Binding Sites of Ribosomal Proteins on Prokaryotic 5S Ribonucleic Acids: A Study with Ribonucleases†

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ABSTRACT: The binding sites of ribosomal proteins L18 and L25 on 5S RNA from *Escherichia coli* were probed with ribonucleases A, T_1, and T_2 and a double helix specific cobra venom endonuclease. The results for the protein–RNA complexes, which were compared with those for the free RNA [Douthwaite, S., & Garrett, R. A. (1981) *Biochemistry 20*, 7301–7307], reveal an extensive interaction site for protein L18 and a more localized one for L25. Generally comparable results, with a few important differences, were obtained in a study of the binding sites of the two *E. coli* proteins on *Bacillus stearothermophilus* 5S RNA. Several protein-induced changes in the RNA structures were identified; some are possibly allosteric in nature. The two prokaryotic 5S RNAs were also incubated with total 50S subunit proteins from *E. coli* and *B. stearothermophilus* ribosomes. Homologous and heterologous reconstitution experiments were performed for both RNAs. The effects of the bound proteins on the ribonuclease digestion of the RNAs could generally be correlated with the results obtained with the *E. coli* proteins L18 and L25, although there was evidence for an additional protein-induced conformational change in the *B. stearothermophilus* 5S RNA, which may have been due to a third ribosomal protein L5.

The complexing of proteins and RNA is an essential part of many biological processes and especially those occurring during protein biosynthesis. Although many protein binding sites have been characterized, for example, on the ribosomal RNAs (Garrett, 1979; Zimmermann, 1980), little is known about either the topography of the RNA binding sites or the basis of chemical specificity of protein–RNA interactions. The 5S ribosomal RNA from *Escherichia coli* can form complexes with the ribosomal proteins L5, L18, and L25 (Garrett et al., 1981), but, whereas proteins L18 and L25 bind strongly to the 5S RNA, protein L5 forms a stable complex only in the presence of L18 (Feunteun et al., 1975; Spierer & Zimmermann, 1978; Newberry & Garrett, 1980). This relatively simple system is especially suited to studying protein–RNA interactions.

Relatively little is known about the binding site of protein L18 (Douthwaite et al., 1979), except that the protein strongly protects guanines 24 and 69 against kethoxal modification (Garrett & Noller, 1979). These guanines are located at the extremities of the helix II in the Fox & Woese (1975) model. The RNA binding site of protein L25 has been partially defined by probing the structure of the L25–5S RNA complex with ribonuclease A and is contained within the resistant fragment extending over nucleotides 69–110 (Gray et al., 1973; Douthwaite et al., 1979).

In the preceding paper, we investigated the accessibility of the 5S RNA structures of *E. coli* and *Bacillus stearothermophilus* to ribonucleases A, T_1, and T_2 and cobra venom ribonuclease (Douthwaite & Garrett, 1981); here, we compare the accessibility of these RNA structures to the ribonucleases in complexes with proteins L18 and L25 and after incubating with homologous and heterologous total 50S subunit proteins. As in the preceding study, mild ribonuclease digestion conditions were employed; digested yet intact complexes were selected electrophoretically. Analyses of the cutting positions were made on rapid sequencing gels, and a distinction was made between primary and secondary cuts.

Materials and Methods

5S RNA was extracted from *E. coli* strain MRE 600 and *B. stearothermophilus* strain NCA 1503 according to Monier & Feunteun (1971) and was either 5′-end labeled with [γ-^{32}P]ATP[1] and polynucleotide kinase, after removing the 5′-phosphate with alkaline phosphatase, or 3′-end labeled with [^{32}P]Cp and RNA ligase as described in the previous paper. The 5′ end labeled 5S RNA was purified from labeled fragments by electrophoresis in a 12% polyacrylamide gel containing 8 M urea, 50 mM Tris-HCl, and 1 mM EDTA, pH 8.3. It was extracted in 0.2 M ammonium acetate, pH 5.5, and renatured as described earlier (Douthwaite & Garrett, 1981). *E. coli* proteins L18 and L25 were prepared according to the procedures of Hindennach et al. (1971) or Chen-Schmeisser & Garrett (1975). Total 50S subunit proteins were extracted by the acetic acid procedure (Hardy et al., 1969), dialyzed against 1% acetic acid, lyophilized, and dissolved at 40 mg/mL in 8 M urea. 5S RNA was complexed with proteins L18 and L25 by incubating 10 μg of 5S RNA with a 3-fold molar excess of protein in ribosomal reconstitution buffer (30 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, and 300 mM KCl) for 40 min at 34 °C (Douthwaite et al., 1979; Garrett & Noller, 1979). Complexes with total 50S subunit proteins were formed by incubating 10 μg of 5S RNA with approximately 0.5 mg of proteins under the same conditions.

Complex formation of 5S RNA with proteins L18 and L25 was assayed for, and quantitated by, electrophoretic mobility in magnesium-containing gels (Douthwaite et al., 1979). For some complexes, bands were excised from the gel, and the bound protein was identified directly on a sodium dodecyl sulfate–polyacrylamide gel as described by Newberry & Garrett (1980). The proteins corresponding to L18, L25, and

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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L5, which were bound to the RNA on incubating with total 50S subunit proteins, were determined as described earlier (Horne & Erdmann, 1972; Chen-Schmeisser & Garrett, 1977; Newberry & Garrett, 1980).

