, pH 7.6/95 mM KCl/2 mM $MgCl_2$ and digested with α-sarcin for 15 min at 30°C. Lanes: 1, alkaline hydrolysate of 5S rRNA; 2, T1 ribonuclease digest of 5S rRNA; 3, digestion of L18–5S rRNA complex with 8 μM α-sarcin; 4, digestion of 5S rRNA with 8 μM α-sarcin; 5, digestion of L18–5S rRNA complex with 4 μM α-sarcin; 6, digestion of 5S rRNA with 4 μM α-sarcin; 7, digestion of 5S rRNA with 0.8 μM α-sarcin in the absence of KCl and $MgCl_2$; 8, 5S rRNA that was not treated with α-sarcin. The 5S rRNA was labeled at the 5′ end and the digests were analyzed by electrophoresis on 10% polyacrylamide gels. Brackets enclose regions of 5S rRNA protected by ribosomal protein L18.

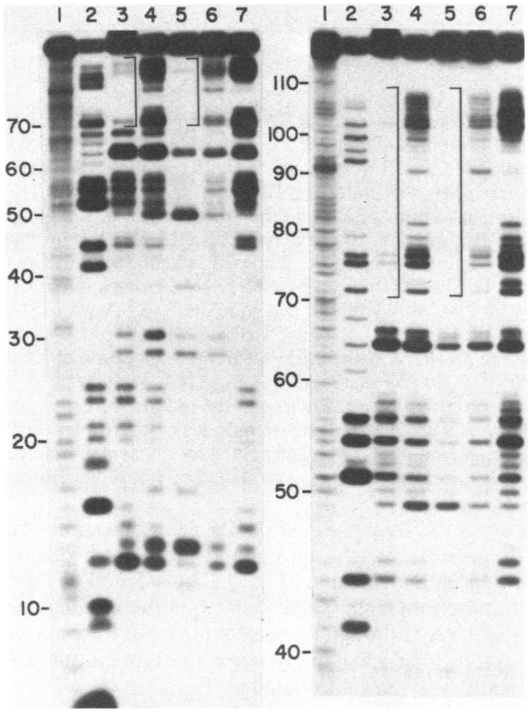

FIG. 2. Protection of 5S rRNA from digestion with α-sarcin by ribosomal protein L25. *E. coli* ribosomal protein L25 (20 μM) was incubated with renatured radioactive 5S rRNA (2 μM) in 50 mM Tris·HCl, pH 7.6/230 mM KCl/1.3 mM $MgCl_2$ for 30–45 min at 33°C and then the mixture was diluted to 50 mM Tris·HCl, pH 7.6/135 mM KCl/0.75 mM $MgCl_2$ and digested with α-sarcin for 15 min at 30°C. Lanes: 1, alkaline hydrolysate of 5S rRNA; 2, T1 ribonuclease digest of 5S rRNA; 3, digestion of L25–5S rRNA with 14 μM α-sarcin; 4, digestion of 5S rRNA with 14 μM α-sarcin; 5, digestion of L25–5S rRNA with 7 μM α-sarcin; 6, digestion of 5S rRNA with 7 μM α-sarcin; 7, digestion of 5S rRNA with 0.8 μM α-sarcin in the absence of KCl and $MgCl_2$. The 5S rRNA was labeled at the 5′ end and the digests were analyzed by electrophoresis on 10% polyacrylamide gels. Brackets enclose regions of 5S rRNA protected by ribosomal protein L25.

