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Structural analyses of E.coli 5S RNA fragments, their associates and complexes with proteins L18 and L25

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ABSTRACT

The structure of Escherichia coli 5S RNA fragments 1-41 and 42-120 has been studied by the read-off gel sequencing technique using S_1 nuclease and cobra venom RNase as probes. Comparison of the digestion patterns with those of reassociated and intact 5S RNA suggests that the structure of both fragments is very similar to that of the corresponding regions in the intact molecule.

Six different fragments obtained by partial digestion with T_1 RNase and S_1 nuclease have been used for reconstitution of 5S RNA, its certain structural regions and complexes with ribosomal proteins L18 and L25. The results presented indicate that in 5S RNA protein L25 recognizes the double-helix consisting of nucleotides 79-97 (i.e. prokaryotic stem), whereas a loop-region around position 40 (possibly positions 39-47) is involved in the interaction with protein L18.

INTRODUCTION

The arrangement of nucleotide residues in polynucleotide chain determines its three-dimensional form. As it was recently established concerning tRNA [1], polynucleotide chain can be sequentally folded as it is being synthesized from the 5' end.

Sequence of the RNA can be rapidly determined using the read-off gel sequencing method [2,3]. The enzymatic method itself provides little data about the RNA secondary structure. But, by this method, more detailed information about the single-stranded and double-stranded regions within the RNA polynucleotide chain can be obtained using the structure-specific nucleases (e.g. S_1 nuclease and cobra venom RNase). This approach, based on the use of S_1 nuclease as probe, was initially proposed by Wurst et al. [4] and its reliability was further supported by the studies of tRNA [5-7]. The structure of several RNAs and RNA fragments have now been mapped by using S_1 nuclease and cobra

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venom RNase [8-12].

We have recently determined the single-stranded and double-stranded regions in E.coli 5S RNA by the same method [13]. In the present paper we describe the structure mapping of fragments 1-41 and 42-120 and reassociated 5S RNA. The aim of this study is to get more insight into the structure of 5S RNA and particularly its fragments. In addition, a number of obtained fragments and reconstituted molecules were tested in the binding of ribosomal proteins L18 and L25. This allowed us to localize the regions in 5S RNA involved in the interactions with these proteins.

MATERIALS AND METHODS

Isolation of 5S RNA and ribosomal proteins L18 and L25

5S RNA was isolated from E.coli (MRE600) ribosomes as described by Monier and Feunteun [14]. The nativity of 5S RNA was probed by arcylamide gel electrophoresis in nondenaturing conditions. It migrated as a single band on a 10% polyacrylamide gel in 40 mM Tris-HCl, pH 7.4, as described by Aubert et al. [15] and it was totally transferred to the form of ribonucleoprotein in REC buffer (30 mM Tris-HCl, 300 mM KCl, 20 mM MgCl, 6 mM β-mercaptoethanol, pH 7.4 at 23° C) by incubating with proteins L18 and L25 at protein: RNA molar ratio 3:1 (see also below).

Proteins L18 and L25 (equimolar mixture; see Fig. 4, line 13) were isolated from 50S subunit proteins (deficient in protein L5) by affinity chromatography on a immobilized 5S RNA-Sepharose 2B column [16]. Proteins, used in rebinding assays were concentrated up to 10^{-5} M solution and dialyzed using dialyses membrane tubing 3 (Spectrapor, M.W. cutoff 3.500) and their approximate concentration was calculated from A_{230} [17].

Preparation of 5S RNA fragments

For 5S RNA fragmentation partial digestions with T_1 RNase (Sankyo) essentially as described by Brownlee and Sanger [18] and S_1 nuclease (SKTB BAV, Glavmikrobioprom) were performed. Fragments 1-41 and 42-120 were obtained by digesting 1 mg of 5S RNA in 1 ml of TMN buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, 200 mM NaCl, pH 7.5 at 0° C) with 20 U T_1 RNase for 6 h at 0° C. The digestion

was stopped by addition of $ZnCl_2$ to a final concentration of 20 mM followed by phenol treatment. A digestion with S_1 nuclease was carried out as follows: 1.5 mg of 5S RNA in 0.3 ml of S buffer (30 mM NaOAc, 1 mM $ZnCl_2$, 100 mM NaCl, pH 4.6) was digested with 150 U S_1 nuclease for 1 h at 23° C. The digestion was stopped by addition of EDTA-Na_2 to the final concentration of 10 mM.

The digestion products were fractionated on the polyacrylamide slab gels (20×30×0.2 cm) containing 8 M urea, localized by UV shadowing, cut out and eluted as described by Donis-Keller et al. [2]. For sequence determination 20 µg of each fragment obtained was dephosphorylated as described by Shinagawa and Padmanabhan [19], labelled at its 5' end and sequenced (ladder + reactions with T_2 and U_2) by the method of Donis-Keller et al. [2] (data not shown). The deduced sequences of the fragments were consistent with the cuts introduced into 5S RNA by T_1 RNase and S_1 nuclease established previously [13].