Ribonuclease digestions were performed essentially as described in the preceding paper, namely, over the enzyme ranges 1:5000–1:500 units/μg of RNA for RNase T_1, 1:5000–1:50 units/μg of RNA for RNase T_2, 1:10000–1:50 μg/μg of RNA for RNase A, and 1:3000–1:100 units/μg of RNA for the *Naja naja oxiana* cobra venom ribonuclease. All digestions were performed at 0 °C. The digested complexes containing purified L18 and L25 were electrophoresed in 12% polyacrylamide gels (15 × 15 × 0.2 cm) containing 40 mM Tris-HCl and 10 mM $MgCl_2$, pH 8, at 4 °C and about 7 V/cm for 16 h with circulated buffer. Bands migrating adjacent to 5S RNA and protein–5S RNA complex markers were excised and extracted as described earlier (Douthwaite et al., 1979). Complexes formed with total 50S subunit proteins showed a strong tendency to aggregate, and, therefore, they were extracted with an equal volume of phenol prior to electrophoresing in the gel. The band coelectrophoresing with the 5S RNA was then analyzed. Chemical modifications were executed as in the preceding paper. Sequencing gels (40 × 40 × 0.03 cm) containing 12% polyacrylamide, 50 mM Tris–borate, 1 mM EDTA, pH 8.3, and 7 M urea were run at 50 W. The locations of the enzyme cuts were determined by comparing the band positions with those of the sequence read from a chemically modified sample.

When protein protection or enhancement of the ribonuclease cuts was partial, the relative intensities of the cuts in the free RNA and complex were estimated by microdensitometry, and the degree of change is presented as a percentage of the cut in the free RNA.

Results

The data for the free RNAs were reported in the preceding paper (Douthwaite & Garrett, 1981). They are summarized in Tables I–III and in Figures 4 and 5 on an extended Fox and Woese secondary structural model. In this study, samples of free RNA were digested under conditions identical with those of the protein complexes and coelectrophoresed on the sequencing gels.

Digestion and Fractionation of Protein–RNA Complexes. *E. coli* and *B. stearothermophilus* 5S RNAs were 5′- or 3′-end labeled, renatured, and then complexed either with *E. coli* proteins L18 and/or L25 or with total 50S subunit proteins from both bacteria. The complexes and control RNA samples were digested with ribonucleases A, T_1, and T_2 and cobra venom ribonuclease, prior to coelectrophoresing with undigested complex in magnesium-containing gels. For each enzyme a range of digestion conditions was employed from very mild digests, producing a few weak bands, increasing until no whole 5S RNA was detected. The RNA was relatively insensitive to the cobra venom ribonuclease concentration, and a more limited digestion range was investigated for this enzyme.

The fractionation of *E. coli* 5S RNA–(L18 + L25), 5S RNA–L25, and 5S RNA, in a polyacrylamide gel, is depicted in Figure 1; the identities of the proteins associated with the bands were ascertained as described under Materials and Methods. *E. coli* RNA was extracted from the bands migrating adjacent to the undigested complex or free RNA. Since the complexes were separated from one another and from the 5S RNA and were resolved as discrete bands (see Figure 1), we inferred that they exhibited integral molar protein:RNA stoichiometry. Our previous studies have indicated that it is a 1:1 stoichiometry (Garrett & Noller, 1979; Newberry & Garrett, 1980). Complexes formed by incubating L18 or L25 with 5S RNA were generally resolved into two bands, a strong protein–RNA complex band and a faster migrating weak band with the same R_f value as 5S RNA. Both bands were analyzed on sequencing gels. The results for the former band demonstrated strong protection or enhancement of ribonuclease cuts by the proteins; they also revealed a positive selection of the proteins for undamaged RNA molecules in the undigested control sample. For the weak band partial protein protection of ribonuclease cuts was observed, which suggested that the protein dissociated from the RNA during either the digestion or the electrophoresis.

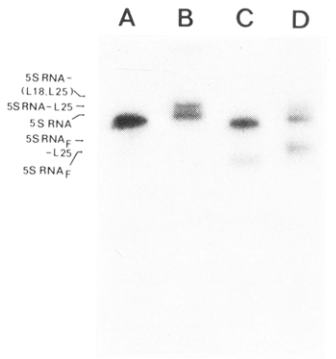

FIGURE 1: Separation of intact digested *E. coli* 5S RNA, fragments, and their complexes with proteins L18 and L25. Ribonuclease A digestion of 5S RNA that was 3′-end labeled with [^{32}P]Cp. (A and C) RNA untreated and digested at an enzyme:RNA ratio of 1:1000 (w/w) for 10 min at 0 °C, respectively. (B and D) 5S RNA–(L18 + L25) complex untreated and digested as in (C). The positions of the complexes, RNAs, and fragments denoted by an F subscript are indicated. Details of the polyacrylamide gel are given under Materials and Methods.

The complexes with *B. stearothermophilus* were also fractionated in the magnesium-containing gels. In general, mobilities were very close to that of the RNA, with the L25 complex migrating slightly behind the L18 complex. Protein L18 tended to dissociate from the RNA during the gel run. RNA incubated with total 50S subunit proteins was extracted with phenol prior to the electrophoresis, and the band comigrating with 5S RNA was examined.

Quantitation of Cutting Data. As argued in the preceding paper (Douthwaite & Garrett, 1981), quantitation of primary cuts in the free RNA was difficult for two main reasons: First, the occurrence of a relatively strong cut near the end label produces weak bands throughout the rest of the sequencing track, and, second, weak primary cuts may be stimulated by secondary cuts resulting from other, stronger, primary cuts. Therefore, we limited our quantitation of the RNA cuts to a simple (+) system. In this study, however, it was possible to estimate, approximately, the degree of protection (or stimulation) for a given sample of RNA and protein–RNA complex, digested under identical conditions, and to correlate this value approximately with the binding stoichiometry, at least for *E. coli* 5S RNA complexes. Nevertheless, there were still various difficulties in normalizing the band intensities, in addition to the usual problems of quantitating bands on photographic film. For example, L25 strongly enhanced the ribonuclease A cut after U_{89} in *E. coli* 5S RNA; consequently, for the 3′ end labeled complex, all the cuts within the sequence 1–88 appeared weak relative to the corresponding free RNA cuts. When possible, bands were normalized relative to neighboring bands that were not altered in the presence of the protein. Protection or enhancement effects estimated in the range ±20% as are mentioned below, for example, for C_{12} and C_{35} in the *E. coli* RNA, were omitted from the tables. The

