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An experimentally-derived model for the secondary structure of the 16S ribosomal RNA from Escherichia coli

Carola Glotz and Richard Brimacombe

Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Berlin-Dahlem, GFR

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SUMMARY

Ribonucleoprotein fragments are isolated by mild ribonuclease digestion of E. coli 30S ribosomal subunits, and are deproteinized and subjected to a second partial digestion. Base-pairing between the resulting small RNA fragments is investigated using the two-dimensional gel electrophoresis procedure already reported (see Ref. 1). The interactions thus found are incorporated into a secondary structure model covering approximately 80% of the 16S RNA.

INTRODUCTION

In a recent paper (1) we described a direct experimental approach for the determination of interacting sequences - and hence secondary structure - in ribosomal RNA molecules. The method involves a two-dimensional gel electrophoresis procedure, similar to that of Vigne and Jordan (2), in which mixtures of interacting RNA fragments are separated under non-dissociating conditions in the first dimension, and are then dissociated in the second dimension. The interacting fragments thus appear as pairs or families of fragments in the second dimension, and can be identified by sequence analysis. The experiments so far reported (1) were made with isolated 16S RNA from E. coli, using nuclease S_1 to generate the fragments, and yielded data on the secondary structure of about half of the 16S molecule.

In this paper we present a further set of data, obtained using ribonucleoprotein fragments generated by partial ribonuclease T_1 digestion of the 30S subunit (3) as starting material. These fragments were deproteinized under mild conditions, subjected to a second partial digestion with ribonuclease T_1, and the interacting sequences analysed on the two-dimensional elec-

trophoresis system. The results have enabled us to modify and extend our model for the secondary structure of 16S RNA so that it now covers about 80% of the molecule. The data are in general extremely self-consistent, and the proposed structure agrees well with information from other sources, such as the location of kethoxal-sensitive sites (4,5), nuclease cutting points (6), or base substitutions in the closely-related 16S RNA from Zea mays chloroplast ribosomes (7). This latter type of data was used by Noller and Woese and their coworkers (8,9) in their recently proposed secondary structure, which also covers about 80% of the 16S RNA. There is very substantial agreement between their structure and the one we propose here, although there are areas of discrepancy. In addition, we have found some evidence for multiple structures, supporting the ribosomal "switches" proposed by Noller (8). Further evidence in favour of our secondary structure model comes from a number of intra-RNA cross-links induced by ultraviolet irradiation of 30S subunits, the localisation of these cross-links being the subject of a separate paper (10).

MATERIALS AND METHODS

^{32}P-labelled 30S ribosomal subunits were prepared from E. coli strain MRE600 as described (11), with the exceptions that no ammonium chloride wash was made, and that the isolated subunits were subjected to an activation dialysis as in ref. 12. The subunits (ca. 3 A_{260} units, 5 x 10^8 counts/min) were partially digested with ribonuclease T_1 in the presence of 2 M urea and 5 mM magnesium exactly as described (11), and the digestion products were separated on 5% polyacrylamide gels (20 cm long, 1 mm thick), using either the 1 mM magnesium, 20 mM potassium phosphate (pH 6) buffer system of ref. 13, or the 1 mM magnesium, 25 mM Tris-HCl (pH 7.8) buffer system of ref. 3, with potassium chloride being omitted in the latter case. Under these conditions, the subunits yield two ribonucleoprotein fragments, "Bands II and III" (13), together with some fragments of free RNA (14). In some experiments, using the Tris-HCl buffer system, 4 - 20% gradient gels 40 cm long were used, and the gel strips containing these small RNA fragments were loaded directly onto second dimension gels, as described below.

Ribonucleoprotein Bands II and III were located by autoradiography, extracted overnight into 5 mM magnesium acetate, 50 mM KCl, 25 mM triethanolamine-HCl pH 7.8, and precipitated at - 40° by addition of 3.5 vol. ethanol. The pelleted fragments were resuspended in 50 µl of 0.3 mM magnesium acetate, 10 mM NaCl, 2 mM EDTA, 2 mM Tris-HCl pH 7.8, and were treated for 30 min at 30° with a few grains of polymer-bound proteinase K (Merck, Darmstadt). In some experiments 0.1% dodecyl sulphate was added during this treatment. The proteinase was removed by centrifugation, and the RNA was then subjected to a further partial ribonuclease T_1 digestion using a total of 2 - 10 units of enzyme for 30 min at 30°. Enzyme and remaining traces of ribosomal protein were destroyed by addition of 2 µl of soluble proteinase K (Merck, 5 mg/ml), and after 30 min at 30° the samples were applied to the two-dimensional gel system (1). The system was modified in that gels 1 mm thick were used, and the first dimension consisted of a 5 - 20% polyacrylamide gradient in the upper 20 cms of the gel, the lower 20 cms being a constant 20%. The dodecyl sulphate-EDTA system (13) was used for the first dimension, the second dimension being a 20% gel in the buffer system of Maxam and Gilbert (15), as described (1).

The gels were autoradiographed, appropriate spots were cut out, and the RNA was extracted with phenol, isolated by ethanol precipitation and fully digested with ribonuclease T_1 as described (11). The oligonucleotides were separated on polyethyleneimine thin-layer plates, using the "mini-fingerprint" system of Volckaert and Fiers (16) with minor modification as in ref. 17. After autoradiography, the oligonucleotides were extracted into 30% triethylamonium carbonate pH 10, lyophilized, digested with ribonuclease A, and the products separated on the "double-digestion" system of Volckaert and Fiers (16).

