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Long range RNA-RNA interactions in the 30 S ribosomal subunit of *E. coli*

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ABSTRACT

We have attempted to identify long-range interactions in the tertiary structure of RNA in the E. coli 30 S ribosome. Native subunits were cleaved with ribonuclease and separated into nucleoprotein fragments which were deproteinized and fractionated into multi-oligonucleotide complexes under conditions intended to preserve RNA-RNA interactions. The final products were denatured by urea and heat and their constituent oligonucleotides resolved and sequenced. Many complexes contained complementary sequences known to be bound together in the RNA secondary structure, attesting to the validity of the technique. Other co-migrating oligonucleotides, not joined in the secondary structure, contained mutually complementary sequences in locations that allow base-pairing interaction without disrupting pre-existing secondary structure. In seven instances the complementary relationship was found to have been preserved during phylogenetic diversification.

INTRODUCTION

One approach to the study of ribosomal structure is through the production and analysis of ribosome fragments. We have used this approach to investigate the 30 S subunit of E. coli ribosomes (1, 2). Intact native subunits were subjected to mild ribonuclease digestion and warmed under controlled conditions, and came apart into fragments whose protein and RNA components were identified. Each fragment proved to be not a random assortment of RNA and protein, but a well-defined region of the ribosome, showing that native ribosomal structure had survived the treatment (1, 2).

We have now extended and refined this technique and have used it in an attempt to isolate smaller fragments consisting of RNA segments still bound together by bonds that existed in the parent ribosome. The aim is to identify long-range RNA-RNA interactions that participate in forming the RNA tertiary structure. We have found a number of cases that may fit into this category: pairs of RNA segments that behave electrophoretically as if associated with each other and contain mutually complementary nucleotide sequences that could interact by base pairing. In seven cases the complementarity has been found

to have been preserved during phylogenetic diversification.

MATERIALS AND METHODS

Fragmentation of 30 S ribosomal subunits

30 S ribosomal subunits were prepared from E. coli MRE 600, subjected to limited digestion with insoluble matrix-bound ribonuclease A, and heated at 57°C for 10 min. The resulting ribonucleoprotein fragments were separated by zonal centrifugation in a convex 9-20% sucrose gradient in a Beckman B-15 rotor (see Results). All operations were carried out in buffer MTK 1-10-20 (1 mM magnesium acetate, 10 mM Tris acetate (pH 7.8), 20 mM potassium acetate). The experimental details were as described previously (1) except that the time of heating at 57° was reduced to 10 min.

Processing of the fragments

Sucrose gradient fractions and their RNA moieties were examined by analytical polyacrylamide gel electrophoresis (1) and were pooled according to the results. Three fragment preparations, denoted A, D and E, were concentrated and freed of sucrose by pressure filtration (Amicon, filter PM-10), dissolved in a small volume of buffer MTK 1-10-20, and kept at -70°. Proteins were extracted and identified by two-dimensional gel electrophoresis (1), and the protein-free RNA moieties were taken for further processing.

Fractionation and sequencing of the RNA moieties

Nucleoprotein fragments were deproteinized in the presence of Mg^{++} (MTK 1-10-20) by three treatments with sodium dodecyl sulfate and phenol (12). The resulting RNA was fractionated by three successive polyacrylamide gel electrophoretic runs: the first in the presence of Mg^{++} and the following two after Mg^{++} ions had been removed to promote partial dissociation of the RNA complexes.

The first run, with Mg^{++}, was conducted in a cold room in a 5% polyacrylamide horizontal slab gel in buffer MTK 1-10-20, and the resulting RNA bands were located by UV shadowing (1). Each RNA band was extracted with MTK 1-10-20, precipitated with three volumes of MTK-1-10-20 in ethanol, and stored at -70° in the ethanolic buffer until taken for sequencing. It was then labelled at the 5' end with (γ-^{32}P)ATP (Amersham) and polynucleotide kinase (P-L Biochemicals) in a medium containing 100 mM potassium acetate, 50 mM Tris acetate (pH 8), 10 mM magnesium acetate, 1 mM EDTA, 6 mM mercaptoethanol and 0.1 mM spermidine. After 1 hr at 37° the mixture was made 0.5 M in ammonium acetate and was chilled in ice to stop phosphorylation. Ten µg of carrier tRNA were added, followed immediately by 3 volumes of cold ethanol. After 2 hrs at -70°, the RNA was pelleted and washed twice with ethanol at room temperature.

In order to ensure that Mg^{++} ions would be replaced by monovalent cations, the RNA was dissolved in 0.3 ml of 0.5 M ammonium acetate and 1 mM EDTA, precipitated and washed with ethanol as before, and again dissolved, precipitated and washed in the same way.

The Mg^{++}-depleted RNA was briefly dried under vacuum to remove residual ethanol and was dissolved in 20 µl of 50 mM ammonium acetate and 5 mM EDTA. After 4-5 hours at room temperature, the sample was made 15% in sucrose and 0.25% in xylene cyanol, cooled for an hour in a cold room, and loaded on a 12% polyacrylamide gel 40 cm long and 0.5 mm thick. The running buffer was 50 mM Tris borate (pH 8.3) and 1 mM EDTA (3). Electrophoresis was carried out overnight in the cold room at 600 volts or less (to prevent heating of the gel) until the dye migrated about 16-18 cm. RNA bands were located by radioautography, cut out, and extracted with 0.3 ml of 0.5 M ammonium acetate and 1 mM EDTA. Extraction was usually overnight at room temperature, but shorter times or lower temperatures were also effective. Each RNA band was precipitated with 30 µg of carrier tRNA and 3 vols. of ethanol at -70^{o} for at least 2 hrs. It was then washed, dried, dissolved in running buffer, and prepared, as for the preceding gel, for the second no-magnesium electrophoresis in a 12% (slow bands) or 20% (fast bands) polyacrylamide gel. The electrophoresis conditions were those of the preceding gel except that different combinations of voltage and time were used for samples of different mobility so as to bring them all approximately to the middle of the gel. The RNA bands were located, extracted and precipitated as before.