Table I: Effect of Protein L18 on the Ribonuclease Digestion of *E. coli* 5S RNA[a]

nucleotide	ribo-nuclease	degree of cut in free RNA	degree of change (%)
protected primary cuts			
U_{14}	A	++	50
C_{38}	A/T_2	++/+	100
C_{42}	A	+++	100
A_{52}	T_2	++	60
C_{63}	cobra	++	100
G_{64}	cobra	++	100
U_{65}	A	++	100
C_{114}	cobra	+++	100
protected secondary cuts			
C_{17} (U_{14})	A	+	100
C_{37} (C_{38})	A	+	100
C_{43} (C_{42})	A	+++	100
C_{47} (C_{42})	A	+	100
A_{53} (A_{52})	T_2	++	60
enhanced cuts			
G_{20}	cobra	++	350
G_{81}	cobra	+	100
C_{93}	cobra	+	160

[a] The data for the free RNA cuts are taken from the preceding paper (Douthwaite & Garrett, 1981). The ambiguity as to whether the strong cut at C_{38} is primary or secondary remains. For the secondary cuts the related primary cut is given in parentheses. The percentage degree of change of ribosomal cutting was estimated relative to the coelectrophoresed free RNA sample by densitometry and is given to within ±20%. The degree of enhancement was determined at an intermediate point in the enzyme range when approximately 50% uncut 5S RNA molecules were still present.

Table II: Effect of L25 on Ribonuclease Cuts in *E. coli* 5S RNA[a]

nucleotide	ribo-nuclease	degree of cut in free RNA	degree of change (%)
(A) protection			
G_{81}	cobra	+	100
C_{93}	cobra	+	100
(B) enhancement			
C_{35}–C_{43}	A/T_2		
C_{63}	cobra	++	30
G_{64}	cobra	++	20
U_{65}	A	++	≃100
U_{89}	A/T_2	++/(+)	250/–

[a] The data are presented, and were derived, as described for Table I. No quantitation of the general stimulation in the C_{35}–C_{43} region by L25 was possible for the reasons cited in the text.

quantitated data are presented in Tables I–III.

E. coli 5S RNA–Protein Complexes. (*A*) *Protection.* The data for proteins L18 and L25 are summarized in Tables I and II, respectively. L18 producted large effects within and adjacent to helix II as depicted in Figure 4A. The ribonuclease A cut at U_{65} was protected, as illustrated in Figure 2, as were the cobra venom ribonuclease cuts at C_{63} and G_{64}. The primary ribonuclease T_2 cut at A_{52}, in the region between helices II and III, was partially protected. Changes were also observed at the extremities of the model drawn in Figure 4A. The cobra venom ribonuclease cut at C_{114} in helix I was strongly protected, and major effects were detected in the C_{38}–C_{42} region where ribonuclease A and T_2 cuts were inhibited; the ribonuclease A effects are shown in Figure 2.

The ribonuclease A hydrolysis after nucleotides C_{12} and C_{35}, in the 3′ end labeled RNA in Figure 2, appears to be stimulated by protein L18. However, the results obtained by digesting 5′ end labeled 5S RNA–L18 in an identical manner

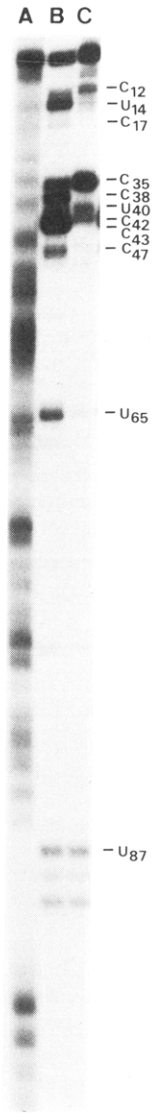

FIGURE 2: Effect of L18 on the ribonuclease A digestion of *E. coli* 5S RNA. The 5S RNA was 3′-end labeled with [^{32}P]Cp. (A) Adenosine cleavage of 5S RNA by diethyl pyrocarbonate–aniline treatment. (B and C) Digestion of 5S RNA and the L18 complex at a ribonuclease:RNA ratio of 1:500 (w/w) for 10 min at 0 °C. The bands of differing intensity in the complex, and a reference band U_{87}, are indicated. The gel electrophoresis conditions are given under Materials and Methods.

revealed either no effect of L18 at C_{12} and C_{35} or a slight protection against ribonuclease A hydrolysis, and these cuts are not included, therefore, in Table I. The increase in the band intensities at C_{12} and C_{35} in Figure 2 could be due to strong L18 protection of nucleotides on the 3′ sides of these two sites.

The protection effects observed with L25 were more localized than with L18 and were limited to the two cobra venom nuclease cuts on opposite sites of helix IV as shown in Figure 4B. The combination of proteins (L18 + L25) produced additive protection effects on the RNA with all the ribonucleases. The homologous and heterologous total 50S subunit proteins also yielded results comparable to those of (L18 + L25). The 5S RNA bound proteins were assayed as described earlier (Horne & Erdmann, 1972; Erdmann, 1976) with essentially the same results as listed in Table III. In the homologous reconstitution L5 bound weakly whereas in the heterologous reconstitution it bound more strongly but the protein equivalent to L25 was not detected and may have dissociated in the gradient. L5 was not tested alone because

Table III: Main Proteins Binding to the Two 5S RNAs When They Were Reconstituted Homologously and Heterologously with Total 50S Subunit Proteins[a]

bound proteins	*E. coli* RNA	*B. stearothermophilus* RNA
E L5	(+)	+
E L18	+	+
E L25	+	+
B L5	+	+
B L22	+	+
B L?	(?)	(?)