Structure mapping with S_1 nuclease and cobra venom RNase as probes

5S RNA, its fragments 1-41 and 42-120 were 5' or 3' end-labelled according to the published procedures [4,20]. In one experiment 200-400 µCi [γ-^{32}P] ATP (specific activity 1000 Ci/mmol, V/O Isotop, USSR) was used. 5'-^{32}P-pCp was synthesized as described by England et al. [20] and used without further purification. All end-labelled RNAs (1-4 µCi/µg) were purified by electrophoresis on the acrylamide slab gels containing 8 M urea and precipitated in the presence of the same unlabelled RNA component as carrier. Before nuclease addition 5S RNA, its fragments and corresponding complexes were heated for 15 min at 60°C in S or TMN buffers followed by slow cooling to the indicated reaction temperature. The complex formation between fragments 1-41 and 42-120 was checked electrophoretically (see the next section).

Structure mapping experiments with S_1 nuclease, which cleaves single stranded RNA regions [21] was performed in S buffer and with cobra venom RNase (a gift by Dr. S.K.Vasilenko), which attacks double-stranded RNA regions [22] in TMN buffer. Experi-

mental details of these digestions are indicated in the legends to the figures. In all control experiments incubation of RNA was carried out exactly under the conditions of enzyme reaction but without nuclease ((-E)-line).

Since Pavlakis et al. [23] have shown the 3' phosphatase activity of S_1 nuclease, 3' end-labelled fragment 42-120 was dephosphorylated [19] before its digestion. Sequencing reactions with T_1 and U_2 RNase were performed as in [2] and the ladder was obtained by incubating 1 µg of end-labelled RNA in 10 µl bidistilled water for 20-60 sec at 100°C. The digestion products were fractionated on thin 20% polyacrylamide gel slabs (400×200×0.4 mm) containing 8M UREA [31]. The gels were freshly prepared, preelectrophoresed for 2 h and run at 10 mA (2.0 mV). To prevent band curvature and to obtain higher temperatures two thin gels were prepared between three glass plates and run together at 20 mA (2.0 kV).

Since the products of both S_1 nuclease and cobra venom RNase terminate with a 3' hydroxyl group and at the same time RNases used in sequencing give rise to the products having a 3' phosphate there raises a problem in determination of precise cuts of these nucleases, and especially in the case of small oligonucleotides [4,24]. We found that the correct assignment can be made, when the specific band spacing of both the ladder and the analyzed region is taken into consideration (see Fig. 2A). Usually a wide space between the bands in the ladder indicates that a band with slower mobility in the case of 5' end-labelled RNA corresponds to the fragment terminated at its 3' end with G residue (see Fig. 1B and ref. [25]). But in the case of 3' end-labelled RNA in the same situation the oligonucleotide having G residue at its 5' end corresponds to a faster migrating band (see Fig. 2B).

Digestion of 5S RNA-L18 and L25 complex with cobra venom RNase was performed as follows: 4 µg of 5S RNA containing 100.000 cpm of 3' end-labelled RNA (or 5' end-labelled RNA) was complexed with ribosomal proteins L18 and L25 in 30 µl of REC buffer at protein: RNA molar ratio 3:1 for 40 min at 37°C followed by slow cooling to 0°C. Complex formation was checked electrophoretically (see the next section). Two digestion with 1.0 ng

and 0.2 ng of cobra venom RNase in reaction volume of 10 µl were carried out for 10 min at 0°C. The reactions were stopped by adding 10 µl of sample buffer (50 mM Tris-borate, 8 M urea, 2 mM EDTA-Na_2 and dyes, pH 8.3), heated for 1 min at 60°C and loaded onto a thin acrylamide slab gel (400×200×0.4 mm). Electrophoresis lasted for 9 h (the first loading) and 2 h (the second loading) at 10 mA (2.0 kV).

Electrophoresis of reconstituted 5S RNA and its protein complexes

This was carried out on the 10% acrylamide slab gels (20×20×0.15 cm) for 10-15 h at 40 mA and at 5°C. Two electrophoresis buffers were used: A - 50 mM Tris-HCl, 20 mM $MgCl_2$ and 50 mM KCl, pH 7.6, essentially as in [26] and B - 50 mM Tris-borate, 10 mM $MgCl_2$, 50 mM KCl, pH 7.7 (omission of KCl did not affect the results). In the former case 5S RNA-L18 and L25 and fragment 42-120-L25 complxes migrated somewhat slower (data not shown) than the corresponding RNA components, while in the latter case the RNP complexes and its respective RNAs had the same mobility.

Complexes between about equimolar amounts of 5S RNA fragments (few µg) were formed by incubating them in 20 µl of TMN buffer for 15 min at 40°C followed by slow cooling. Before electrophoresis, 5S RNA fragments (3 µg of each) were also incubated in the same way. Electrophoresis was in buffer B and the RNA was made visible by staining it with methylene blue.