Finally, each RNA band was denatured and its component oligonucleotides were separated and sequenced. To accomplish this, the band was dissolved in 10 µl of 8 M urea, 5 mM Tris borate (pH 8.3) and 0.1 mM EDTA. It was left at room temprature overnight, heated 4 min at 55^{o}, and run at 1800 volts in a hot 20% gel. Each oligonucleotide band was extracted, precipitated and washed as before. Its radioactivity was measured, and it was dissolved in a small volume of water and sequenced in a 12% or 20% gel run at 1800 volts, as described previously (1).

Treatment of data

Data on the identity of co-migrating oligonucleotides were obtained from the present experiments and also from an earlier set of similar experiments. The sequences of co-migrating oligonucleotides were scanned, usually with a computer (4), for mutual complementarity, counting A-U, G-C and G-U as complementary base pairs. Information on the accessibility of nucleotides to enzymes or modifying reagents in the intact subunit was taken from the litera-

ture (5,6,7) and our own unpublished data on sites of cleavage by matrix-bound RNase A (8). Searches for the evolutionary conservation of complementarity relationships made use of a comparative compilation of the secondary structures of small subunit RNAs from 14 sources: 3 eubacteria, 1 archaebacterium, 3 kinds of chloroplast, 1 mitochondrion and 6 eukaryotes (9). Interpretation of E. coli data is based on the published sequence and secondary structure models (5, 10,11). The notation a-b/c-d indicates that the RNA segment that starts at nucleotide number a and ends at position b migrated with the segment c-d as a single electrophoretic band. When the segments are very short (of the order of 3-10 nucleotides) the notation signifies that they are complementary to each other (10).

RESULTS

Ribosome fragments

The first step in our procedure is to break the ribosomal subunit into nucleoprotein fragments after partial nucleolytic cleavage of the RNA in the intact subunit (1,2,12). After cleavage, the subunit is still held together by non-covalent interactions among its macromolecular components, and a mild heat treatment is applied to make it come apart into fragments, each held together by non-covalent interactions that survived the heating. At the heating temperature and in the buffer employed, the production of fragments and their further dissociation are time-dependent (12). In the present work we were interested in preserving not only the strongest RNA-RNA interactions but weaker ones as well. We therefore reduced the heating time to 10 min, in contrast to the times of 30 and 60 min used in earlier experiments (1,2).

The effect of different heating times is shown in Fig. 1 (sucrose gradient centrifugation patterns of heated ribosome digests) and Fig. 2 (gel electrophoresis patterns of the digests and the RNA extracted from them). Fragments A, D and E, isolated by sucrose gradient centrifugation (Fig. 1, III), were studied in the present work. Their proteins were identified and their RNA moieties were fractionated and sequenced as described in Materials and Methods.

Fragment D

The RNA moiety of fragment D covered most of domains I and II of the 16 S RNA (Fig. 3), being similar to that of an earlier version of fragment D prepared by 30 min heating (2). Owing to the shorter heating time, the present preparation contained more proteins than the earlier one: S4, S5, S6, S8, S15 and S20, found previously; S12, not present in all of the earlier preparations; and S13 and S16, previously absent.

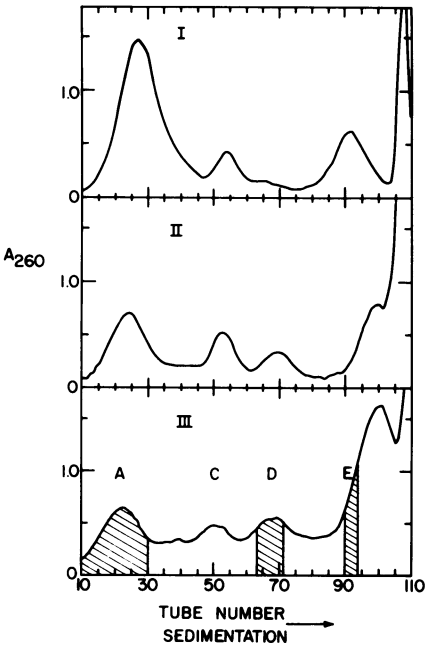

Fig. 1. Effect of heating time on sedimentation pattern of RNase A-digested 30 S ribosomes. Heating time; I, 60 min; II, 30 min; III, 10 min. The patterns are normalized to represent equal amounts of ribosomes. Conditions: convex 9-20% sucrose gradient in MTK 1-10-20; 19 hrs at 30,000 rev/min in a B-15 zonal rotor at 5°.

Fragments A and E

Fragment A, the most slowly sedimenting material released from nuclease-treated subunits in the presence of 1 mM Mg^{++}, had previously been shown to be a mixture of protein-free oligonucleotides (13). In the present investiga-

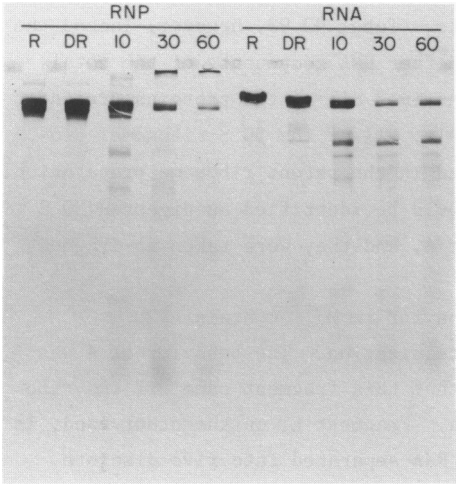

Fig. 2. Effect of heating time on gel electrophoretic pattern of RNase A-digested 30 S ribosomes. RNP, ribonucleoprotein complex; RNA, RNA extracted from RNP; R, intact subunit; DR, digested subunit, not heated; 10, 30, 60, digested subunit heated for 10, 30 or 60 min, respectively. Conditions: 5% polyacrylamide slab gel 2 mm thick; run 2 hrs in a cold room at 10 volts/cm; stained for RNA.