[a] The results are based on the studies of Horne & Erdmann (1972) and Erdmann (1976). Prefixes E and B refer to *E. coli* and *B. stearothermophilus*, respectively. RNA and proteins were incubated in ribosomal reconstitution buffer and separated on sucrose gradients, and the bound proteins were identified on two-dimensional gels. B L5 and B L22 were shown to be equivalent to E L5 and E L18, respectively (Erdmann, 1976). No protein equivalent to E L25 was detected in the *B. stearothermophilus* protein complexes. + indicates strong binding and (+) weak binding.

it aggregates rapidly in ribosomal reconstitution buffer and binds very weakly to the RNA (Garrett & Noller, 1979; Newberry & Garrett, 1980). No additional protein effects were observed that could be attributable to the weakly binding protein L5.

(*B*) *Enhanced Cuts.* Although no new ribonuclease cuts were observed in the protein–RNA complexes, enhanced cutting was evident at several sites. L18 stimulated the cobra venom ribonuclease hydrolysis at G_{20} in helix II and at G_{81} and C_{93} in helix IV (see Table I), whereas L25 enhanced the cutting of cobra venom ribonuclease in helix II, of ribonuclease A at U_{65} and U_{89}, and of ribonucleases A and T_2 in the C_{35}–C_{43} region (see Table II). The degree of stimulation of the primary cut at U_{65} was deduced approximately from both the increased yield of the RNA fragment on the magnesium-containing gel (Figure 1) and the increased yield of the secondary cut at C_{68} within the fragment (Douthwaite & Garrett, 1981). When both proteins L18 and L25 or the homologous and heterologous total 50S subunit proteins were complexed to the 5S RNA, the protective effects of L18 in the region C_{35}–C_{43} (although not at C_{38}) were overridden by L25 to give an overall increase in digestion; this increase in digestion kinetics with ribonucleases A and T_2 prevented a more exact determination of the effects of L25 in this region.

B. stearothermophilus 5S RNA–Protein Complexes. (*A*) *Protection.* The individual *E. coli* proteins L18 and L25 were less effective in protecting *B. stearothermophilus* 5S RNA than they were in protecting the homologous 5S RNA. Strong protection was obtained, however, with the heterologous total 50S subunit proteins and significantly less with the homologous total 50S subunit proteins. We were unable to determine the protein:RNA stoichiometry from the magnesium-containing gels of the type illustrated in Figure 1 because the mobility differences between the RNA and complexes were very small and we were limited to estimating the changes on the sequencing gels. The effects of proteins L18 and L25, and of total heterologous and homologous 50S subunit proteins, on the *B. stearothermophilus* RNA are summarized in Table IV and depicted in Figure 5.

As shown in Table III the heterologous reconstitution gave strong binding of all the *E. coli* proteins; even L5 bound at a higher stoichiometry than to *E. coli* 5S RNA. In the homologous reconstitution strong binding of the proteins equivalent to L5 and L18 occurs; as for the experiment with *E. coli* 5S RNA, no binding of a protein equivalent to L25

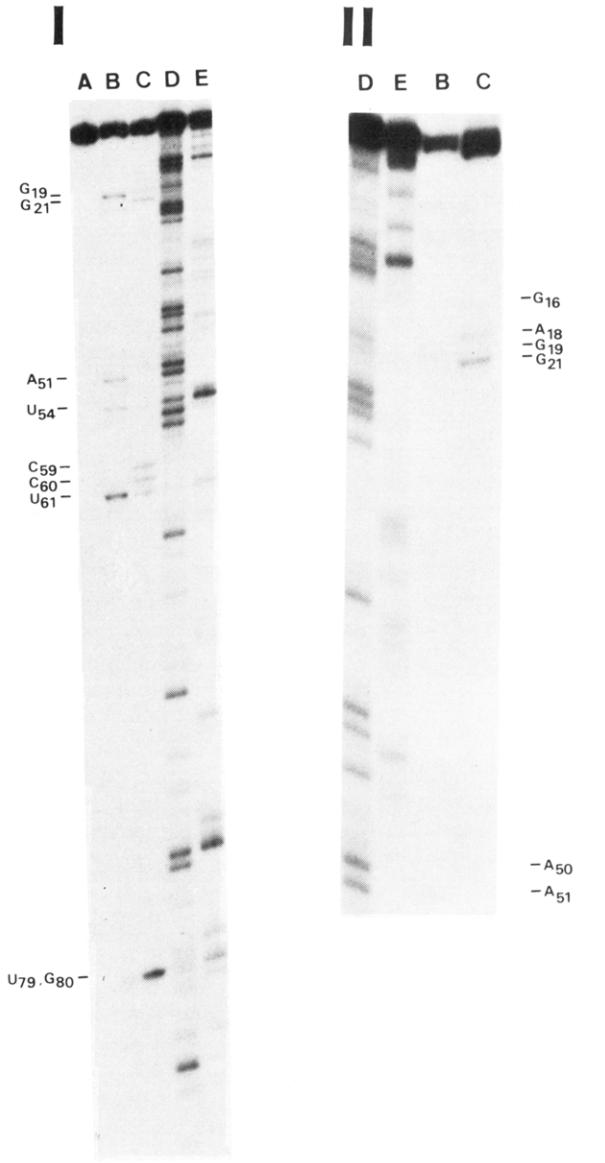

FIGURE 3: Protection of *B. stearothermophilus* 5S RNA against cobra venom ribonuclease digestion by *E. coli* 50S subunit proteins. 5S RNA was 3′-end labeled with [^{32}P]Cp. (A) Untreated 5S RNA. (B and C) Digested protein–5S RNA complex and 5S RNA, respectively, with 1:100 units of cobra venom ribonuclease/μg of RNA for 20 min at 0 °C. (D and E) Chemical cleavage of adenosines and uridines by the diethyl pyrocarbonate–aniline and hydrazine–aniline reactions, respectively. Sequencing gel I was run for 1.5 h, and gel II was run for 3 h. The identities of the ribonuclease cutting positions are indicated.

was detected, although the protection effects attributable to L25 were observed.