RESULTS AND DISCUSSION

Mild digestion of 30S subunits with ribonuclease T_1 under appropriate conditions leads to the formation of two ribonucleoprotein particles of unequal size, "Bands II and III", together with some small fragments of free RNA (3,11,14). The RNA content of these particles is well documented, Band II containing the

first 920 nucleotides from the 5'-end of the 16S RNA (14), whereas Band III contains the next 470 nucleotides (11). The remaining 150 nucleotides from the 3'-end appear at least in part in the free RNA fragment fraction (14), although this latter fraction also contains RNA fragments from many parts of the 16S RNA (see below).

When RNA was isolated from Bands II or III in preliminary experiments and applied to the two-dimensional gel system to search for interacting fragments (1), surprisingly few dissociated structures could be observed. Almost all the RNA species lay on the gel "diagonal" (cf. ref. 1), implying either that the secondary structure was already destroyed before the electrophoresis, or that the fragments consisted mainly of uncut hairpin loops. Since the first of these possibilities seemed very unlikely under the mild conditions used, a second partial ribonuclease T_1 digestion step was introduced at this stage (as detailed in Materials and Methods), and this led as expected to the appearance of families of dissociated fragments lying below the diagonal in the second dimension of the gel system (1). The conditions of the second enzyme treatment were varied in order to generate different sets of fragments, and the free RNA fragment fraction (see above) also proved a useful source of interacting fragments, when applied directly to the second dimension gel system (see Materials and Methods).

Some parts of the gel patterns obtained were rather complex, whereas other regions showed simple sets of fragment pairs or triads, examples being presented in Fig. 1. Fig. 1A shows a part of a two-dimensional gel pattern from Band II RNA, using very mild conditions in the second ribonuclease T_1 digest, and it can be seen that here most of the RNA still lies on the diagonal. The gel section in Fig. 1B was also obtained from Band II RNA, under rather more vigorous conditions, whereas the gel of Fig. 1C was from the free RNA fragments liberated by the initial ribonuclease T_1 digestion. In each of these cases the arrowed fragments were subjected to sequence analysis. Gel patterns derived from Band III tended to be rather more complex, but nevertheless also showed clear sets of fragment pairs.

A series of two-dimensional gels were run with RNA digests

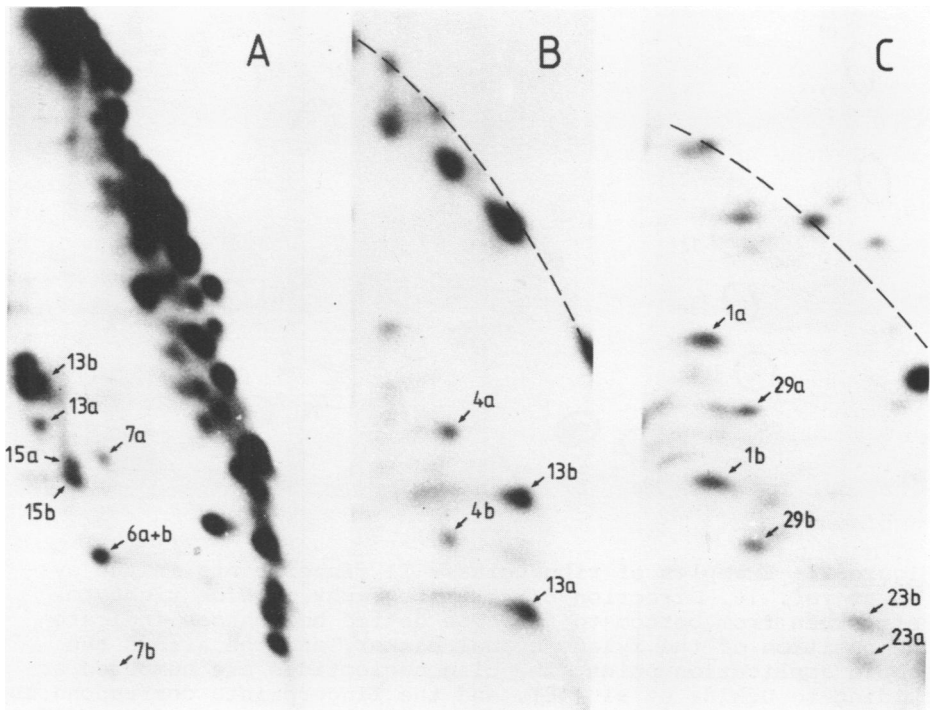

Figure 1: Two-dimensional gel separations of RNA fragments. Direction of electrophoresis is from left to right, then from top to bottom. The dotted lines indicate the "diagonal" (cf. ref. 1), and the numbers assigned to the fragment pairs correspond to those of Fig. 3. The fragments are from ca. 15 to 30 bases in length. A: Fragments derived from ribonucleoprotein Band II, after a mild second digestion with ribonuclease T1. B: As for "A", but with a stronger second digestion. C: RNA fragments liberated from 30S subunits by the first ribonuclease T1 digestion. See text for details.

derived from Band II, Band III and the free RNA fraction. Large numbers of fragments from these gels were subjected to oligonucleotide analysis, and for this purpose the "mini-fingerprint" system of Volckaert and Fiers (16) was found to be the most convenient. Examples of the ribonuclease T_1 fingerprints obtained in this system from a pair of interacting fragments are shown in Fig. 2. In every case the oligonucleotides were identified by secondary digestion with ribonuclease A, and the fragments fitted