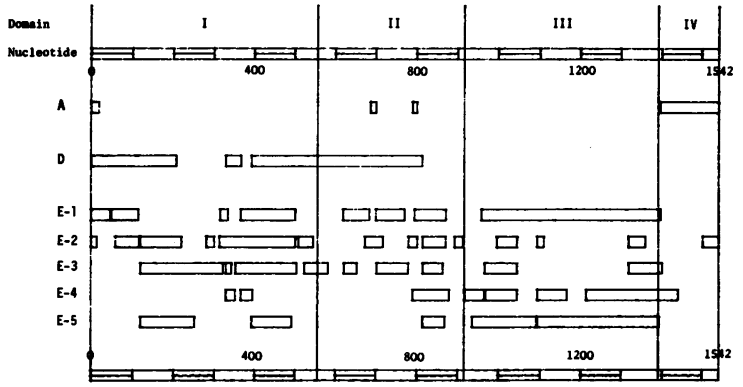

Fig. 3. RNA sequence found in the RNA moieties of fragments A and D and in the five RNA complexes derived from fragment E.

tion preliminary experiments showed these oligonucleotides to come almost entirely from RNA domain IV, the 3' terminal 150 nucleotides. It was therefore of interest to examine the particle from which A had been detached, since this particle would be a 30 S subunit without the 3' end of its RNA. The particle was sought in the sucrose gradient near the location of entire 30 S subunits, namely, in the region of tubes 90-100 (Fig. 1,III). The way in which this was done is illustrated in Fig. 4. Samples from each tube were deproteinized and their RNA components were examined by gel electrophoresis. Tubes 94-98 contained all of the RNA components of the total unfractionated subunit digest, including the rapidly migrating components of fragment A, and were discarded as complete subunit digests. Tubes 90-93, however, lacked the RNA of component A but contained all the other RNA components of the total digest. Furthermore, these fractions contained all of the proteins present in the total unfractionated digest: namely, all of the 30 S ribosomal proteins except S21, which was also not found in the parent ribosome preparation. Thus, sucrose gradient fractions 90-93 could be identified as digested 30 S subunits lacking only the RNA of fragment A, and they were taken as fragment E.

Fig. 5 shows the electrophoretic behavior in Mg^{++}-containing gels of fragments A and E before and after deproteinization. The behavior of A was not affected by this treatment, showing that this fragment came off the ribosome as free RNA and not as nucleoprotein. Fragment E, on the other hand, is seen to be a nucleoprotein complex whose RNA separated into five discrete bands when the proteins were removed.

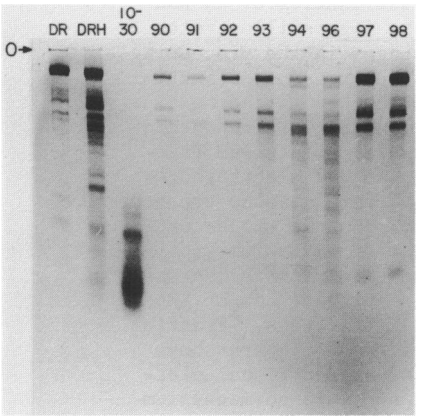

Fig. 4 Gel electrophoretic patterns of RNA extracted from RNase A-digested 30 S ribosomes and nucleoprotein fragments derived from them. DR, total unfractionated digest, not heated; DRH, DR heated for 10 min. The others are sucrose gradient fractions derived from DRH; the numbers refer to the tube numbers of Fig. 1, III. Conditions as for Fig. 2.

The RNA bands of fragment E contained nearly all of RNA domains I, II and III, plus some bits of domain IV that had not yet become detached (Fig. 3). Fragment A contained the 5' end of the 16S RNA (sequence 1-20) and small segments of domain II (sequences 687-701 and 790-801), but the major part of its RNA (71 oligonucleotides out of 82) came from domain IV and covered the entire domain. Thus, fragment E is essentially a 30S subunit minus the 3' end of its RNA.

In a dark field electron microscope study performed in this laboratory by Dr. A.P. Korn, positively stained specimens of fragment E appeared as 30 S subunits lacking one prominent feature: the sharply protruding "collar" which stains as a band of RNA (14). This identifies the collar with RNA domain IV, the 3' end (see Discussion). A full account of the electron microscopic

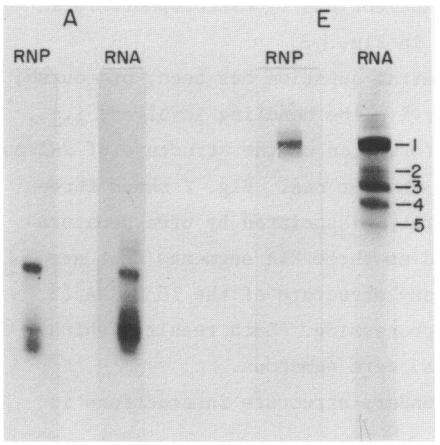

Fig. 5. Gel electrophoretic patterns of fragments A and E before deproteinization (RNP) and after (RNA). Conditions as for Fig. 2.

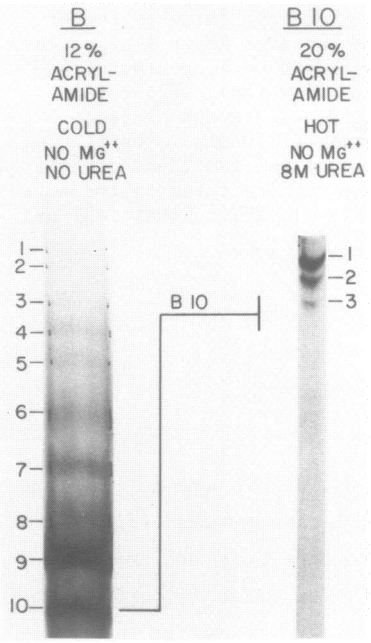

Fig. 6. Fractionation of an RNA complex. The figure illustrates the final stages in the processing of an RNA complex. In the initial stage, as described in the text, a ribosome fragment was deproteinized and its RNA moiety was fractionated by two gel electrophoretic runs, the first in the presence of magnesium ions and the second after removal of magnesium (not shown). The figure shows the subsequent processing of one of the resulting RNA fractions, "Fraction B". The left hand pattern shows the result of an additional electrophoretic run in a no-magnesium gel, which separated B into ten bands. The right hand pattern shows the three oligonucleotides derived from one of the bands, B 10, after it was denatured. The oligonucleotides cover the sequences 996-1045, 1169-1211 and 1318-1359, and are compatible with items 4 and 15 of Table 2.

study will appear elsewhere (15).