Extensive protection against cobra venom ribonuclease digestion was afforded to helix II by both L18 and the total proteins. The effects of the total heterologous proteins are displayed in Figure 3, where all the helix II cuts, with the exception of C_{19} and U_{61}, are inhibited. The effect of L18 in inhibiting cobra venom ribonuclease hydrolysis in helix I, as found in *E. coli* 5S RNA, was also observed in *B. stearothermophilus* RNA, but to a lesser degree. However, no comparable protection against ribonuclease A or T_2 was detected in the region neighboring C_{40} with either L18 or the total proteins. This might be a consequence of (a) the lack of a primary cutting site in *B. stearothermophilus* RNA equivalent to the C_{35} site in *E. coli* RNA or (b) the weaker binding of the heterologous L18 when used alone and the overriding of

Table IV: Effect of Ribosomal Proteins on the Structure of *B. stearothermophilus* 5S RNA[a]

nucleotide	ribo-nuclease	degree of cut in free RNA	proteins	degree of change (%)
(A) protection				
G_{16}	cobra	+	L18/tp	
A_{18}	cobra	++	L18/tp	
G_{21}	cobra	++	L18/tp	
G_{22}	cobra	+	L18/tp	
A_{27}	cobra	+	L18/tp	
C_{28}	cobra	+	L18/tp	
C_{30}	cobra	+	L18/tp	
A_{50}	cobra/T_2	+/(+)	L18/tp	
C_{59}	cobra	+	L18/tp	
C_{60}	cobra	++	L18/tp	
U_{79}	cobra	+++	L25/tp	
G_{80}	cobra	++	L25/tp	
C_{93}	cobra	+	L25/tp	
G_{111}	cobra	+++	L18/tp	
C_{112}	cobra	++	L18/tp	
(B) enhanced cuts				
U_{54}	cobra	(+)	E tp	25
G_{19}	cobra	+	E tp	45
U_{61}	cobra	++	E tp	60
(C) new cut				
A_{51}	cobra	+	E tp	

[a] The data for the free RNA derive from the preceding paper (Douthwaite & Garrett, 1981). The very weak T_1 ribonuclease cuts have been omitted as mentioned in the text, as have the weak cobra venom cuts at C_{92}, U_{110}, and U_{113}, which were generally not observed in the enzyme range tested in this study. tp indicates the effect of total 50S subunit proteins; prefixed by an E it refers exclusively to the *E. coli* proteins. Quantitation of the protection effects was not made because of the relatively low and sometimes uncertain protein:RNA stoichiometries compared with the *E. coli* protein-RNA complexes. Stimulation effects were estimated as described in the legend to Table I.

an L18 protection effect when complexed together with L25, as was observed in the *E. coli* RNA.

The total proteins, and to a lesser extent L25, also protected the cobra venom ribonuclease digestion in helix IV at U_{79} and G_{80} (Figure 3), and C_{92} and C_{93} (not shown); equivalent effects were observed in the *E. coli* 5S RNA.

All of the weak T_1 ribonuclease cuts at G_{14}, G_{42}, G_{65}, and G_{67}, detected in the free RNA in the preceding paper (Douthwaite & Garrett, 1981), were not observed in the protein–RNA complexes, suggesting the probability that they arose from minor conformational heterogeneities; they are not included in Figure 5.

(B) Enhanced and New Cuts. Quantitation of enhanced cuts was difficult for the *B. stearothermophilus* RNA because of the variable and nonunitary stoichiometry of the protein–RNA complexes. The data are less complete, therefore, than for the *E. coli* RNA. A few clearly enhanced cuts were observed, however. As shown in Table IV, three cobra venom ribonuclease cuts were enhanced in and adjacent to helix II. A new cobra venom ribonuclease cut was observed at A_{51} in the presence of the total proteins (see Figure 3) and the cut in the free RNA at the preceding nucleotide was inhibited. This result indicates a protein-induced change in the RNA structure. Since it was only observed for the heterologous total protein–RNA complex when L5 bound strongly (see Table III), the effect could be mediated by the latter.

Methodological Limitations. The problems of (a) identifying ribonuclease cuts, (b) establishing whether they are primary or secondary, and (c) determining the possible levels of alternate conformers, which may give rise to weak artifact bands, were considered in detail in the preceding paper