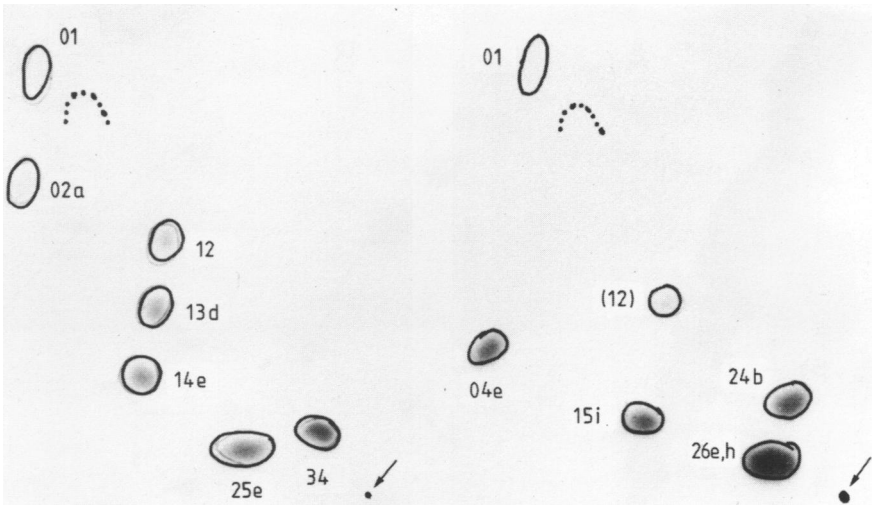

Figure 2: Examples of ribonuclease T1 fingerprints in the system of ref. 16. Direction of chromatography is from right to left, then from bottom to top. The dotted horseshoes indicate the position of the xylene cyanol marker, and the arrows the sample application point. The oligonucleotides are numbered according to Uchida et al (18), and the fingerprints correspond to fragments 13a (bases 584 - 605, cf. Fig. 3) and 13b (627 - 653) respectively.

to the nucleotide sequence of 16S RNA (19,20). The complete set of nucleotide sequences found, combined with those already reported (1), is summarized in Fig. 3.

It should be noted that, in contrast to the fragments obtained with the single-strand specific nuclease S_1 which tended to have "ragged ends", the ribonuclease T_1 fragments have very clean ends. However, when the same sets of fragments appeared in different experiments, they were often cut at slightly different points (compare for example the relative mobilities of fragments 13a and b which occur in both Figs. 1A and B). Consequently, as before (1), the 3' and 5' ends of the fragments listed in Fig. 3 should be taken as approximate to the extent of 2 or 3 bases. The specificity of ribonuclease T_1 (as opposed to nuclease S_1) tended to give rise to fragment pairs where each component was of a different length, sometimes extending over more than one helical region in cases where there was no intervening accessible

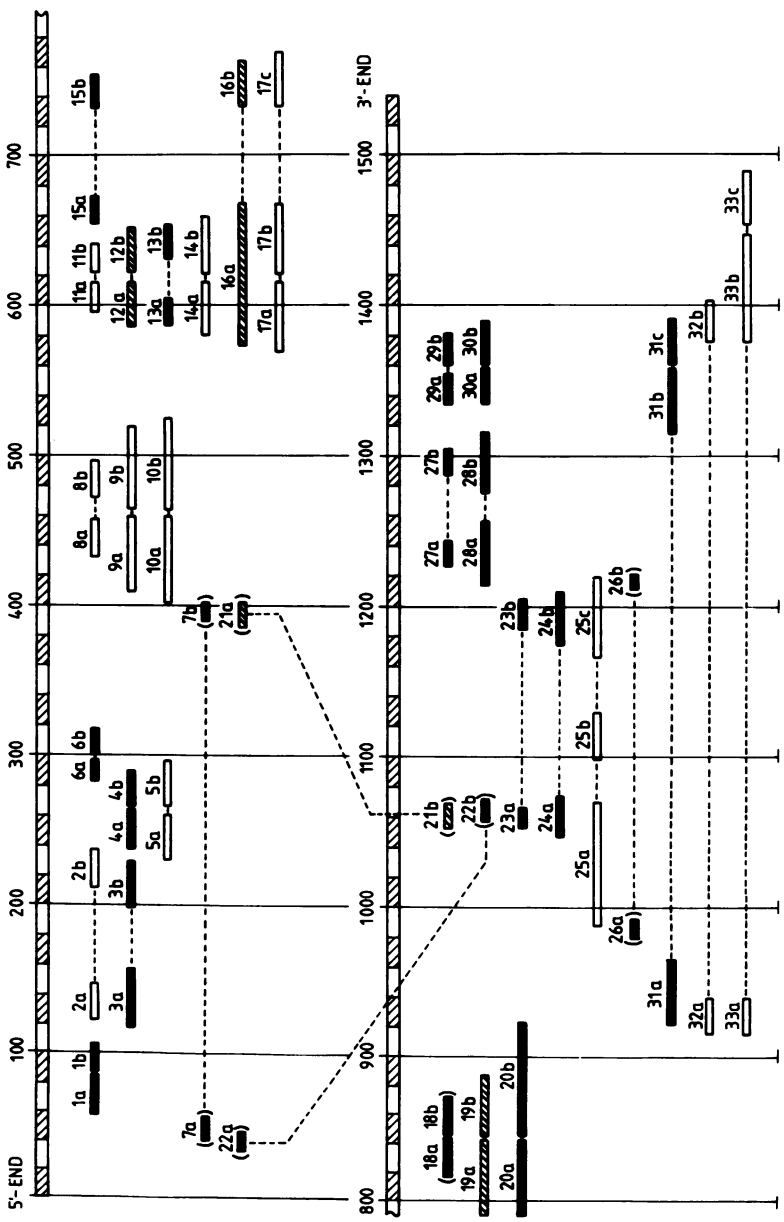

Figure 3: Location of fragment families in the 16S RNA (19, 20). The scale of nucleotides is numbered from the 5'-end. Fragment sets with the same number and joined by dotted lines constitute interacting pairs or triads (cf. Fig. 1). Fragment families in empty boxes are from ref. 1, those in filled boxes are the new data, and those in striped boxes have been found both here and in ref. 1. Brackets denote tentative interactions.

guanine residue. Further, since the fragments were produced by two successive partial enzyme digestions (with a possible loss of parts of the secondary structure during the separation procedures between the two digestions), it is important to note that the cutting points observed do not necessarily occur only in single-stranded regions of the original structure.