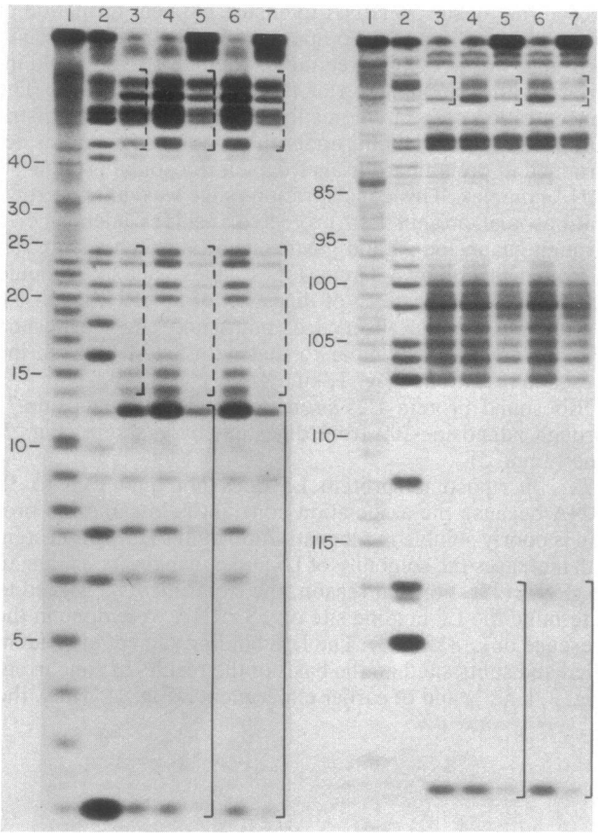

FIG. 3. Protection of 5S rRNA from digestion with α-sarcin by ribosomal proteins L5 and L18. *E. coli* ribosomal proteins L5 (5.8 μM) and L18 (3.9 μM) were incubated with renatured radioactive 5S rRNA (0.6 μM) in 50 mM Tris·HCl, pH 7.6/200 mM KCl/5 mM $MgCl_2$ for 30–45 min at 33°C and then the mixture was diluted to 50 mM Tris·HCl, pH 7.6/100 mM KCl/2.5 mM $MgCl_2$ and digested with α-sarcin for 15 min at 30°C. Lanes: 1, alkaline hydrolysate of 5S rRNA; 2, T1 ribonuclease digest of 5S rRNA; 3, digestion of L18–5S rRNA with 25 μM α-sarcin; 4, digestion of 5S rRNA with 25 μM α-sarcin; 5, digestion of L5, L18–5S rRNA with 25 μM α-sarcin; 6, digestion of 5S rRNA with 20 μM α-sarcin; 7, digestion of L5, L18–5S rRNA with 20 μM α-sarcin. The 5S rRNA was labeled at the 5′ end (*Left*) or at the 3′ end (*Right*) and the digests were analyzed by electrophoresis on 20% polyacrylamide gels. Interrupted brackets enclose the region of 5S rRNA protected by ribosomal protein L18; solid brackets enclose the region protected by ribosomal protein L5.

region of 5S rRNA protected by L5 from digestion with α-sarcin is guanosine-2 through guanosine-13 and guanosine-116 through adenosine-119 (Fig. 3, lanes 5 and 7). We assume that the molecular stalk (helix I) is the binding site for L5 on 5S rRNA, although we are cognizant of the possibility, albeit remote, that L5 actually alters the L18 binding site so as to encompass the two domains.

We tested the effects of additional proteins, *E. coli* ribosomal protein L7, rat ribosomal protein S8, and bovine serum albumin, on the hydrolysis of 5S rRNA by α-sarcin. None of these proteins protected the nucleic acid from digestion by the toxin (results not shown).

The binding sites for L5, L18, and L25 are collated on a diagram of the secondary structure of 5S rRNA (Fig. 4) modified (14) from the proposal of Fox and Woese (27).

DISCUSSION

Digestion with α-sarcin of ribonucleoprotein particles containing RNA labeled at a terminus followed by analysis of the products of hydrolysis on sequencing gels is a rapid and efficient means of determining the protein binding sites on the nucleic acid. The method has been authenticated by confirming the binding sites for *E. coli* ribosomal proteins L18 and L25 on 5S rRNA, binding sites that had been tentatively established before (14), albeit not without substantial differences (cf. refs. 11, 15–17), by relatively laborious and cumbersome biochemical and physical techniques. Moreover, the utility of the technique has been demonstrated by identification of the region where L5 associates with 5S rRNA.