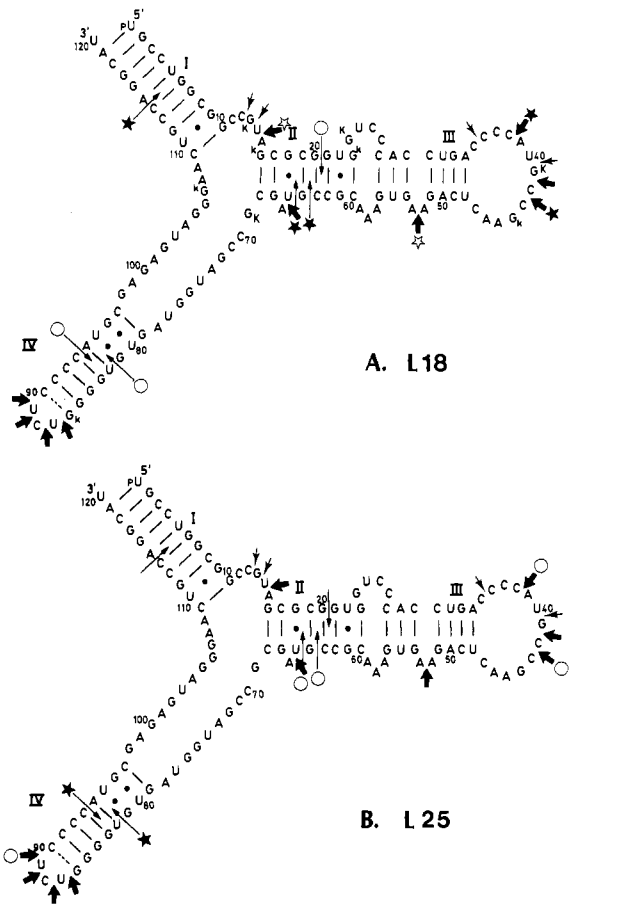

FIGURE 4: *E. coli* 5S RNA: protection and stimulation of ribonuclease cuts in the presence of (A) L18 and (B) L25. *E. coli* 5S RNA is drawn according to the Fox and Woese secondary structural model with extensions to helices II–IV. The arrows indicate the primary cuts in the free RNA as determined by Douthwaite & Garrett (1981). Secondary cuts are not shown. The cut at C_{38}, which may be primary or secondary, is included. Thick arrows correspond to strong cuts and thin arrows indicate weaker cuts. If a cut is strong with one ribonuclease and weak with another, it is represented by a thick arrow. Arrows drawn into the helices, across the polynucleotide chain, represent the sites of cobra venom ribonuclease cuts; again the arrow size defines the degree of cutting. The effects on the ribonuclease cutting of (A) L18 and (B) L25 are represented as follows: (solid star) strong protection by the protein, (open star) weak to medium protection, and (open circle) stimulation of cutting. The sites of rapid and slow kethoxal modification (Garrett & Noller, 1979) are denoted by large and small K's, respectively.

(Douthwaite & Garrett, 1981). The only serious ambiguity discerned was whether the strong cut at C_{38} in the *E. coli* 5S RNA was primary or secondary. In this study, as far as we could determine, the nucleotide cuts, previously defined as either primary or secondary enzyme target sites in the free RNA, fell into the same categories after complex formation. The problems associated with determining protein:RNA stoichiometries, which were exclusive to this study, were considered above.

Discussion

In the preceding paper we investigated the structure of the *E. coli* and *B. stearothermophilus* 5S RNAs using ribonuclease probes. The evidence in favor of the Fox and Woese secondary structural model was described, and base-pairing extensions to the helices II–IV were also considered (Noller & Garrett, 1979; Garrett et al., 1981; Peattie et al., 1981; Studnicka et al., 1981). These structural features are presented for the two RNAs in Figures 4 and 5. The ribonuclease digestion data also revealed a high degree of structure, in

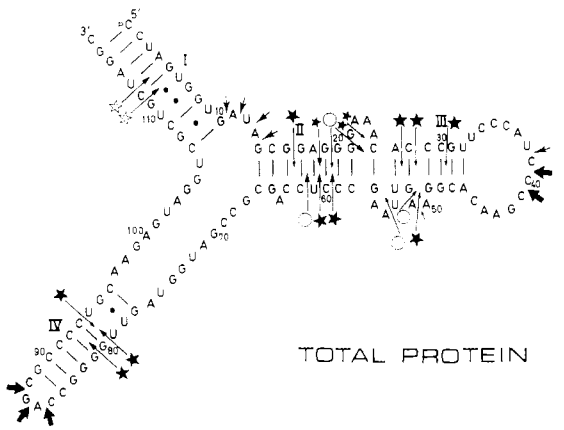

FIGURE 5: *B. stearothermophilus* 5S RNA: protection and stimulation of ribonuclease cuts in the presence of L18, L25, and total 50S subunit proteins. The secondary structural model corresponds to that presented in Figure 4 with a base-pairing difference in the region between helices II and III as described by Studnicka et al. (1981). Arrows indicate the cutting positions of ribonucleases A, T_1, T_2, and cobra venom; the same system of arrows and the same denotations for protection and stimulation by the proteins are used as described for Figure 4.

addition to the base pairing shown in the figures, especially within the region 69–110.

The approach of studying both 3′ and 5′ end labeled 5S RNAs in the various complexes was found to be an essential aid in establishing the identities of primary and secondary cuts and in the interpretation of ambiguous or misleading effects observed in the sequencing gels. This approach helped to clarify the effect of L18 on nucleotides C_{12} and C_{35}, as already described for Figure 2, and to elucidate exactly enzyme cutting positions that occurred in regions of sequence compression, such as those in the G-rich regions in helices II and IV of *B. stearothermophilus* RNA (see Figure 3).

Interpretation of the Protection Effects. The following general rules were evolved during the analysis and interpretation of the data. (a) Primary-cut protection coincides with protection of the dependent secondary cuts but not necessarily vice versa; the protected secondary cuts listed in Tables I and II are compatible with this. (b) The degree of protection of a cut within a protein binding region is proportional to the protein:RNA stoichiometry. (c) A low level of protection relative to the protein:RNA stoichiometry indicates, probably, that accessibility to the ribonuclease is partially hindered because the cutting site is close to the protein binding region. Interpretation of the enhancement effects is more complex and is considered below.

(*A*) *L18 Binding Region.* There has been some uncertainty about the location of the L18 binding site. Evidence was presented that L18, like L25, could protect nucleotides 69–120 and 1–11 of the *E. coli* RNA against ribonuclease A digestion (Gray et al., 1973). More recently, however, this result was shown to be an artifact of the protection approach used to isolate RNA binding sites (Douthwaite et al., 1979), the L18 being displaced prior to RNA-fragment formation. Kethoxal modification studies revealed that guanines 24 and 69, at opposite ends of helix II, were protected by L18 against modification, and L18 rebinding studies indicated that one or both guanines were directly involved in the protein–RNA interaction site (Garrett & Noller, 1979). The present data provide strong additional evidence that the protein binds to this helical region in both RNAs, as outlined in Figures 4A and 5. Cobra venom ribonuclease cuts in helix II were protected, as was the ribonuclease A cut preceding the putative bulged A_{66} in the *E. coli* RNA and the weak cuts that occur at C_{17} and G_{65} in the *E. coli* and *B. stearothermophilus* RNAs, respectively (see Tables I and IV). The region between helices II and III was also involved. All of the weak cobra venom ribonuclease cuts observed here in the *B. stearothermophilus* RNA were inhibited by the protein, as were the ribonuclease T_2 cuts at A_{52} and A_{53} in the *E. coli* RNA and the A_{50} cut in the *B. stearothermophilus* RNA. These results receive further support from a dimethyl sulfate modification study, which showed that L18 protects guanines 51, 54, and 56 from modification, all of which are situated in this region of the *E. coli* RNA (Peattie et al., 1981).