RNP complexes were prepared by incubating the 5S RNA fragment or reconstituted 5S RNA with proteins L18 and L25 in 50 µl of REC buffer for 40 min at 37°C. Both RNA and protein concentrations were 5×10^{-6} M. After electrophoresis in buffer B, the RNA was localized by UV shadowing and the corresponding RNP complex (in the same gel) by staining with Coomassie brilliant blue. The stained proteins were identified as follows: the gel-piece containing the stained complex was excised, soaked in SDS to dissociate the complex and electrophoresed directly into a SDS containing acrylamide slab gel [27].

RESULTS

Structure mapping of fragments 1-41, 42-120 and reassociated 5S RNA

5' end-labelled 5S RNA fragment 1-41 was partially digested with S_1 nuclease at 0°C and the resulting digestion products were analyzed by the read-off gel sequencing technique (Fig. 1A, line S_1 2). The comparison with reassociated 5S RNA (line S_1 1) and previous studies [13] shows no apparent difference between the digestion patterns of this fragment and the corresponding region of intact and reassociated 5S RNAs. Therefore, it seems that at 0°C S_1 nuclease did not discriminate between the conformations of fragment 1-41 and the corresponding region in 5S RNA.

In the second experiment a double-strand specific RNase from cobra venom was used to probe RNA structure. Fig. 1A, lines CE1 and CE2 show the digestion patterns by this enzyme of the region 1-41 in the reassociated 5S RNA and free fragment 1-41, respectively. The only difference at positions 18-20 was found to indicate that in the fragment this region is differently arranged from the corresponding region in the reassociated 5S RNA.

The structure of free fragment 42-120 and its structure in the complex with fragment 1-41 was probed in a similar way. At 0°C S_1 nuclease hydrolyzes both the fragment and the complex (Fig. 1B, lines $S_1$2 and $S_1$1 respectively) predominantly at the same positions: 89 and 42-46 (data not shown). In addition, at 40°C a much larger region (42-59) was cleaved by this nuclease (Fig. 1C, line S_1). The major cleavage sites of the fragment and the complex introduced by cobra venom RNase were found to be identical. However, in the fragment the minor cleavages by this RNase occured at positions 110-112 and 118 (Fig. 1B, line EC1), which were absent in the digest of the complex (Fig. 1B, line EC2). Also, position 98 was cleaved only in the complex. It is important to note that cobra venom RNase digestion patterns of the intact 5S RNA and the reassociated molecule are identical, while the fragments digestion patterns differ from them in a few regions.

The structure of 5S RNA at 60°C

Since at 0°C S_1 nuclease did not show differences in the conformation of the fragment and the corresponding region in 5S

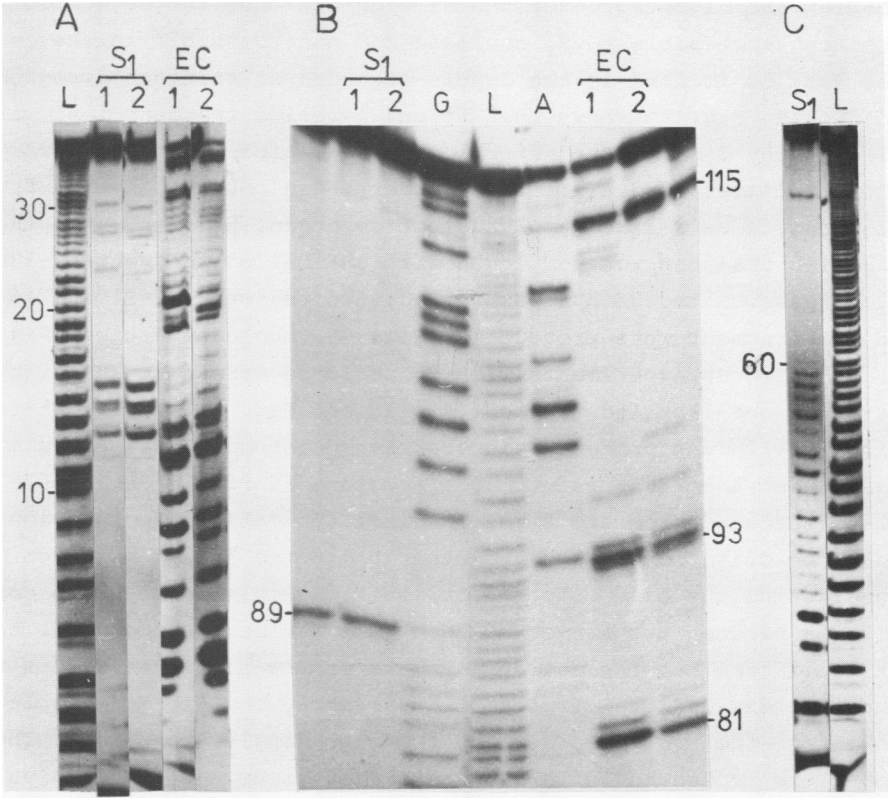

Figure 1. Structure mapping of fragments 1-41 and 42-120 and reassociated 5S RNA.