Our interpretation of the fragment data of Fig. 3 is given in Figs. 4 to 7. Fig. 4 is a schematic representation of the whole structure, indicating the approximate locations of fragment pairs from the various regions. The principle difference from our previous data (1) is that the long-range interaction corresponding to fragments 21a and b, which was designated as tentative (1), has been removed from the structure. Although this fragment pair was seen again in the present set of experiments, it appears to be a special case, which will be discussed later in connection with other fragment pairs involving the same sequences (7,22 and 23, Fig. 3). Some other minor modifications to the previous data have been made, which will be described in the appropriate context below. The new data consist of a detailed structure for the whole of the region between bases 920 and 1395, some additional fragment pairs in the 5'-region including the long-range interaction comprising fragments 7a and b, as well as data which confirm the structures already proposed for the central region (bases 570 to 900).

In addition, structures have been assigned to several "loop tops" (e.g. bases 670 - 730, Fig. 4), although we have no experimental data for them. These assignments have been made on the grounds that such loop tops must have a self-contained structure, since extensive base-pairing with other regions of the sequence would lead to topological knots in the RNA chain. These proposed structures should be verifiable by further experiment, and do not of course imply that single-stranded sequences in the regions concerned are unable to form short helices (up to ca. 6 base-pairs in length) with other parts of the RNA. For the sake of completeness, the hairpin-loop structure proposed for the 50 nucleotides at the 3'-terminus of the RNA (21,22) has also been included, although we have no data for this region.

The remaining regions with no assigned structure consist of

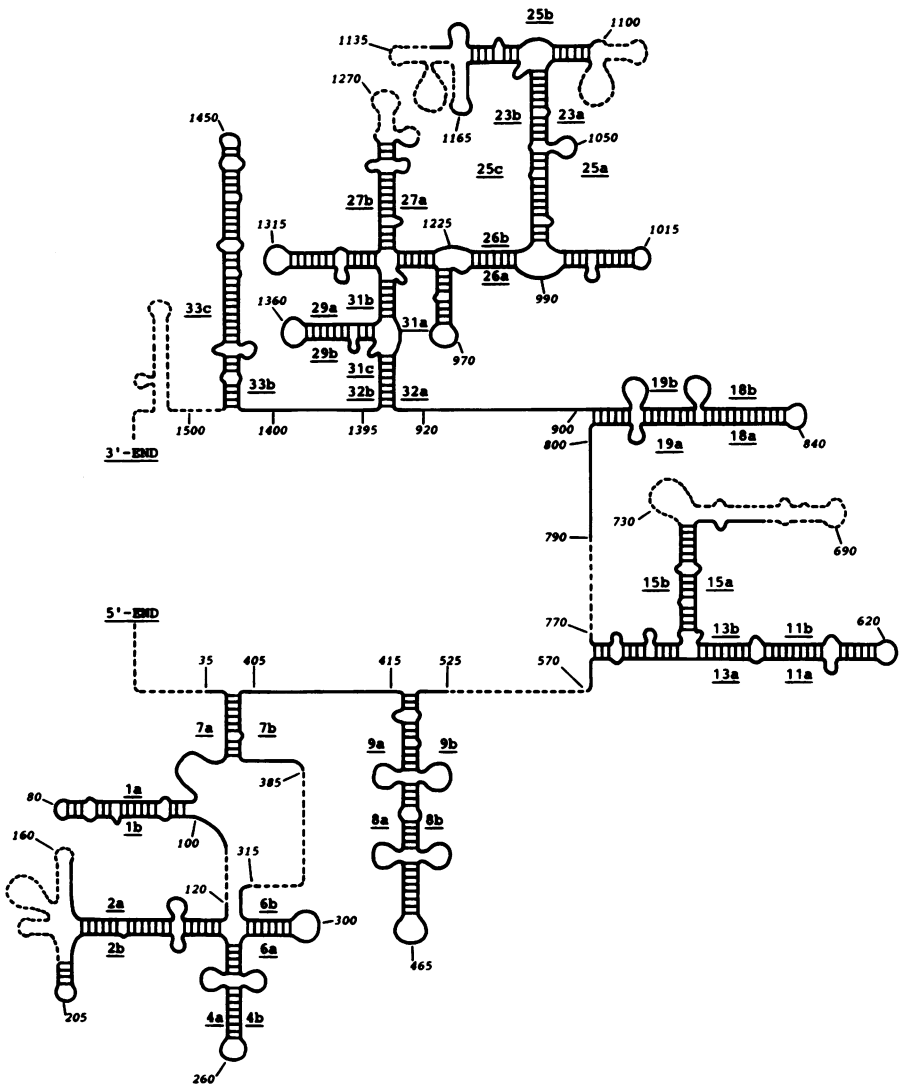

Figure 4: Scheme of the secondary structure proposed for 16S RNA. The nucleotides are numbered (in italics) from the 5'-end, and the underlined numbers indicate the positions of selected sets of fragments, to orientate the diagram to the data of Fig. 3. Dotted lines indicate regions where we have no data, and base-pairing is indicated by the bars. See text and Figs. 5 to 7 for details.

parts of the 5'-one third of the molecule (the largest section being bases 315 to 385), and the "central rectangle" in Fig. 4. For these regions, more experimental data will be required, although there is already evidence for some long-range interactions within the "rectangle", notably between bases 1 - 500 and 800 - 900 in the RNA protected by protein S4 (6), and between bases 20 - 70 and 400 - 500, as evidenced by an RNA-RNA cross-link (10). The psoralen-induced cross-links reported by Cantor and his co-workers (23) do not fit at all with our data, and we suggest that these cross-links may reflect very short base-paired structures between looped-out regions of the structure depicted in Fig. 4, as mentioned above.