Other parts of the RNA were also affected by L18. The cobra venom ribonuclease cuts in helix I were protected by L18 in *E. coli* and, to a lesser extent, by L18 and total protein in *B. stearothermophilus*. The ribonuclease A cuts in the *E. coli* RNA region C_{38}–C_{43} were also protected by L18.

(*B*) *L25 Binding Region.* Earlier studies have implicated a ribonuclease A resistant fragment of 5S RNA as the binding site of L25, extending from G_{69} to U_{87} and C_{90} to C_{110} (Gray et al., 1973; Douthwaite et al., 1979). The protein remains bound to this RNA fragment after digestion and can be dissociated and reassociated, although it appears to have a lower binding affinity to the fragment than the whole RNA. Results from both ribonuclease digestion (Douthwaite et al., 1979; Douthwaite & Garrett, 1981) and kethoxal modification studies (Garrett & Noller, 1979) all emphasize the high degree of structuring in this fragment. In addition to forming helix IV, it probably either exhibits a complex tertiary structure or has interactions with other parts of the RNA molecule (Garrett et al., 1981). Our results provide further insight into the localization of the L25 binding site, which is drawn in Figures 4B and 5; the cobra venom ribonuclease cuts that occur on opposite sides of helix IV in the free RNAs are protected by both L25 and the heterologous proteins. The results implicate this helical region as one attachment site for L25.

Conformational Heterogeneity and Conformational Change. There have been several indications of protein-induced conformational changes in 5S RNA. For example, a large increase in the RNA circular dichroism band at 267 nm occurs on complexing with protein L18; a smaller increase was detected with L25 (Bear et al., 1977; Spierer et al., 1978). Increases in the ribonuclease A sensitivity of the nucleotide bonds after C_{68} and U_{89} were also observed during the digestion of L25–5S RNA complexes (Douthwaite et al., 1979). In this study, there were several examples of enhanced cutting in the complexes. They can be grouped into three classes. (a) Those that occurred within the binding domain of the protein; for example, L18 stimulates one cobra venom cut in helix II of both *E. coli* and *B. stearothermophilus* RNA, and L25, as found earlier (Douthwaite et al., 1979), stimulates the ribonuclease A cutting of *E. coli* RNA at U_{89}. (b) Those cuts that occurred outside the protein binding domain. In the *E. coli* RNA, L18 stimulates the cobra venom cuts in the L25 binding site, and L25 stimulates two of the cobra venom cuts in helix II and the ribonuclease A cut after U_{65} [this is primary to the secondary cut at C_{68}, which is, consequently, also stimulated (Douthwaite et al., 1979)]. L25 also produces enhanced cutting in the C_{35}–C_{43} region, which has also been implicated in L18 binding. A precise quantitation of these data, as pointed out earlier, is difficult because of the strongly enhanced cut at U_{65} resulting in fragment formation. (c) The third group constitutes one new cut, namely, the cobra venom cut occurring at A_{51}, and stimulation of the U_{54} cut in the *B. stearothermophilus* RNA. These effects were only observed with the heterologous 50S subunit proteins.

Whether the above-mentioned effects reflect changes in conformational heterogeneity, especially in localized flexible RNA regions such as short single-stranded regions, or whether they result from the formation of new structural features within the secondary or tertiary structure is difficult to decide. Although our preselecting for the A conformer, after ribonuclease digestion, minimized the possibility of stable conformers coexisting with widely differing secondary structures, local heterogeneities could still occur within the A conformer, and the disappearance of weak cuts in the presence of the proteins, especially some ribonuclease T_1 cuts and the weakest cobra venom ribonuclease cuts in the *B. stearothermophilus* 5S RNA, supports this inference [see Douthwaite & Garrett (1981)].

However, there are other observations that appear more profound, for example, the dynamic interplay between helices II and IV, which constitute the respective binding sites of L18 and L25. The effects are large for the *E. coli* RNA (see Tables I and II), suggesting a marked effect of the proteins on the RNA structure; they may also provide a structural explanation of the observed cooperative RNA binding of the two proteins (Feunteun et al., 1975). At present, we cannot establish whether these effects are due to a physical interaction of the two helices or to an allosteric effect of the proteins transmitted through the RNA. Similar problems arise in interpreting the opposing effects of L18 and L25 in the region around C_{38} and C_{42} in the *E. coli* RNA. Again some of the effects are large, and there is the possibility of allosteric effects at least for L25. An unambiguous conformational change is indicated by the appearance of a new cobra venom ribonuclease cut at A_{51} in the *B. stearothermophilus* 5S RNA in the presence of the *E. coli* total 50S subunit proteins, but this may be attributable to protein L5.

As discussed above, the interpretation of several of the aforementioned protein-induced structural changes is ambiguous, but it does raise the general question of the role of the ribosomal proteins, and the answer may be that they have more than one role. One possibility, which is supported by the above data, is that the proteins may be required to establish and maintain conformational homogeneity in certain parts of the RNA molecule. Moreover, they may also be required to induce and/or control reversible conformational changes in the RNA that are of functional significance.