A - Digestion of 5' end-labelled fragment 1-41 (0.5 µg) with 2 U S_1 nuclease ($S_1$2) and 0.4 ng of cobra venom RNase (EC2). Digestion of reassociated 5S RNA (0.5 µg of 5' end-labelled 1-41 + 1.0 µg of 42-120) with 2 U S_1 nuclease ($S_1$1) and 0.4 ng of cobra venom RNase (FC1).

B - Digestion of 5' end-labelled fragment 42-120 (1.0 µg) with 6 U S_1 nuclease ($S_1$2) and 1.0 ng of cobra venom RNase (EC1). Digestion of reassociated 5S RNA (1.0 µg of 5' end-labelled 42-120 + 0.5 µg of 1-41) with 6 U S_1 nuclease($S_1$1) and 1.0 ng of cobra venom RNase (EC2).

C - Digestion of reassociated 5S RNA (1.0 µg of 5' end-labelled 42-120 + 0.5 µg of 1-41) with 3 U S_1 for 1 min at 40°C (S_1).

All reactions were in 10 µl of the corresponding buffer (see Methods) and in A and B ($S_1$1,2, EC1,2) for 30 min at 0°C. Sequence reactions with RNases T_1 (G) and U_2 (A) and the ladders were made as described under Materials and Methods.

RNA we repeated experiments at 60°C. Here, the strategy used is based on the assumption: when the fragment and the corresponding

complex are digested under the same conditions, the region in the fragment accessible to S_1 nuclease and nonaccessible in the complex must be located in the double-stranded or structured regions of 5S RNA. Fig. 2A shows that 5' end-labelled fragment 1-41 was digested by S_1 nuclease after 1 min incubation at 60°C at every phosphodiester bond. Therefore, under these conditions this fragment exists as a linear polynucleotide chain. However, when the labelled fragment was complexed with unlabelled fragment 42-120, i.e. reassociated 5S RNA was formed, two regions - 2-11 and 16-22 in the fragment were protected against S_1 nuclease action (Fig.2A).

Analogous experiments with S_1 nuclease were carried out by using 3' end-labelled fragment 42-120 and Fig. 2B shows that region 109-118 was protected in the reassociated 5S RNA molecule. Because of the high resistance of regions 90-98, 77-86 and 67-76, 61-64 (deduced from the first loading), no definite conclusion could be drawn concerning the other protected sequences. Even considering the band compression it is possible to show the connection between two former regions (see the next paragraph). As it is difficult to select appropriate enzyme-substrate ratios for the fragment and the complex it is a fairly complicated problem to assign the protected regions from the single experiment. Therefore, the protection of certain 5S RNA regions was observed only in the kinetic experiments.

Band compression as evidence for loop-back structures

The occurence of the bands representing nucleotides unusually close to one another in the gel (compression) is caused by persistence of the RNA secondary structure during electrophoresis [28,29].

5S RNA reveals in the case of 5' end-labelled intact RNA or fragment 42-120 the band compression in region 90-94 ([13] and Fig. 1B, line L). But in the case of 3' end-labelled 5S RNA the other region 81-85 is compressed in the sequencing gel (Fig. 3, line L_1 and ref. [30]). Partial resolution of this region can be obtained by running the gel at high voltage (2.0 kV) (line L_2) as recommended by Sanger and Coulson [31]. It is interesting to point out that at lower gel temperatures nine last nucleotides of 5S RNA are also compressed (Fig. 3, line L_1) and they can be