A fragment derived from 5S rRNA by limited nuclease digestion and containing the sequences 69–87 and 90–110 binds L25 specfically and with high affinity (28). In addition, the guanosine-81 and cytosine-93 residues are protected from cleavage by the double-strand-specific cobra venom endonuclease on addition of L25 to 5S rRNA (29), further implicating helix IV as part of the binding site. Moreover, analysis by NMR spectroscopy of the downfield proton spectra of 5S rRNA taking advantage of nuclear Overhauser effects reveals perturbations in the presence of L25 that also suggest a binding site for the protein in helix IV and exclude the molecular stalk (helix I) as part of the site (30). We find that L25 protects residues 72–109 from digestion by α-sarcin (Fig. 4). This domain contains a sequence of seven consecutive pyrimidines (residues 87–93). Because α-sarcin is pyrimidine blind, we cannot determine whether they are part of the L25 binding site. However, nuclease protection experiments have indicated that three of the pyrimidines (bases 87–89) remain sensitive in the presence of L25 (28, 29). Thus, our definition of the L25 binding site on 5S rRNA (Fig. 4) is consistent with a subset of the previous data (28–30), indeed resolves previous conflicts (11, 15, 16), and hence may more precisely delineate the contact site.

There is a body of evidence suggesting the central helix (helix II) of 5S rRNA as the primary attachment site for L18 (summarized in ref. 14). Chemical modification studies using kethoxal (26) and dimethyl sulfate (31) have shown that the reactivity of guanines 16, 23, 24, 54, 56, 64, 67, and 69 is decreased in the presence of L18. In addition, L18 protects phosphodiester bonds at positions 14, 38, 42, 52, 63, 64, 65, and 114 of 5S rRNA from hydrolysis by various ribonucleases (29). Chemical alteration of adenosine-66 prevents binding of L18 to 5S rRNA (31). It has been proposed that adenosine-66, which is probably bulged out of the central helix, constitutes a recognition signal for the binding of the protein (31). Our results confirm that L18 protects the central helix of 5S rRNA from digestion by α-sarcin; moreover, we find the attachment site extends from the central helix through the common arm (helix III) to at least guanosine-44 (Fig. 4) in accord with the finding (29) that L18 protects residues 38–42 against nuclease digestion. Once again then, our data corroborate a set of previous experiments (14, 26, 29, 31) while helping to resolve uncertainties or discrepancies (11, 15–17).

The L5 binding site on 5S rRNA had not been identified before. Although L5 is found in association with 5S rRNA in reconstitution assays using the nucleic acid and all of the 50S ribosomal subunit proteins (10), the association seems weak and the stoichiometry is less than one. Binding of the single protein to 5S rRNA is particularly difficult and is not saturable (10, 26). However, L5 is necessary for incorporation of 5S rRNA into 50S subunits (32), which suggests that it does interact with the nucleic acid. Our advantage was that we could determine the attachment site for L5 in the presence of L18, which enhances the association of the former with 5S rRNA. When the L18 binding site is factored out, it is evident that L5 protects residues guanosine-2 through guanosine-13 and guanosine-116 through adenosine-119 (Fig. 3) in the molecular stalk (helix I) (Fig. 4). Unfortunately, we cannot determine whether the L5 attachment site extends from guanosine-116 to the edge of the L25 binding site at residue 109 because that region of 5S rRNA is resistant to α-sarcin hydrolysis. Nonetheless, we have established that L5 does