Comparison of the Effects of Proteins on the E. coli and B. stearothermophilus RNAs. The main protection effects occur for proteins L18 and L25 in helices II and IV, respectively, in both RNAs as summarized in Figures 4 and 5. The main protein binding regions that appear to be defined by these effects are directly comparable. The main differences concern the protection effects at the extremities of the model in *B. stearothermophilus*, including the weaker protection of the cut in helix I, and in the C_{38}–C_{43} region by L18. Also the corresponding stimulation effects of L18 in helix IV and L25 in helix II were not observed. While this may reflect some important differences in the protein binding regions, it is probably due to the weaker binding of the L18 and L25 proteins and especially the former to the *B. stearothermophilus* RNA. In a study of the binding of *B. stearothermophilus* protein S8 to the *E. coli* 16S RNA a similar conclusion was reached that the protein binding was less strong than in the homologous complex (Stanley & Ebel, 1977).

More effects were observed in the region between helices II and III in the *B. stearothermophilus* RNA, and this is the region for which different base-pairing schemes have been proposed for Gram-negative and Gram-positive bacteria (Studnicka et al., 1981). Although our data are unable to define the correct base pairing in this region, they are compatible with the pairing in both RNAs except for the intercalated base pair C_{26}-G_{57} in the Gram-positive bacterium (Figure 5); the data would favor the alternative pair G_{22}-U_{54}. However, the formation of a new cut at A_{51} in the *B. stearothermophilus* 5S RNA in the presence of total heterologous 50S subunit proteins suggests that some structural rearrangements may occur in this region.

The possible involvement of the nucleotide looped out from helix II in L18 recognition (Peattie et al., 1981; Garrett et al., 1981) is supported by the protection of the cut preceding A_{66} in the *E. coli* RNA; no cut occurred in the corresponding position in the *B. stearothermophilus* RNA, and possible reasons for this were considered earlier (Douthwaite & Garrett, 1981).

In conclusion we have defined further the RNA sites of the two primary binding proteins on prokaryotic 5S RNA. They have separate binding domains, with that of L18 being the more extended. Evidence is also provided for protein-induced structural changes in the RNA, which may be important in protein–5S RNA assembly and function in the translation process.

Added in Proof

A recent report has suggested that protein L25 does not complex with 5S RNA under the conditions of the two-step reconstitution procedure used for *E. coli* 50S subunits (Röhl & Nierhaus, 1982). Our conditions are those used for reconstituting active *B. stearothermophilus* 50S subunits (Erdmann, 1976) and are almost identical with those of the second step of the *E. coli* procedure. The report contradicts a large body of evidence, from different laboratories, indicating that L25 forms a site-specific complex with *E. coli* 5S RNA under the conditions of both steps of the reconstitution procedure, by various criteria [e.g., Erdmann (1976), Spierer & Zimmermann (1978), and Garrett & Noller (1979)]. While the reason for their negative binding result is unclear, errors have resulted because two *E. coli* proteins comigrate in the L25 spot on the standard 2-D gel identification method (Brosius, 1976). We, and others, distinguish between these proteins *by the capacity of one of them to bind to 5S RNA*; it is possible that Röhl and Nierhaus have taken the other protein.

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Enzymatic and Chemical Structure Mapping of Mouse 28S Ribosomal Ribonucleic Acid Contacts in 5.8S Ribosomal Ribonucleic Acid†

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ABSTRACT: Secondary structure mapping experiments using S1 nuclease, RNase T_1, and diethyl pyrocarbonate as conformational probes have identified those regions in mouse 5.8S rRNA containing major sites of interaction with 28S rRNA. One site encompasses the 3′-terminal 20 nucleotides and corresponds to the region identified previously as a component of an RNase-resistant 5.8S/28S rRNA junction complex. A second site, located at the 5′ terminus, has not been defined precisely but is believed to involve approximately 20–30 nucleotides. The existence of these sites of interaction is supported by comparing sequences of eukaryotic 5.8S and 28S rRNA with those of the prokaryotic 23S rRNA. Evidence for the occurrence of at least three helical regions in the central portion of the mouse 5.8S rRNA molecule is also presented.

Intermolecular RNA–RNA interactions mediate reactions that occur during protein synthesis, and probably other cellular functions. Such interactions have been difficult to explore because they generally are very tenuous. An RNA–RNA interaction of unknown role occurs in the large subunit of eukaryotic ribosomes, where 5.8S rRNA exists in the form of a specific complex with 28S rRNA (Pene et al., 1968). The noncovalent association presumably is maintained by hydrogen bonding and base stacking, and the complex is readily isolated by the usual extraction procedures. The stability of the 5.8S/28S rRNA complex affords an opportunity to investigate the details of an intermolecular RNA–RNA interaction.

We have demonstrated previously that the 5.8S/28S rRNA complex of mouse ribosomes can be dissociated by heating and reconstituted into a structure which exhibits the same thermal denaturation properties as those of its native counterpart (Pace et al., 1977). Limited ribonuclease digestion of the reconstituted complex between radioactive 5.8S rRNA and unlabeled 28S rRNA, and subsequent fractionation of the hydrolysis products, led to the identification of a specific "junction fragment" that contained part of the interacting regions of 5.8S and 28S rRNAs. The 5.8S moiety of the RNase-resistant fragment consisted of 42 nucleotides derived from the 3′ terminus of 5.8S rRNA. It was proposed that the 3′-terminal 20 nucleotides of the 5.8S molecule engage with 28S rRNA. The remaining 22 residues form an intramolecular GC-rich helix which, if allowed to engage in coaxial helix stacking, could in principle enhance the stability of the bimolecular interaction.

The isolated junction fragment was considerably less stable to thermal denaturation than the intact complex, however (Pace et al., 1977). This suggested that it is not the only region of interaction between 5.8S and 28S rRNAs. In an effort to define additional points of interaction and to assess conformational changes in 5.8S rRNA induced by annealing to 28S rRNA, we have exploited the structure mapping techniques described by Wurst et al. (1978) and Peattie & Gilbert (1980). In these procedures the sensitivity of a 5′- or 3′-end-labeled RNA molecule to nuclease or structure-specific reagent attack is analyzed by a modification of the rapid RNA gel sequencing

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