The details of the structure are given in Figs. 5, 6 and 7, and include the positions of the ends of the various fragment pairs (Fig. 3), the positions of kethoxal-sensitive sites (4,5), ribonuclease cutting points (6), and heterogeneities in the sequence (24). The base substitutions found in the 16S RNA from Zea mays chloroplast ribosomes (7) are also indicated. Here, three types of base substitution can be distinguished. Firstly, there are clear double mutations leading to substitution of one base-pair by another (e.g. A-U to G-C), implying a high degree of conservation in the secondary structure between the two species. Secondly, there are groups of substitutions which at first sight appear contradictory, but which can be fitted to a base-paired helix simply by slipping one of the RNA chains one or two bases along, and making corresponding slight alterations to the looped-out regions. Thirdly, there are substitutions which do not fit in terms of double-helix formation, implying either that our proposed structure is incorrect, or that there is no conservation of the structure in the region concerned. These will be referred to as substitutions of the "first, second and third type" respectively, in the following discussion.

The central region of the RNA (bases 570 to 900) is the least complicated, this region being divided into two distinct domains as shown in Fig. 5. The structure in the upper region was previously designated as tentative (1), but now appears rather more certain, being substantiated by the short fragment pair 18. The data agree well with the sites of nuclease action

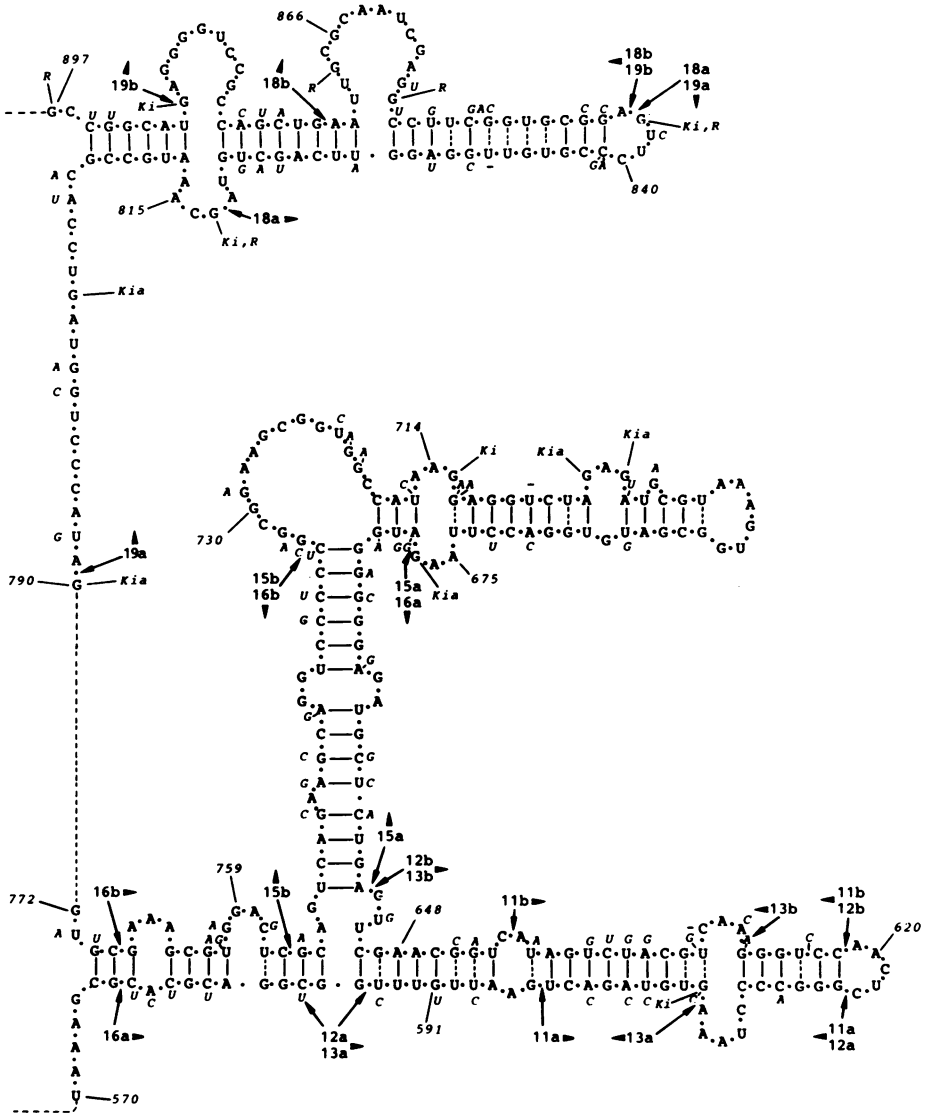

Figure 5: Details of the central region of the structure (bases 570 to 897). Numbers in italics show the distance in nucleotides from the 5'-end. Numbers in bold type denote the approximate 3'- and 5'-ends of the fragments (Fig. 3), with the arrowheads indicating the direction in which each fragment runs. Letters in italics are base substitutions in chloroplast 16S RNA (7), with dashes denoting deletions. Ki and Ka denote kethoxal sites in active (4) or inactive (5) 30S subunits respectively, and R denotes a ribonuclease sensitive site (6).