Identification of interacting RNA segments

Our procedure for elucidating RNA-RNA interactions is based on the isolation of multi-oligonucleotide complexes which contain the interacting segments. As described above, we begin with a complex that is stabilized by Mg^{++} ions. The complex is destabilized by removing the Mg^{++}, is fractionated into simpler complexes by gel electrophoresis, and is finally denatured with urea and resolved into its component oligonucleotides by electrophoresis in urea-containing gels. An example is given in Fig. 6.

In working out this procedure, the central question has been, of course, whether native RNA-RNA interactions can survive the handling involved; i.e., whether the procedure can provide valid information on the structure of RNA as it is in the ribosome. Figs. 7 and 8 show that it can. Fig. 7 shows three oligonucleotides that migrated together until dissociated by urea denaturation. When sequenced, they were identified as three RNA segments that are bound directly to each other in the secondary structure of the 16 S RNA (5, 10, 11) and remained bound throughout the processing. Such results, which conform with the secondary structure models, were numerous.

A result that is not explained by secondary structure interactions is

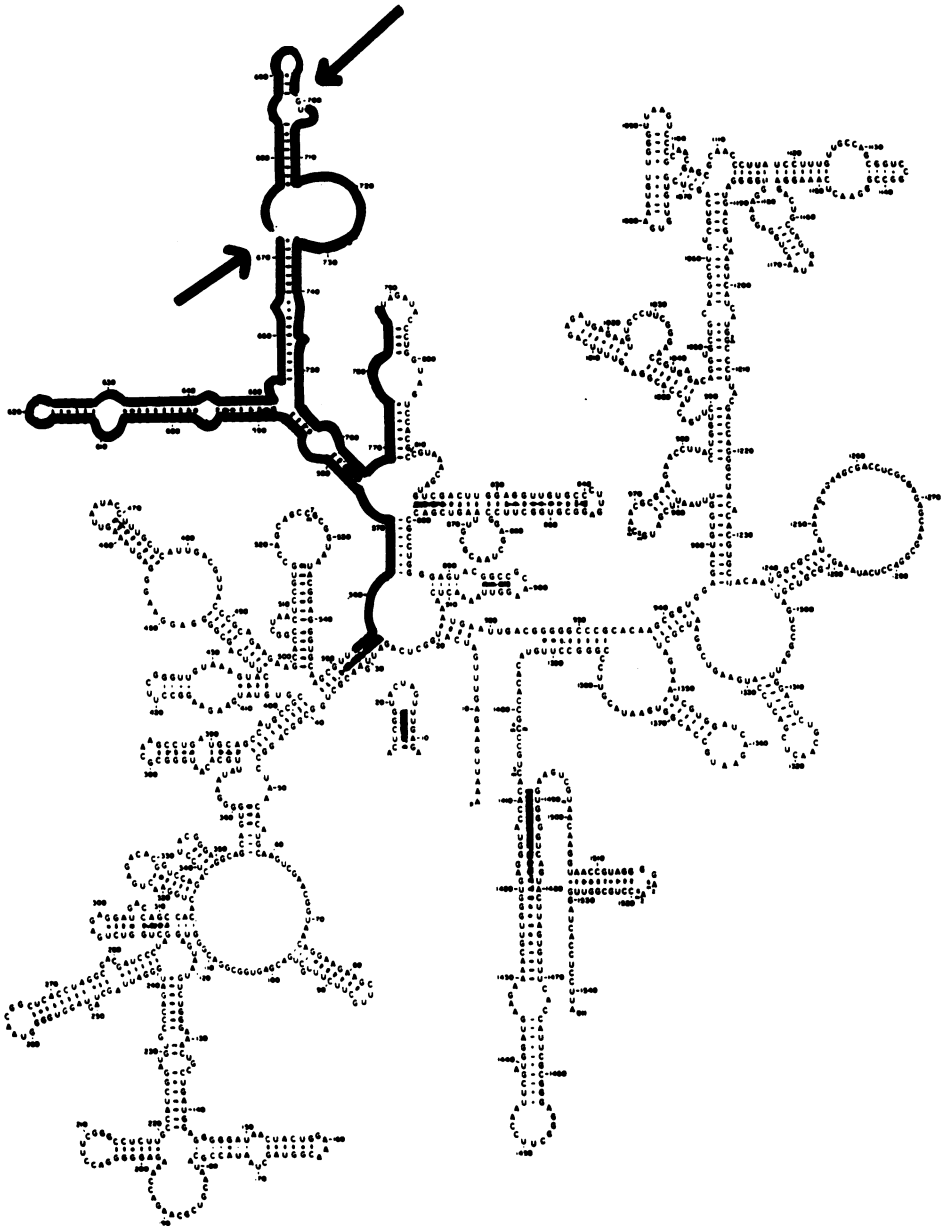

Fig. 7. Secondary structure interactions. Three RNA segments that migrated together in gel electrophoresis until denatured by urea. The arrows show nuclease cleavage sites.