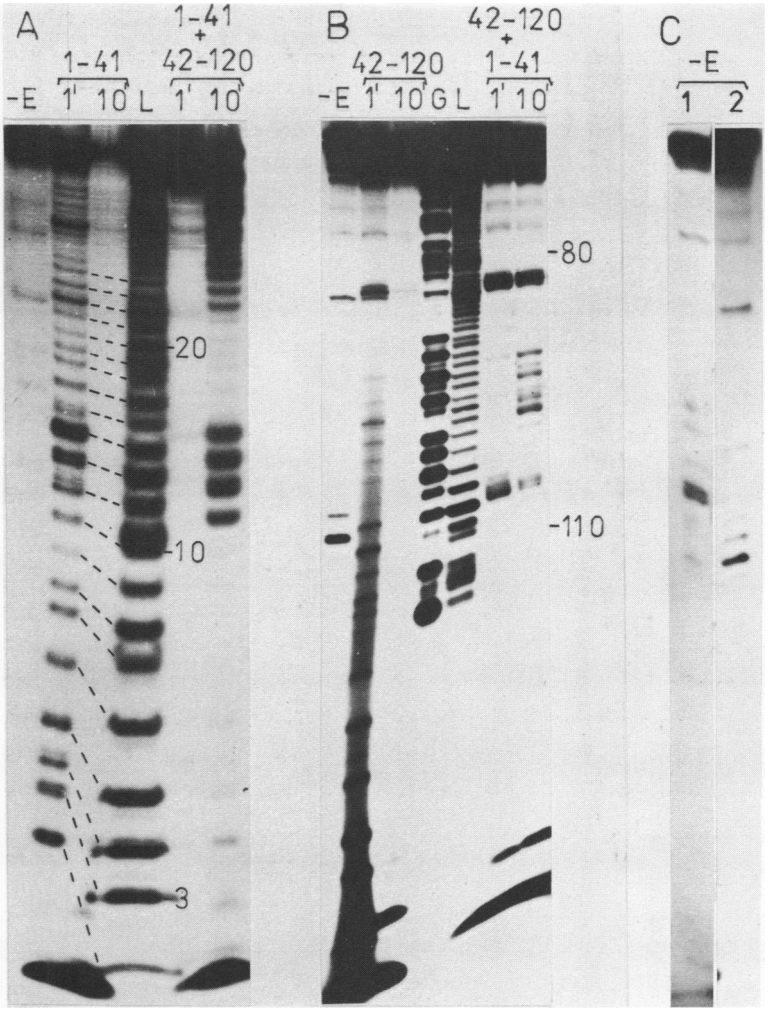

Figure 2. S_1 nuclease digestion kinetics of 5' end-labelled fragment 1-41 (A), 3' end-labelled fragment 42-120 (B) and the corresponding regions in reassociated 5S RNA at 60°C.

Each digestion was carried out with 1 U S_1 nuclease in 12 µl of buffer containing the following amounts of fragment(s): A - 1-41 (0.3 µg) and 1-41 + 42-120 (0.3 + 0.6 µg); B - 42-120 (1.0 µg) and 42-120 + 1-41 (1.0 + 0.5 µg); C - 5' and 3' end-labelled reassociated 5S RNA controls (-E1,2). At indicated times an aliquot (6 µl) was withdrawn and digestion was stopped by adding of 6 µl of sample buffer. For the other details see Materials and Methods. The assignment of S_1 nuclease products is shown by the dashed lines.

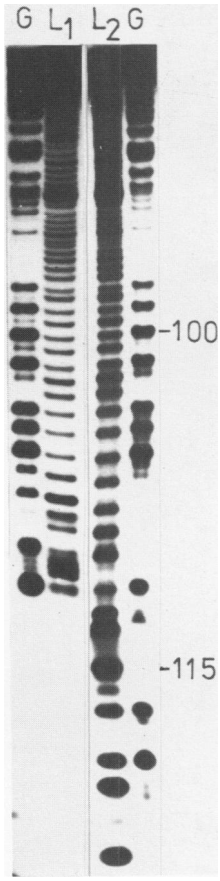

Figure 3. Comparison of the ladders of 3' end-labelled 5S RNA fragment 42-120 in different electrophoresis conditions:
L_1 - 10 mA (2.0 kV one gel);
L_2 - 20 mA (2.0 kV two gels, prepared between three glass-plates).

completely resolved by running the gels at higher temperatures (line L_2). Consequently, sequencing of 5S RNA from opposite directions, using 5' and 3' end-labelled samples shows in both cases the different compressed regions, which together indicate the existence of hairpin structure covering positions 81-94.

Reconstitution of 5S RNA from its fragments and binding of proteins L18 and L25 detected by electrophoresis in nondenaturing conditions

Six 5S RNA fragments: 1-37(38), 1-41, 42-120, 48-88(89), 48-119(120) and 90-119(120) (the last was slightly cross-contaminated with 1-32 and 48-77) obtained in partial hydrolysis conditions by means of S_1 nuclease and T_1 RNase (see Materials and

Methods) were used in reconstitution experiments. All these fragments and possible reconstituted molecules were also tested to bind ribosomal proteins L18 and L25. As an illustration of these results some of 5S RNA fragments, their reassociates and RNP complexes are presented in Fig. 4. Almost all 5S RNA fragments appeared in the nondenaturated gel as single bands (lines 1, 2, 3 and 6, the faster migrating band) having the mobility in agreement with their chain length. It was possible to reconstitute 5S RNA from its three fragments 1-37(38), 48-77 or 48-88(89) and 90-119(120) lines 4 and 5). Moreover, the complexes between fragments 1-41 and 90-119(120) and between 48-88(89) and 90-119(120)

