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Intra-RNA cross-linking in *Escherichia coli* 30S ribosomal subunits: selective isolation of cross-linked products by hybridization to specific cDNA fragments

Wolfgang Stiege, Michael Kosack*, Katrin Stade and Richard Brimacombe+

Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, D-1000 Berlin-Dahlem, FRG

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ABSTRACT

M13 clones were constructed with cDNA inserts corresponding to specific regions of E. coli ribosomal RNA. The DNA from the clones was immobilized by coupling to diazobenzyloxymethyl cellulose, and was used for the selective isolation by hybridization of cross-linked RNA complexes containing the complementary sequences. Immobilized DNA samples with inserts complementary to four different regions covering bases 735-1384 of the 16S RNA were hybridized with a mixture of 16S RNA fragments generated by partial digestion of 30S subunits that had been cross-linked by ultraviolet irradiation in vivo. After dehybridization, the individual RNA fragments and cross-linked complexes were separated by gel electrophoresis and analysed by our usual procedures. Nine cross-links are described; four of these are hitherto unobserved "secondary structural" cross-links, and one is a new "tertiary structural" cross-link between positions 243-247 and 891-894 of the 16S RNA.

INTRODUCTION

Intra-RNA cross-linking has become an established method for the investigation of the tertiary folding of large molecules such as ribosomal RNA (1-6). The aim of these studies is to analyse sites of cross-linking within the RNA, and thus to identify points of contact between different regions of the RNA sequence in the three-dimensional structure. In our laboratory we have analysed a number of cross-link sites within both the 16S and 23S ribosomal RNA from Escherichia coli, generated by in situ cross-linking of 30S and 50S ribosomal subunits (1,2,5,6), or by in vivo cross-linking of intact bacterial cells (7).

The mixture of cross-linked products that one obtains in this type of experiment is inevitably complex, for two reasons. First, the spectrum of cross-links formed is itself qualitatively and quantitatively heterogeneous, regardless as to whether the

cross-links are generated by (for example) simple ultraviolet irradiation (2,5,7) or treatment with a bifunctional chemical reagent (1,3,6). Secondly, at some stage during the analysis the RNA must be subjected to a partial digestion procedure so as to cut it into fragments of a size suitable for sequence determinations. This has the effect of compounding the degree of heterogeneity of the products, since any given sequence region of the RNA is likely to appear in a range of different fragments with varying lengths and at varying intensities, according to the nature of the partial digestion procedure being used. In our experiments (1,2,5-7), the partially digested RNA fragments and cross-linked complexes are separated by two-dimensional gel electrophoresis, and it has for a long time been clear that important data is being lost, simply as a result of this complexity and heterogeneity. Some cross-linked complexes cannot be analysed because they lie in "overcrowded" areas of the two-dimensional gels, and others may be "overshadowed" by a relatively much stronger (i.e. more frequently occurring) neighbouring complex on the gel.

Here we describe an approach to circumvent this problem, by selective hybridization of the digested RNA samples with complementary DNA sequences immobilized on cellulose. For this purpose a library of M13 clones has been constructed, containing E. coli ribosomal DNA inserts of ca. 40 to 250 nucleotides in length. Any one of these clones should only be capable of hybridizing to a small proportion of the RNA fragments or cross-linked complexes in the partially digested mixture, with the result that, after elution of the hybridized RNA from the immobilized DNA, the subsequent patterns of two-dimensional gel electrophoresis should be considerably simplified. A related approach, for the isolation of RNA-protein cross-linked complexes, has been reported by Chiaruttini et al (8), although these authors did not use immobilized DNA. Immobilized DNA has the dual advantage that the hybridized samples are very easy to handle, and that the DNA-cellulose matrix can be re-used several times. In order to demonstrate the method, we describe in this paper the analysis of a number of intra-RNA cross-linked complexes that were isolated by hybridization to a series of DNA clones containing inserts complementary

to regions from the 3'-proximal half of the E. coli 16S RNA. The cross-linked substrate was a partial digest with cobra venom nuclease (9) of 30S ribosomal subunits that had been cross-linked by ultraviolet irradiation in vivo of intact E. coli cells (7).