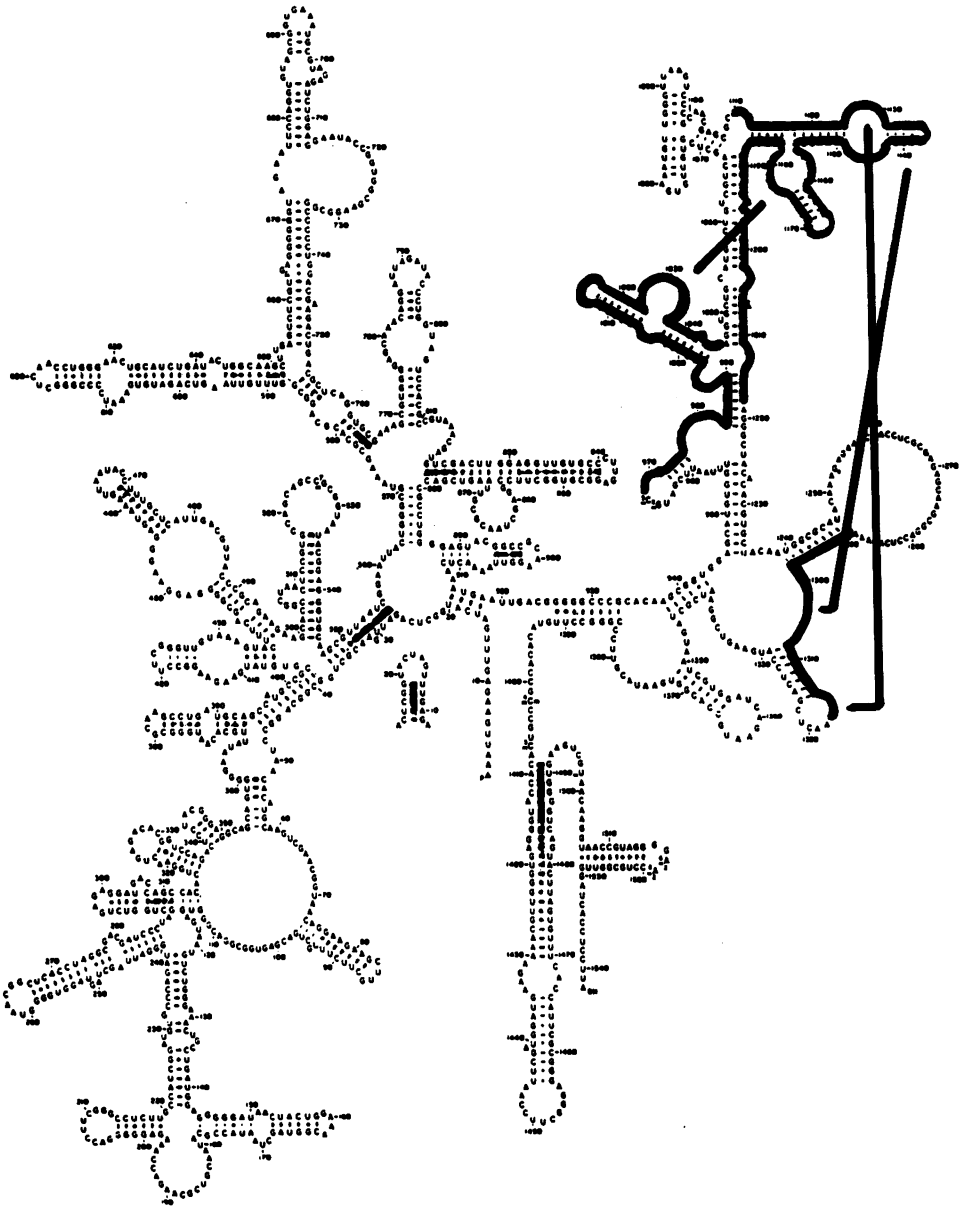

Fig. 8. Possible tertiary interactions. Three RNA segments that migrated together in gel electrophoresis until denatured by urea. The straight lines indicate pairs of mutually complementary sequences that might join the segments together by base-pairing interactions.

Table 1. Secondary structure interactions

Item	Times seen	Co-migrating complementary sequences (a)	Domain (b)
1	1	39-47 / 394-403	I
2	1	52-54 / 357-359	I
3	10	576-580 / 761-765	II
4	11	584-587 / 754-757	II
5	5	588-617 / 623-651	II
6	9	655-659 / 747-751	II
7	7	660-662 / 743-745	II
8	5	666-672 / 734-740	II
9	4	677-684 / 706-713	II
10	2	926-933 / 1384-1391	III
11	2	938-943 / 1340-1345	III
12	2	946-955 / 1225-1235	III
13	1	984-990 / 1215-1221	III
14	2	1046-1067 / 1189-1211	III
15	2	1068-1071 / 1104-1107	III
16	2	1086-1089 / 1096-1099	III
17	6	1113-1117 / 1183-1187	III
18	3	1239-1247 / 1290-1298	III
19	1	1308-1314 / 1323-1329	III
20	3	1350-1356 / 1366-1372	III
21	6	1409-1445 / 1457-1491	IV

a. Contained in oligonucleotides that migrated together until denatured.
b. Defined in Fig. 3.

shown in Fig. 8. These three co-migrating oligonucleotides are not attached to each other in the secondary structure, but they were found to contain mutually complementary sequences that might bind them together by three long-range RNA-RNA interactions. That these three interactions may actually take place is strongly supported by evolutionary considerations: equivalent complementarities are found in each of 14 different small ribosomal subunit RNAs (9) despite evolutionary changes in nucleotide sequence.

Results such as those of Fig. 7 and 8 show that our procedure is capable of providing valid information on the structure of ribosomal RNA *in situ*.

Our findings are summarized in Tables 1 and 2, which list not only the results of the experiment described in this communication but also those obtained in several earlier experiments. Table 1 shows interactions that are part of the secondary structure (5, 10, 11). These results are confirmatory and their main value to us is that they demonstrate the validity of our experimental approach.