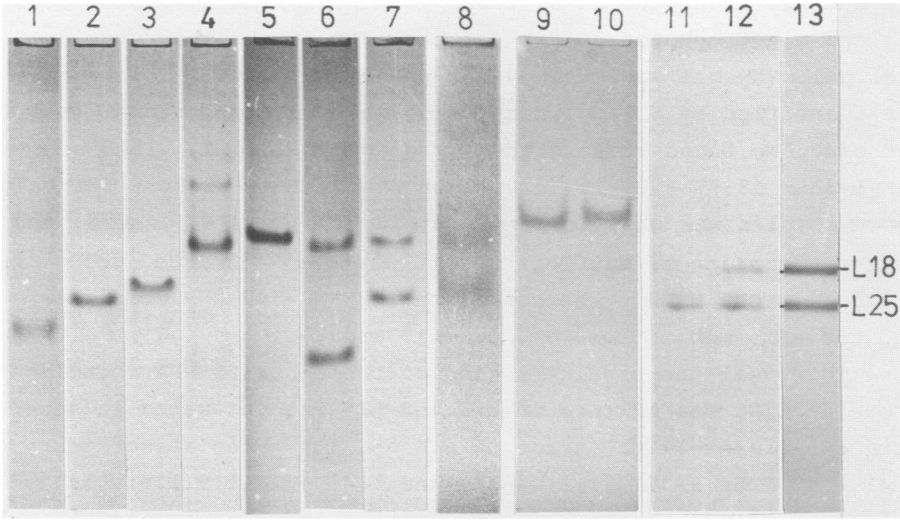

Figure 4. Electrophoresis of the 5S RNA fragments (1-3,6), its associates (4,5,7), complexes with proteins L18 and L25 (8-10, staining for protein) and proteins L18 and L25 (11-13).
1 - 1-41; 2 - 48-119(120); 3 - 42-120; 4 - 1-37(38) + 48-77 + 90-119(120) also small amounts of aggregates were present; 5 - 1-37(38) + 48-88(89) + 90-119(120); 6 - the faster migrating band - 90-119(120), the slower migrating band - 90-119(120) + 1-32 + 48-77; 7 - the faster migrating band - 90 -119(120) + 48-88(90), the slower migrating band - 90-119(120) + 48-88(89) + 1-32; 8 - the complexes of L25 with the same associates as in 7; 9 - 1-37(38) + 42-120-protein complex; 10 - 1-41 + 42-120-protein complex; 11 - identified protein from 9; 12 - identified proteins from 10; 13 - proteins isolated by affinity chromatography and identified as in [16].
For details of electrophoresis and complex formation see Materials and Methods.

(line 7, the faster migrating band) were observed. No complex formation between fragments 1-41 and 48-88(89) was found.

The affinity chromatography technique showed before that protein L25 is able to bind to immobilized 5S RNA fragment 42-120 [32]. In the present study the fragment 42-120-L25 complex was also proved electrophoretically. A remarkable result of Douthwaite et al. [26] shows that after 5S RNA-L25 complex digestion L25 remained bound to the RNA fragment containing nucleotides 69-87 and 90-110. In this paper we made an attempt to narrow down the binding region of this protein. For this reason fragments 90-119(120) and 48-89(90) individually and their complex in separate experiments were mixed with equimolar amounts of proteins L18 and L25 incubated at 37°C for 40 min, followed by slow cooling and electrophoresed in adjacent slots of a polyacrylamide slab gel. The gel was stained for protein and stained protein was identified by SDS polyacrylamide gel electrophoresis (see Methods). We found that neither fragment 90-119(120) (and complex 1-32 + 48-77 + 90-119(120)) nor fragment 48-89(90), but only their complex was able to bind protein L25 (Fig. 4, line 8, the faster migrating band). This result was also confirmed using individual protein L25. The corresponding RNA and RNP complexes had the same mobilities (cf. lines 7 and 8).

To reveal the binding region of the other 5S RNA "binding protein" L18, the mixture of L18 and L25 was incubated in separate experiments with intact 5S RNA and its three associates: 1-41 + 42-120; 1-37(38) + 42-120 and 1-41 + 48-119(120). As detected by gel electrphoresis only intact and reassociated 5S RNA (Fig. 4, RNP - line 10, proteins - line 12) was capable of binding protein L18. At the same time the associates, which contain excision either at 38(39)-41 (RNP - line 9) or at 42-47 bind merely L25 (line 11).

Digestion of 5S RNA-L18 and L25 complex with cobra venom RNase

End-labelled 5S RNA was complexed with ribosomal proteins L18 and L25 and the resulting RNP complex was digested using cobra venom RNase (see Methods). Simultaneously, the digestion of end-labelled free 5S RNA was performed under the same conditions. Both 5S RNA and 5S RNA-L18-L25 complex gave exactly the

same digestion patterns, without any observed protection by proteins L18 and L25 (data not shown). Cleavage at all previously established positions [13] occured simultaneously and at different rates (from comparison of the 5' end- and 3' end-labelled 5S RNA digestion patterns). We note that by this RNase a cut at positions 65 (see ref. [13]) instead of right positions 63 and 64 was due to misreading of an autoradiograph.