(6), bases 851 - 868 being excised from the RNA protected by protein S4 (6). Comparison with the chloroplast 16S RNA sequence (7) shows base substitutions of the "first type" (see above) in the central helical region, and substitutions of the "second type" in the helical region on the right, which forms the closed end of the hairpin loop. Schwarz and Kössel (7) propose a different structure for this region, but their structure is not thermodynamically very favourable, and does not take account of the nuclease data just mentioned (6). On the other hand, Woese et al. (9) propose the same structure as that of Fig. 5, with the exception that the base of the hairpin loop (residues 808 - 813 and 890 - 895) is different. The base substitutions in this region are of the "third type", and it is possible that other regions of the RNA are involved in base-pairing here. The longer fragment pair 20 (Fig. 3) from this region could not be used to extend the base-paired structure, supporting the idea that a third piece of RNA is involved at the base of the structure, which was lost in our analysis.

This type of situation has already been observed in our experiments, notably with fragments 14a and b (9 and 10 in ref. 1), which could be base-paired together along their whole length in a weak structure, but which subsequently were shown to involve a third region of RNA at the base of the hairpin. Such weakly-paired structures may simply be artefacts, but they could provide a mechanism for "storing" RNA sequences during biosynthesis of the ribosomal RNA, prior to incorporation of the sequences into long-range interactions as the transcription proceeds.

The lower domain in Fig. 5 (bases 570 to 772) shows very close agreement with the structure proposed by Woese et al. (9), again however with a discrepancy at the extreme left-hand end (bases 570 - 578 and 765 - 772). The main features of the structure are confirmed very clearly by the fragment pairs 13 and 15, and a host of other pairs or triads were found (too numerous to include in Figs. 3 and 5), all consistent with this base-pairing scheme, which has also been put forward by other workers as a result of protein-binding studies (25,26). The base substitutions (7) are of the first and second type, including those in the region between bases 670 and 730. Here we have no fragment

data, but a reasonable structure can be drawn, which agrees well with the kethoxal studies (4,5). Both structural domains shown in Fig. 5 are supported by intra-RNA cross-link data (10).

The next and most complex region of the RNA is depicted in Fig. 6. Here the base of the structure is defined by the long-range interaction already reported (1), comprising the fragment pair 32. The structure proposed in ref. 1 for this interaction has been opened slightly to incorporate the hairpin loop in the fragment pairs 29 and 30, which includes base substitutions (7) of the second type. It is very interesting to note that in the partial sequence of 18S ribosomal RNA from Bombyx mori (27), this same loop can be drawn with three base-pair substitutions of the first type. Another hairpin loop, also with second-type substitutions (7), is defined by the fragment pair 27. (This loop contains incidentally the cross-link site to protein S7, at residue 1239 (12)). These two regions are prolonged by the fragment triad 32 and the fragment pair 28, respectively. In each case, weakly-paired structures could be drawn, between the whole length of 28a and 28b, or between 31a and 31b, but the thermodynamic stability is much higher when the two parts of the structure are put together as shown in Fig. 6. This generates a loop (bases 1307 to 1328) which has base substitutions of the first type, although the continuation of the loop has a kethoxal site (residue 1337) and also some base substitutions of the third type, suggesting that these few base-pairs (1334 - 1338 and 1300 - 1304, and in addition 1296 - 1299 and 1233 - 1236) may not exist in the subunit.

Further into the structure is the base-pair defined by fragment pair 26. This was assigned as tentative in Fig. 3, as it was only seen twice, in nuclease digests which were contaminated with ribonuclease A activity. The fingerprint data were nevertheless clear, and the base substitutions (7) here are of the first type. The whole of the region just discussed is very similar up to this point to that proposed by Woese et al. (9), with some minor discrepancies similar to those mentioned in connection with Fig. 5. However, the rest of the region (bases 990 to 213, Fig. 6) is not in agreement. Our structure is defined by the fragment pairs 23 and 24, and by the triad 25 already re-