Table 2 lists combinations that are not imposed by the secondary structure and may therefore represent long-range RNA-RNA interactions in the tertiary structure. This is not the total list of such combinations that we

Table 2. Possible tertiary interactions

Item	Times seen	Co-migrating segments (a)	Complementary sequences (b)	Domains (c)	Notes
1	1	50-114 / 802-862	61-69 / 810-820	I-II	d,e
2A	6	329-463 / 673-813	417-429 / 722-732	I-II	d,f
2B	8	366-434 / 1318-1359	420-428 / 1330-1339	I-III	d,g,h
3	7	790-899 / 1360-1404	858-864 / 1376-1383	II-III	d
4	3	968-1045 / 1169-1218	1025-1031 / 1178-1184	III	d
5	7	1092-1190 / 1287-1400	1124-1130 / 1315-1320	III	d
6	"	"	1139-1144 / 1301-1307	III	d
7	2	313-365 / 408-440	322-326 / 420-424	I	
8	2	430-508 / 621-653	479-482 / 619-622	I-II	
9	3	435-501 / 802-870	462-465 / 801-804	I-II	i
10	1	408-440 / 1502-1542	414-417 / 1538-1541	I-IV	i
11	2	621-672 / 802-899	618-622 / 842-846	II-III	
12	6	789-879 / 1091-1161	788-791 / 1090-1093	II-III	
13	4	620-662 / 1405-1495	651-655 / 1451-1455	II-IV	h
14	4	937-967 / 1092-1169) / 1250-1317) ←	942-945 / 1264-1267) 1158-1161 / 1276-1279)	III	
15	4	991-1045 / 1318-1359	991-995 / 1334-1338	III	
16	6	1227-1298 / 1318-1404	1285-1288 / 1390-1393	III	j
17	1	998-1045 / 1441-1495	1033-1036 / 1448-1451	III-IV	i

a. Migrated together until denatured.
b. See text for explanation.
c. Defined in Fig. 3.
d. Complementary relationship conserved during phylogenetic diversification; see Discussion.
e. Both segments are in the S4 RNA (31).
f. Interaction may be stabilized by protein S20 (32,33).
g. Interaction may be stabilized by proteins S4 and S12 (30,31,34).
h. Psoralen crosslink, located by sequencing (50).
i. Psoralen crosslink, located by electron microscopy (51,52).
j. Protein S7 was crosslinked to both segments (32).

have seen, but includes those that have turned up repeatedly plus certain others which we have seen only once but whose existence is supported by other evidence: e.g., data from other laboratories, phylogenetic conservation, etc.

As is discussed in greater detail below, we feel that stable secondary structures are likely to remain intact when tertiary structure is formed. This would require that tertiary base pairing should be between sequences that are not located in secondary structural helices of high stability. All of the entries in Table 2 can fulfill this condition; they all have mutually complementary sequences that are centered on single-stranded loops or bulges or are in regions of secondary structure that is unstable because the row of base pairs is short or is interrupted. Only one pair of complementary sequences is shown for each entry, but most of them have several.

So far, we have found seven instances of phylogenetic conservation (the

first items in Table 2). We have examined the sequences and secondary structures of 14 different small subunit RNAs (9) and find, in these seven cases, that equivalent tertiary base-pairing interactions are possible in all of them.

DISCUSSION

The elucidation of the three-dimensional structure of the ribosome is a project that has occupied many research groups for many years and will continue to do so for years to come. The complexity of the problem is such that no single approach can provide more than a partial solution. Even x-ray crystallography, the ultimate technique for structure determination, will require extensive input from other techniques for interpretation of its data. Consequently, it is important to apply to this problem as many methods as can be devised. It is also important that, in the end, the structural knowledge obtained should relate to the integrated structure of the whole ribosome. Although studies of isolated ribosomal components are essential, there is no assurance that an isolated protein or RNA molecule has the same structure as in the ribosome, where it interacts with the various macromolecules and cations that make up the biological complex.

There is evidence that ribosomal proteins affect the RNA structure (16,17) and are required for the formation of its compact tertiary structure in the ribosome (17,18). Treatments such as unfolding or deproteinizing the ribosome cause large changes in shape and size, as measured by hydrodynamic methods (19). However, such massive changes are often accompanied by surprisingly small structural changes of the kind detected by, e.g., hypochromicity measurements (20-22). This can best be interpreted by distinguishing between short-range and long-range RNA-RNA interactions (23). Short-range interactions occur between adjacent complementary sequences in the RNA chain; they are intrinsically stable and if disrupted, re-form spontaneously. Since most of the secondary structure of ribosomal RNA is of this durable type, much fundamental knowledge of RNA secondary structure in the ribosome has come from experiments performed not only on intact ribosomes but also on purified RNA or ribosomes in various degrees of disarray (5,10,11). In contrast, the tertiary structure depends entirely on long range interactions, which are easily disrupted and not easily restored. In this case, therefore, it is necessary to study the intact ribosome.

This consideration has influenced our approach to the search for tertiary interactions. Our method involves the nucleolytic cleavage of the RNA chain followed by its stepwise dissociation into progressively simpler com-