DISCUSSION

About possible conformational rigidity of fragments 1-41 and 42-120

In the present study it was unexpected to find the phenomenon that S_1 nuclease cleaves at 0°C 5S RNA fragments 1-41 and 42-120 at the same positions as the corresponding regions in the intact and reassociated molecules.

Recent studies [1,4,43] allow to provide a possible interpretation to this phenomenon. First, Boyle et al. [1] have shown by NMR study that three $tRNA^{Phe}$ fragments (1-16, 1-36 and 1-45), plus the intact molecule all have correct folding, without no *de novo* generated structures in the fragment molecules, which in turn allow to make a conclusion about sequential folding of tRNA. In subsequent studies [4,43] the structure of different $tRNA^{Phe}$ fragments was probed with S_1 nuclease and base-specific RNases. It can be seen from the gel autoradiographs in Fig. 1-3 (see ref. [43]) that sequences C_2-U_7, C_{11}-U_{12}, C_{27}-C_{28}, m^5C_{40}-C_{43} in different fragment molecules exhibit considerably lower accessibility toward S_1 nuclease than it can be anticipated from fragment structures.

One should also consider the size of S_1 nuclease (Mw 32.000) that restricts the extent to which the enzyme is able to act in different regions of an RNA [5].

Nevertheless, apparent similarity in the folding of certain free fragments of 5S RNA with what they possess in the intact molecule agrees with the idea of sequential folding of an RNA during its biosynthesis [1].

Recent studies by Magarill et al. [33] on homopolyribonucleotides by using cobra venom RNase have revealed the correlation

between the hydrolysis parameters (rate, length of the linear parts of the kinetic curves) and the C 3' endo conformation of the nucleotides in the substrate. The identity of the major cleavage sites introduced by this RNase at the 3', 5' stem region of 5S RNA and the corresponding single-stranded regions (110-120 and 1-12) of the fragments suggests that these cuts may correspond to the nucleotides with the C 3' endo conformation. In contrast to S_1 nuclease, cobra venom RNase shows some difference in minor cleavage between the fragments and intact molecule (see above) and therefore can be used as a probe for subtle conformational changes.

Secondary structure and possible role of nucleotides 24-35/47-59 in the structural arrangement of 5S RNA

The results presented here together with our previously published data [13] allow us to draw a secondary structure model of E.coli 5S RNA shown in Fig. 5. Among the large number of proposed 5S RNA secondary structures it seems that Fox and Woese's model [34] is a very good approximation and all prokaryotic 5S RNAs, sequenced up to date can be arranged according to this form. On

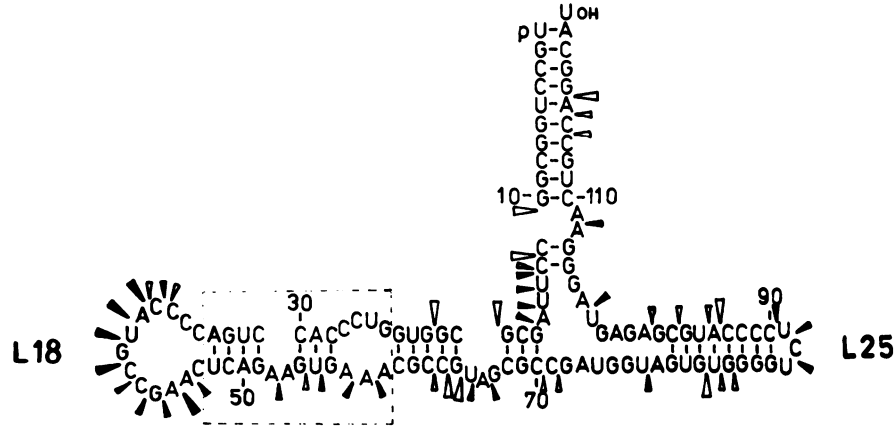

Figure 5. Secondary structure model of F.coli 5S RNA consistent with S_1 nuclease (black arrows) and cobra venom RNase (light arrows) cleavage sites [13]. A region surrounded by a dashed line is probably involved in tertiary folding of the molecule. Proteins L18 and L25 were placed near their possible binding sites (for explanation see the text).

the basis of our nuclease digestion data we found that tuned helix (nucleotides 18-23/60-65) in Fox and Woese's model can be enlarged in a manner as shown in Fig. 5. An enlarged helix, covering positions 16-23 and 60-70 is about twice more energetically favoured (according to Tinoco rules [35]). Also this arrangement is possible for almost all prokaryotic 5S RNAs on the level of the compensatory sequences.

Recently Farber and Cantor reported that nearly 20% of certain E.coli 5S RNA nucleotides were accessible to slow tritium exchange [38]. These results are in agreement with the nuclease cleavage data presented here with an exception of A U_{40}, which is highly accessible to S_1 nuclease. Therefore, we do not support their long-range interactions involving C_{37}-U_{40}.