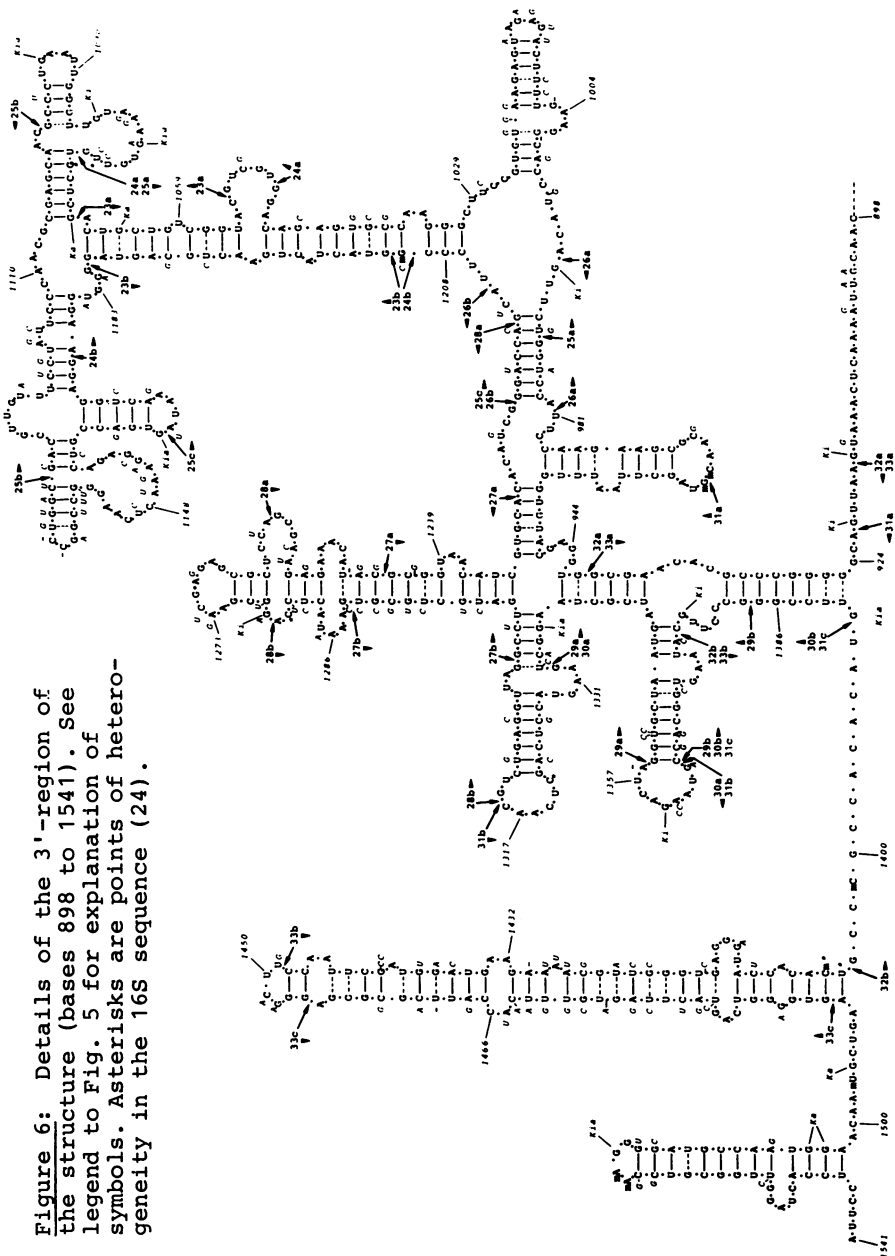

Figure 6: Details of the 3'-region of the structure (bases 898 to 1541). See legend to Fig. 5 for explanation of symbols. Asterisks are points of heterogeneity in the 16S sequence (24).

ported (1). Bases substitutions in the region of fragment pairs 23 and 24 are of the second type, and, most important, there is a very well-defined intra-RNA cross-link in this region (10). The base-pairing proposed for the top of the region (bases 1067 to 1183) is less certain, although fragment 25b must clearly be involved here. The two kethoxal sites (4,5) at positions 1063 and 1067 suggest some opening of the structure in this area, and the structure proposed for the left-hand loop end (bases 1121 to 1159) contains a number of base substitutions of the third type; however, this loop end is obviously not a highly conserved region.

In addition to the "colicin fragment" at the extreme 3'-end of the RNA (21,22), Fig. 6 also indicates the loop structure already published (1) for the intervening bases (1400 - 1500). Woese et al. (9) propose a similar loop (again however slightly different at the base of the structure), and although there are numerous base substitutions here (7), they are in fact all of the second type.

The remainder of the structure is shown in Fig. 7. Here the most important feature is the long-range interaction comprising fragments 7a and b. This fragment pair is listed as tentative in Fig. 3, since it was only seen once, but the data were very clear, and there is also a base-substitution (7) of the first type. The same interaction is deduced by Woese et al. (9), although their structure for the neighbouring loop, defined by the fragment pair 1, is different. The main part of the region is determined by fragment pairs 2, 4 and 6, the structure which we previously proposed (1) being opened up at the 3'-end (bases 286-300) to incorporate the last of these pairs (6a and 6b). Pairs 3 and 5 are also consistent with the base-pairing scheme, which fits well with the kethoxal (4,5) and nuclease data (6), in particular bases 246 to 277 being excised from the RNA protected by protein S4 (6). The structure of Woese et al. (9) is similar, although it differs in the region from bases 140 to 220, but the base substitution data (7) are not easy to interpret throughout this area. There are many changes of the third type, several blocks of bases are deleted, and the 16S RNA from E. coli itself has several heterogeneities (24), indicated by asterisks.

The remaining domain in Fig. 7 is the region from bases 400-520. Here, we have opened up four base pairs from our previous structure (400 - 403 and 525 - 528, ref. 1), to allow incorporation of the long-range interaction with the 5'-end (fragment pair 7), and in the structure of Woese et al. (9) the next part of the hairpin is also opened up as far as bases 436 and 496. The rest of the loop structure is rather clear, with base substitutions of the first type, as well as the complete deletion of the end of the loop (7). Intra-RNA cross-links have been found