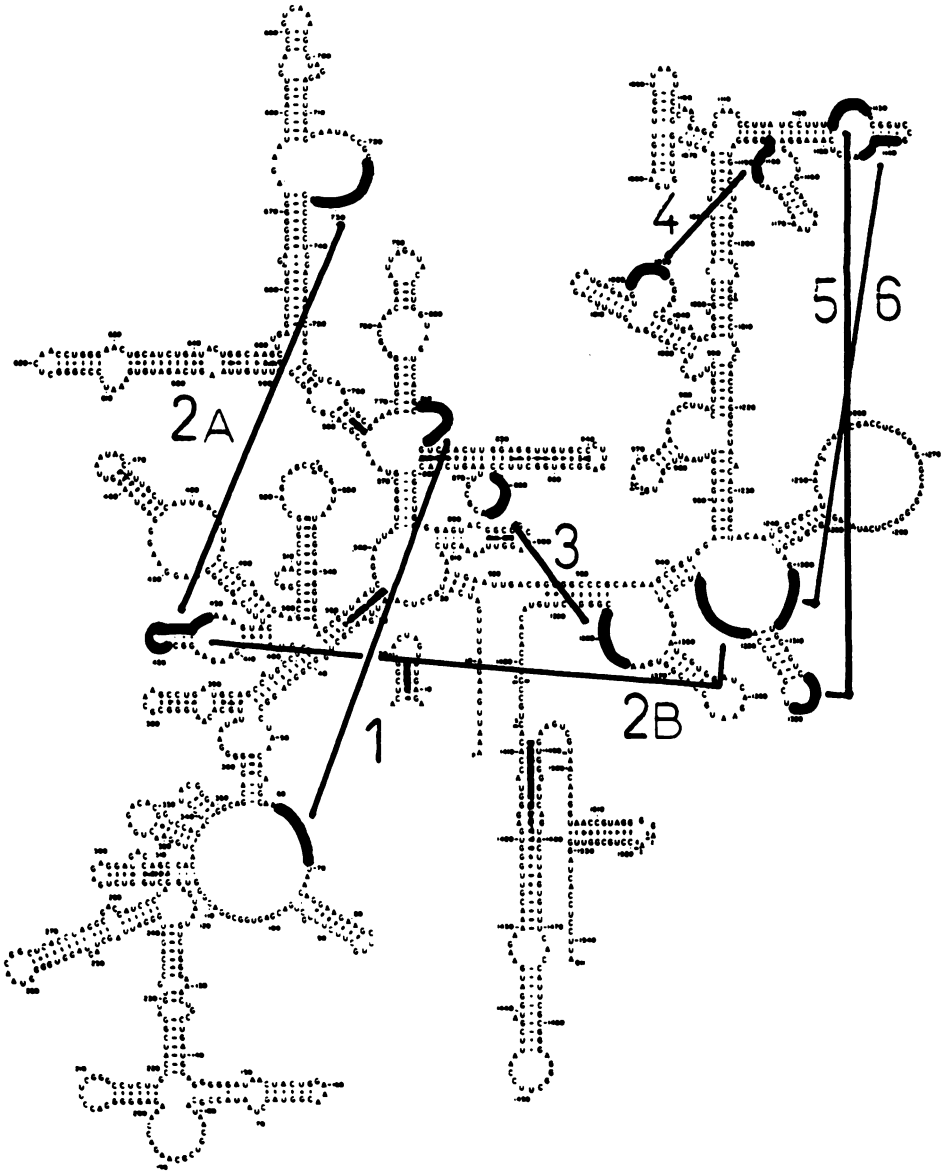

Fig. 9. Possible tertiary base-pairing interactions that are conserved phylogenetically.

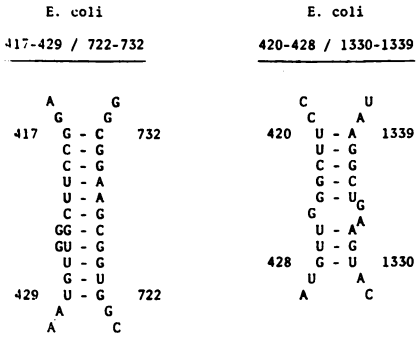

Fig. 10. Possible tertiary interactions. Two base-pairing interactions involving the sequence 420-428. Both are conserved phylogenetically (see text).

plexes of interacting oligonucleotides. The intention is to preserve and identify RNA-RNA interactions that exist in the native ribosome. For this reason the nuclease is applied to intact 30 S subunits in a stabilizing medium, so that the cuts are made in the RNA chain while it is held in its normal in situ tertiary conformation by the ribosomal proteins and cations. We consider this point to be critical.

Once the RNA chain has been converted in situ into a complex of oligonucleotides, the ribosome is separated into nucleoprotein fragments and the RNA in each is dissociated into smaller, more manageable complexes by stepwise destablization of the structure: first, by removing the proteins, and second, by replacing Mg^{++} ions with monovalent cations. Both types of cation stabilize nucleic acid interactions by reducing phosphate-phosphate electrostatic repulsion, but bivalent Mg^{++} is more effective than monovalent cations (discussed in ref. 24) and its replacement causes further dissociation. After each step the RNA complexes are separated by gel electrophoresis. The denaturing factors of heat and low ionic strength are avoided, and the end products of this series of separations are a number of gel electrophoretic bands, many of which contain two, three or more oligonucleotides that remained together. After denaturation by heat and urea, these are separated and sequenced. The strategy of cleavage and separation is similar to that used by Vigne and Jordan (25) with 5 S RNA and successfully employed by Brimacombe and his colleagues in deciphering the secondary structure of E. coli 16 S and 23 S rRNAs (11,26-28). The major differences from our procedure were that the separation and denaturation were carried out in a single two-dimensional gel and, perhaps more significant, that the starting material was usually either purified RNA or ribosomes partly denatured by 2 M urea.

Our object in this investigation is to identify tertiary RNA-RNA inter-

actions in the ribosome. That the technique used can give valid results is shown by the fact that it identified a significant proportion of the known secondary interactions (Table 1). When co-migrating oligonucleotides did not fit into the secondary structure, several criteria were applied in order to assess whether they may be partners in a long-range tertiary interaction. These criteria are: (a) Do the co-migrating oligonucleotides have mutually complementary sequences? (b) Are the complementary sequences in single-stranded regions, or at least in helices that are of low stability because they are short or interrupted by looped out nucleotides; that is, can the base-pairing interaction take place without serious destruction of secondary structure? (c) Are the complementary sequences relatively inaccessible to nucleases and modifying reagents, as expected if they are base-paired? (d) Is the long-range complementarity conserved from species to species, despite evolutionary changes in nucleotide sequence (10)? Since we have used 14 different RNAs (9) in applying this criterion, it is a stringent criterion.

Eighteen candidates for tertiary reactions are listed in Table 2 above. All have mutually complementary sequences at sites that do not significantly interfere with secondary structure. Screening has so far revealed seven examples of phylogenetic conservation. The accessibility of these seven proposed helices to nucleases and modifying reagents (5-7) is varied (data not shown); some are shielded and some are not. When the data are limited to those obtained with intact subunits, most of the vulnerable sites are at the end of a row of base pairs, or at or opposite a looped out nucleotide; but three helices were hit at internal sites, as is true on occasion of secondary structure helices. In any event, phylogenetic conservation is a powerful argument for taking these seven possible helices seriously. Their positions are shown in Fig. 9. There follow some brief comments on some of them.