MATERIALS AND METHODS

Preparation and identification of M13 clones. Restriction fragments of ribosomal DNA were prepared from plasmid pKK3535 (10) in E. coli strain HB101 by standard procedures (11). 50 g of the plasmid was first cut with Dra I and Eco RI (New England Biolabs), and with Pvu I (GIBCO), the latter being added to avoid interference from a Dra I restriction fragment from an unwanted region of the plasmid. The fragments were separated on a 2% agarose gel, and the 674 bp fragment between the Dra I cut at position 1517-18 in the plasmid and the Eco RI cut at position 2191-92 (cf. Fig. 1) was isolated; this fragment covers the 674 bases at the extreme 5'-end of the 16S RNA. A further aliquot of pKK3535 was cut with Sma I and Xba I (Boehringer, Mannheim), and a 770 bp fragment (between the Sma I cuts at positions 2131-32 and 2901-02) and a 340 bp fragment (between the second of the Sma I cuts and the Xba I cut at positions 3241-42) were isolated. The first of these fragments corresponds to positions 615-1384 of the 16S RNA, and the second to a fragment extending to 182 bases beyond the 3'-end of the molecule. The 674 and 770 bp fragments were cut further with Hae III (Boehringer), and the fragments (designated A to K, see Fig. 1 and Table 1) were separated on 2% agarose gels. These fragments, as well as the 340 bp fragment (designated L) were cloned into M13 mp19 RF DNA (Boehringer), the latter being linearized with Sma I for cloning all of the fragments except for D and L; for fragment D the M13 DNA was linearized with Sma I and Eco RI, and for fragment L with Sma I and Xba I.

The rDNA fragments were ligated to the M13 vector, and the ligation mixtures used to transform competent E. coli cells, strain JM103, according to the procedure of Messing (12). Positive clones were identified by white plaque formation (13), and single-stranded M13 DNA was prepared from these plaques using 2 ml cultures of E. coli JM103 (12). The isolated DNA was baked

onto nitrocellulose filters, and hybridized (14) with uniformly ^{32}P-labelled E. coli 16S RNA (see below). Non-hybridized regions of the 16S RNA were digested with ribonuclease T_1 (2 g/ml in 300 mM NaCl, 30 mM trisodium citrate (2 x SSC buffer)) for 30 min at 37 , and, after washing the filters, the hybridized fractions were eluted by heating for 5 min at 90 in 0.4 ml of water containing 20 g of unlabelled tRNA. The eluates were rapidly cooled in ice water, and subjected to fingerprint analysis (refs. 1,15, and see below), in order to determine which ribosomal cDNA regions were present in the individual inserts.

Preparation of immobilized DNA. M13 clones containing appropriate inserts were grown in 500 ml cultures of E. coli JM103, and the single-stranded M13 DNA was extracted as just described (12), with the exceptions that two polyethylene glycol precipitations were made, and the DNA was then extracted with phenol in the presence of 10 mM Tris-HCl pH 7.8, 2 mM EDTA, 100 mM sodium acetate and 0.1% SDS. The DNA was finally precipitated twice with ethanol, giving a yield of ca. 500 - 1000 g in a typical preparation.

Aminobenzyloxymethyl cellulose (Miles Chemical Corp.) was diazotized as described by Goldberg et al (16), and 100 mg of the diazotized mixture was coupled with the M13 DNA sample (ca. 800 g), again as described (16,17). The yield of the coupling reaction was of the order of 40 - 50%, as determined by hydrolysis of an aliquot with deoxyribonuclease I, followed by measurement of the absorption (260 nm) of the oligonucleotides released. The immobilized DNA was used for hybridization with the cross-linked ribosomal RNA samples.

Preparation, hybridization and analysis of cross-linked RNA fragments. ^{32}P-labelled cultures of E. coli strain MRE 600 were grown and subjected to ultraviolet irradiation as previously described (7). 30S ribosomal subunits were isolated from these cross-linked cultures and partially digested with cobra venom nuclease (9), according to our usual procedures (5-7). After removal of ribosomal proteins and nuclease by proteinase K treatment (1), the 16S RNA fragments were extracted with phenol (as described above for the isolation of M13 DNA) and precipitated with ethanol. Before hybridization of these fragments with the

immobilized DNA, the appropriate M13 DNA aminobenzyloxymethyl cellulose preparation (ca. 100 mg) was pre-treated by gently rotating for 1 hr at 42 in 5 ml of 50% deionized formamide containing 5 x SSC buffer, 0.1% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin (5 x Denhardt's solution), 50 mM sodium phosphate pH 6.8, 0.1% SDS and 0.1 mg/ml of unlabelled bulk tRNA. The DNA-cellulose was recovered by centrifugation, and hybridization was then carried out with the ^{32}P-labelled RNA fragments for 2 hr at 42 in 5 ml of the same milieu, but with 1 x Denhardt's solution, 20 mM sodium phosphate pH 6.8 and 0.2% SDS (cf. ref. 18). The cellulose was washed five times for 10 min at 42 with 5 ml of 50% deionized formamide containing 2 x SSC buffer, and the hybridized RNA was then eluted by washing twice at 65 for 30 min with 5 ml of 99% formamide containing 0.1% SDS. The combined eluate fractions were made 300 mM in sodium acetate, and the RNA was precipitated by addition of 2 volumes of isopropanol overnight at -20 .