To gain further insight into the folding of 5S RNA, its structure was mapped by using S_1 nuclease at 40°C and 60°C, i.e. in conditions where tertiary structure probably melts out [36,37]. The results obtained show that at 40°C a new region (24-35 and 47-59) (Fig. 5) was rendered accessible to S_1 nuclease. In addition to this easily accessible region the other region, nucleotides 98-108, was slightly cleaved at 60°C. We believe that the region covering nucleotides 24-35/47-59 plays a crucial role in the formation of 5S RNA tertiary structure, because this region is the first to melt out. This conclusion is supported by the fact that no association between fragments 1-41 and 48-88 was found.

Similar structural features of 5S RNA and tRNA

Some of the results of the present study are rather similar to those obtained by others [4,24,43] for tRNA. Firstly, at electrophoresis conditions 10 mA (2.0 kV) the ladders (only in the case of 3' end-labelled 5S RNA) did not resolve the last nine oligonucleotides near the 3' end. This compression is possibly caused by strong stacking interactions between the 3' end nucleotides, since it is eliminated by running the gels at higher temperatures (see Fig. 3). Similar unusual behaviour of five last oligonucleotides also near the 3' end of $tRNA^{Phe}$ and $tRNA^{Val}$ was observed by Favorova et al. [24], and they think that it seems to be a general property of all tRNAs. Secondly, the 3' side of the amino acid acceptor stem of $tRNA^{Phe}$ and $tRNA^{Val}$ [24] and the

3' side of the 5S RNA stem, nucleotides 113-115 (present and previous studies [13]) are the most susceptible regions of these RNAs to cobra venom RNase. The region most accessible to S_1 nuclease locates around position 38 in both tRNA [6] and 5S RNA [13]. Finally the fragments of tRNA and 5S RNA possibly bear similar features with respect to accessibility to S_1 nuclease (see above). In summary these three features found between tRNA and 5S RNA show their structural similarity.

Binding sites of proteins L18 and L25

A criterion that the presence of a protein binding site in a given RNA fragment can only be ascertained with confidence by confirming its ability to reassociate specifically with the protein [39] was taken as the basis in studying the 5S RNA-protein complexes. Since 5S RNA containing excision either in positions 38(39)-41 or in positions 42-47 did not bind protein L18 it is evident that the loop nucleotides around position 40 are essential for its binding. Douthwaite et al. [26] have earlier demonstrated that in 5S RNA the sequences in the loop regions (35-42 and 87-88) are excised prior the release of L18 and therefore, it is concluded that L18 may have a fairly extended binding site. In contradiction to their conclusion we found that possibly only the nucleotides around position 40 are involved in the interaction with L18. It must be kept in mind that the method of polyacrylamide gel electrophoresis used in both studies allows the detection of RNP complexes which dissociation constant, $K_d \leq$ [RNA] or [protein]. Since the RNA and protein concentrations used in the present study were in the order of 10^{-6} M the possible 5S RNA-protein complexes having $K_d > 10^{-6}$ M cannot be detected. In contrary to the above mentioned results Zimmermann & Erdmann [42] have shown that primary binding site for protein L18 locates in positions 58-100.

Douthwaite et al. [26] have suggested that the binding site of protein L25 on the 5S RNA should be located in the region in positions 69-110. Our results show that after restoration of a helical stem in this region (see Fig. 5) binding of protein L25 was recovered. Thus, it is very likely that the recognitation of protein L25 by 5S RNA occurs through its double-helix containing nucleotides 79-97. In summary, it follows that proteins L18 and

L25 occupy distant regions of 5S RNA, which is consistent with the findings of Fanning and Traut [41].

In the present study special attention was paid to the experiments where the digestion of 5S RNA-L18 and L25 complex by using cobra venom RNase was performed. Recently, Favorova et al. [24] have successfully used the same RNase for studying the tRNA-synthetase complexes. Despite the similarities in several parameters (K_d, concentrations of RNA and protein, and complexes that can be electrophoresed) of the both above mentioned complexes in 5S RNA-L18 and L25 complex proteins L18 and L25 did not afford the protection of any 5S RNA regions accessible to cobra venom RNase, at the same time in the tRNA-synthetase complexes nearly 100% shielding by synthetase in the anticodon stem of both tRNAs studied was observed (see ref. [24]). In conclusion, probably the nature of the interactions of the components of these two complexes are rather different.

We found that cobra venom RNase can be used as a probe for changes in 5S RNA double-helical regions. From comparison of digestion patterns of free 5S RNA and 5S RNA in complex with proteins L18 and L25 we conclude that after the complex formation 5S RNA did not undergo any major conformational changes detectable by cobra venom RNase. Therefore, we believe that conformational changes induced by protein L18 [8,37,40] can be restricted to the single-stranded 5S RNA regions and consistent with the present study, possibly to a region around position 40.

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