61-69 / 810-820 (Fig. 9;1). This is a proposed interaction between RNA domains I and II. The sequence in domain II, 810-820, is in a region that can assume two different secondary structures, each conserved (9,29); the complementary sequence is not base paired in either structure. A residue in this sequence, G818, is accessible to kethoxal and is essential for subunit association (6), suggesting that the proposed interaction site may be located in the 30 S-50 S subunit interface. Protein S4 is reported to be associated with both of the complementary segments (30, 31) and might play a role in facilitating the inter-domain interaction.

417-429 / 722-732 (Fig. 9;2A). This is also an interaction between domains I and II. A protein identified as either S19, S20 or S21 has been

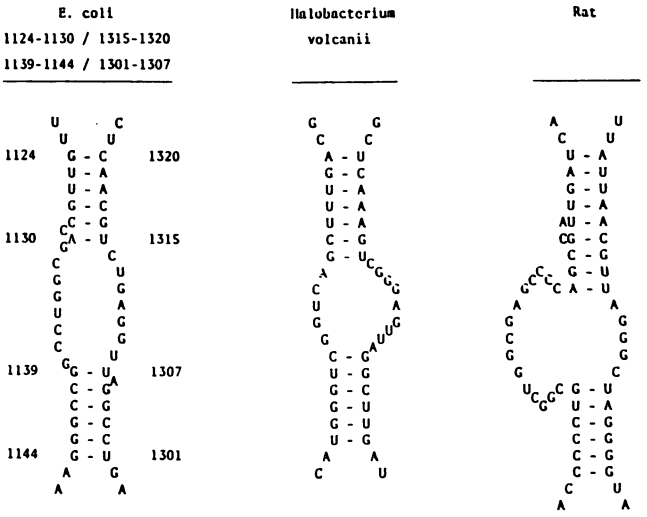

Fig. 11. Possible tertiary interactions: phylogenetic conservation. Base-pairing potential at equivalent sites is shown for three different species out of the fourteen in which it was sought and found (see text).

crosslinked to position 723-724 (32). If S20, it might be involved in the RNA-RNA interaction, since this protein is also associated with domain I (33).

420-428 / 1330-1339 (Fig. 9;2B). Supporting evidence for this possible interaction between domains I and III comes from observations that protein S4 is associated with the first segment (30), protein S12 has been crosslinked to sequence 1316-1322 near the second segment (34), and the two proteins are close neighbors in the 30 S subunit (35).

(Fig. 9;2A and 2B). The domain I segment 417-429 is shared by both of the two preceding interaction schemes (Fig. 10). Several possibilities may be considered: a. It is possible that this rather long sequence might interact with both partners at the same time (e.g., 420-424 / 1335-1339 and 425-429 / 722-726). b. It is possible that it interacts with both, but at different times, i.e., in alternative ribosomal conformations. c. It is possible, and perhaps most probable, that it interacts with only one, even if both complementarities are conserved. If the last supposition is correct, then scheme 2B, 420-428 / 1330-1339, appears to be favored since it is less vulnerable to enzymic attack.

858-864 / 1376-1383 (Fig. 9;3). In this interaction between domains II and III, the segment 858-864 is in the region mentioned above which can assume two alternative secondary structures (9,29). The proposed interaction

is based on the structure that is shown in the published models of E. coli 16 S RNA (5,10,11) and can also be formed in the other RNAs under consideration (9). The segment appears as a single-stranded bulge in the side of a very long helix. In E. coli a significant part of the sequence is also single stranded in the alternative form (29) and it is not clear whether a transition from one structure to the other would nullify the interaction. Studies on vulnerability to kethoxal (6) and RNase TI (36) indicate that the proposed interaction site may lie in the subunit interface.

1124-1130 / 1315-1320 and 1139-1144 / 1301-1307 (Fig. 9;5,6). These two neighboring interactions, involved in the folding of domain III, are shown in Fig. 11 as they appear in a eubacterium, an archaebacterium and a eukaryote, as a partial example of phylogenetic conservation. The lower numbered segments of the two pairs are located in a region of unstable secondary structure, which we have treated as if it were a single-stranded loop. The partner segments, 1301-1307 and 1315-1320 in E. coli, are separated by a stable universally present secondary structure helix (10) which we have avoided opening up.

In general, the technique we have developed and applied to the 30 S ribosomal subunit appears capable of producing data relevant to the three-dimensional structure of the RNA chain in the ribosome. The long range RNA-RNA interactions proposed in Fig. 9 indicate how domain III may be folded upon itself and how domains I, II and III may interact with each other. The data are still under examination and additional complementary relationships may still be encountered.

Fragments A and E and the 3' end of 16 S RNA

As described above, fragment A is made up of those oligonucleotides that are the most rapidly detached from the nuclease-treated 30 S ribosome when it is warmed in the presence of Mg^{++} ions. The detached material contained small pieces of RNA from the 5' end and center of the 16 S RNA, but was chiefly made up of domain IV, the 3' terminal 150 nucleotides. The residual 30 S ribosome, lacking domain IV, was isolated as fragment E. Using a combination of dark field electron microscopy and positive staining, Korn has confirmed that fragment E is a 30 S subunit lacking a band of RNA (15), and has shown that the missing RNA corresponds to a protruding structural feature called the "collar" (14).

These observations are confirmatory of many earlier findings concerning the surface position of the 3' RNA terminus, its interaction with extra-ribosomal components of the protein-synthesizing apparatus, and the nature of the oligonucleotides detached after nuclease digestion (6, 36-45); and the

location by immuno-electron microscopy of the 3' end on or near the "collar" (46-49). Korn's observations add to the prevailing picture. They are the first to visualize the 3' terminal domain directly rather than through the mediation of an attached antibody and thus to provide visual information not only on its location but also on its spatial disposition on the subunit surface. The data presented in Table 2 show probable sites of attachment of domain IV to the rest of the particle.

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