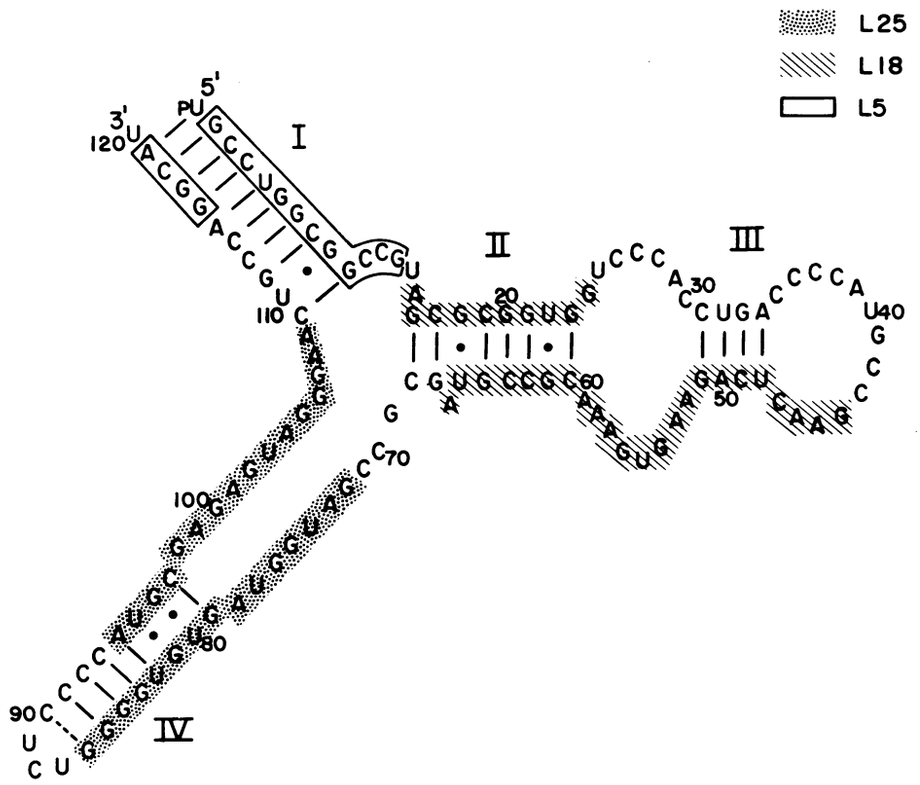

FIG. 4. Proposed secondary structure of *E. coli* 5S rRNA indicating the binding sites for ribosomal proteins L5, L18, and L25. The secondary structure is that proposed by Fox and Woese (27) and modified by Garrett *et al.* (14).

associate with a specific region of 5S rRNA. We note in passing that the binding sites for the three proteins encompass most of the 5S rRNA molecule (Fig. 4).

The two features that distinguish α-sarcin, and account for its utility for nuclease-protection analysis of ribonucleoprotein complexes, are its ability to hydrolyze purines in both single- and double-stranded regions of RNA and optimal activity at near neutral pH. The former characteristic accounts for the relatively uniform digestion ladder generated from RNA substrates by the enzyme, the latter allows digestion to be carried out under conditions favorable for stable protein–RNA interactions. There are, however, limitations to the procedure. α-Sarcin cleaves only after purines. Thus, unlike digestion of DNA with DNase I, a complete hydrolysis ladder is not obtained. Although this means there are blind spots on the RNA, it need not seriously jeopardize determination of the location of protein attachment sites. A sequence of seven pyrimidines interrupts the L25 site on 5S rRNA; nonetheless the attachment region is reasonably well defined. It needs noting that occasionally α-sarcin will cleave after a pyrimidine, especially if the nucleotide is flanked on both sides by purines. A second, and potentially more serious, shortcoming in the technique is the sensitivity of α-sarcin to inhibition by the very cations that are frequently necessary to stabilize protein–nucleic acid complexes. But again, in practice, it has been possible to overcome this problem by forming the complex under optimum conditions and then reducing the concentrations of cations to the minimum that will maintain stability and using higher concentrations of α-sarcin in the subsequent digestion reaction. The final problem is the resistance of some purine residues to hydrolysis by α-sarcin. The basis for the resistance, which is not only vexing but in some instances surprising, is not known. For example, guanosine-41 is the nucleotide most susceptible to chemical modification even when the 5S rRNA is in the ribosome (33), yet it is not cleaved by α-sarcin. There are a number of purines (guanosines-41, -67, -69, and -112 and adenosine-115) in *E. coli* 5S rRNA that are relatively insensitive to hydrolysis by α-sarcin, and one entire region, adenosine-29 to guanosine-41, that is resistant. There is no evidence from other experiments that either the latter region or any of the resistant residues, guanosines-67 and -69 excepted, are part of the attachment site for any one of the three proteins. Thus, the resistance of those residues has not seemed to materially affect our definition of the protein binding sites. Nonetheless, the phenomenon needs to be borne in mind.

We are grateful to R. A. Zimmermann for providing *E. coli* ribosomal proteins L5, L18, and L25 and for his advice on how to construct complexes of those proteins with 5S rRNA; we are indebted to A. Lin for *E. coli* ribosomal protein L7 and rat ribosomal protein S8. The expenses of the research were met by Grants CA 19265 and GM 21769 from the National Institutes of Health.

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