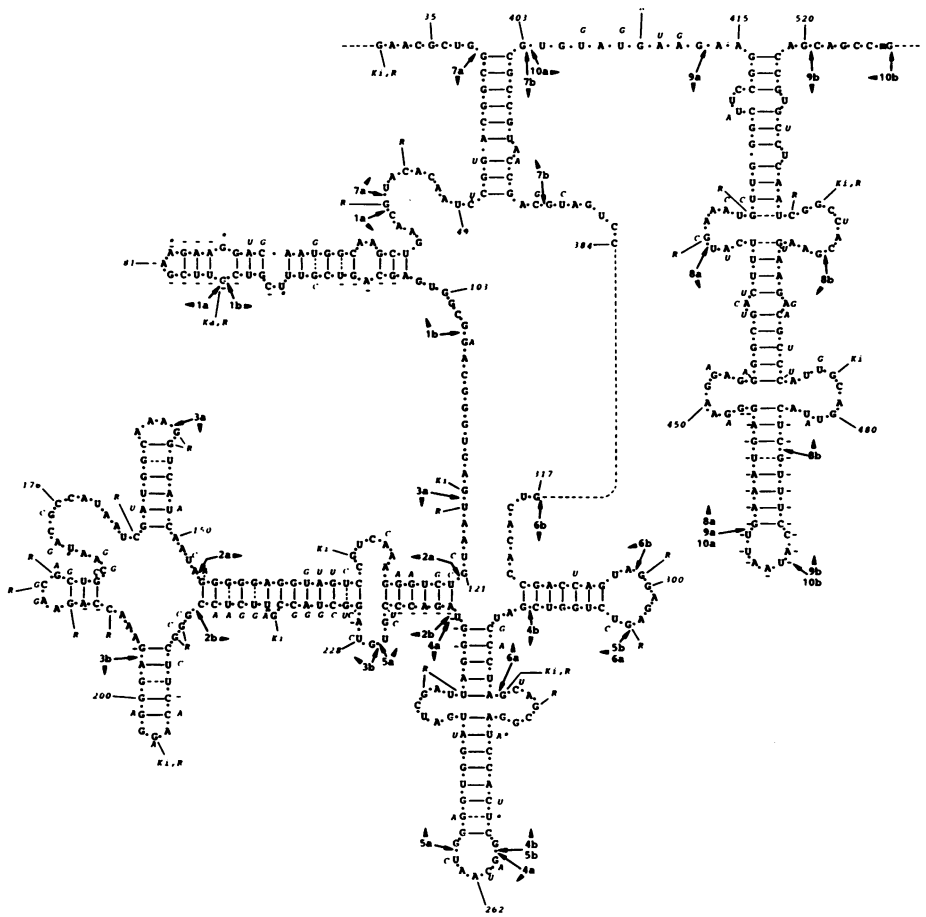

Figure 7: Details of the 5'-region of the structure (bases 31 to 526). See legends to Figs. 5 and 6 for explanation of symbols.

in this domain, including a cross-link to the 5'-end of the RNA (bases 20 to 70) (10).

Finally, it remains to discuss the fragments found in multiple structures. These are depicted in Fig. 8, and comprise the fragment pairs 7, 21, 22 and 23 (cf. Fig. 3). In this set of fragments, the sequence 1050 - 1070 occurs three times, and the sequences 380 - 400 and 30 - 50 each occur twice. Pairs 7 and 23 are the ones which we have incorporated into the structure of Figs. 4 to 7. This is because the structure in pair 23 is supported by intra-RNA cross-linking data (10), as already mentioned. Further, the 1050 to 1070 region is already within a closed loop of the 16S RNA structure as defined by the long-range interactions in Fig. 6, and it follows that incorporation of either

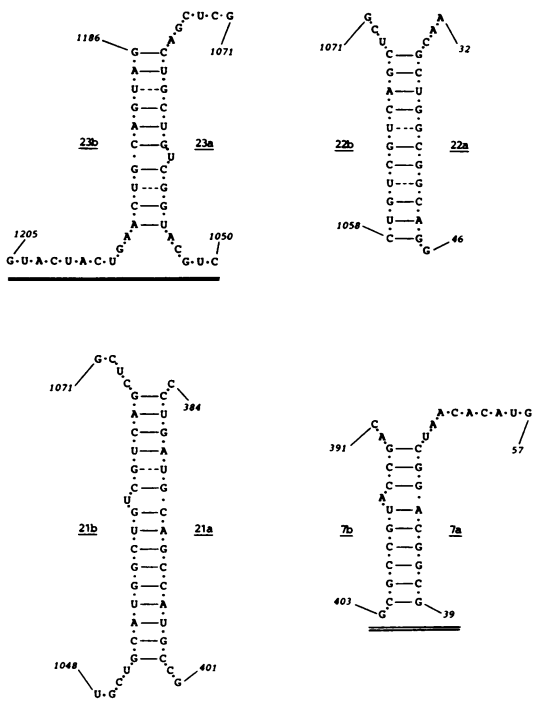

Figure 8: Fragments found in multiple structures. The fragment numbers correspond to those in Fig. 3, and the two underlined structures are those included in Figs. 6 and 7. See text for details.

of the other pairs (21 or 22) would lead to topological knots in the RNA, since each of the structures in Fig. 8 is approximately one full helix turn in length.

It cannot be excluded that the extra fragment pairs (21 and 22) are simply artefacts of our experimental system, but if this were the case it is strange that these are the only ones which we have found. Further, there is no evidence that structures can renature in our system, since fragment mixtures denatured by heat or phenol treatment show no fragment families lying below the diagonal on the two-dimensional gels (cf. Fig. 1). It is tempting to consider the possibility that the extra pairs have some functional significance, and that the sequence regions concerned lie close together in the tertiary structure of the RNA. Clipping with nuclease during the course of our experiment could then allow the release of the fragments as the base-paired entities shown in Fig. 8. Noller (8) has proposed some very similar structures as possible ribosomal "switches", connected with formation of 70S particles. However, in order to avoid the problem of topological knots, formation of these structures within the intact RNA would necessitate the concomitant formation of a corresponding "anti-helix" somewhere in the structure. Such long-range conformational changes could generate highly co-operative "twitches" running through the entire 16S structure, which would probably be rather strained and therefore transient, and which could represent the basis of the translocation process. Further experiments are in progress to try to obtain more data on these possible interactions, as well as to fill the remaining gaps in the structure which we have described here.

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