The precipitated RNA was washed thoroughly with 80% ethanol and re-dissolved, and the individual fragments and cross-linked complexes were separated by two-dimensional gel electrophoresis exactly as described (1,7), using a 5 - 10% polyacrylamide gradient gel as the first dimension and a 10% gel as second dimension. Individual complexes were extracted from the gels, subjected to total digestion with ribonuclease T_1, and analysed by the fingerprint method of Volckaert and Fiers (15) on polyethyleneimine cellulose thin-layer plates, according to our standard procedures (1,2). As usual, the ribonuclease T_1 oligonucleotides were further characterized by secondary digestion with ribonuclease A (1,15), and the oligonucleotide data were fitted to the 16S RNA sequence of Brosius et al (19).

RESULTS

Figure 1 shows the positions of the restriction sites in the pKK3535 plasmid (10), together with the Hae III sites in the three fragments that were isolated. The various restriction fragments were cloned into M13 DNA as outlined in Materials and Methods, so as to produce a series of non-overlapping ribosomal DNA inserts covering the whole of the *E. coli* 16S ribosomal RNA

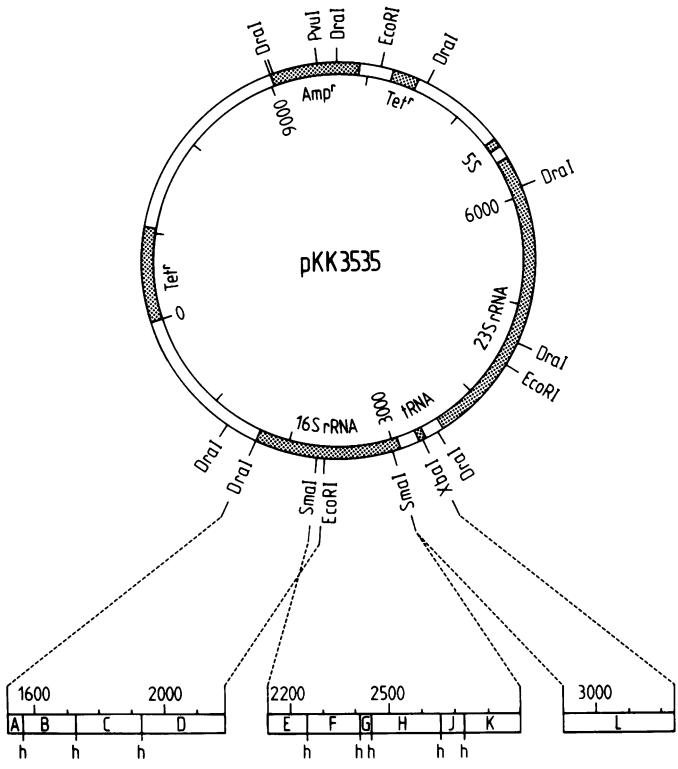

Figure 1: The plasmid pKK3535 (10), showing the positions of the restriction sites used to generate the three principal DNA fragments for this study. In the lower part of the diagram the positions of the secondary restriction sites for Hae III (see Materials and Methods) are denoted by "h". The locations of all the DNA fragments "A" to "L" in the numbering system of the 16S RNA sequence (19) are given in Table 1.

sequence. The sequence of the ribosomal DNA insert was checked in each clone by hybridizing the single-stranded M13 DNA to ^{32}P-labelled 16S RNA, trimming the hybrid by mild digestion with ribonuclease T_1, and analysing the hybridized sequence by total ribonuclease T1 digestion and fingerprint analysis. The sequence regions identified in the M13 DNA inserts are summarized in Table 1.

A number of methods are available for the covalent coupling of DNA to solid supports, and these have been systematically compared by Bünemann et al (20). Two of the methods, namely the use of CNBr-Sepharose (21) or the use of diazotized cellulose

Table 1: Fragments cloned into M13 DNA. The three sub-sections of the Table correspond to the three principal restriction fragments of Fig. 1. Fragment L extends to 182 bases beyond the 3'-terminus of the 16S RNA.

Fragment	Position in pKK3535	Length	Position in 16S RNA
A	1518-1563	46	1-46
B	1564-1730	167	47-213
C	1731-1934	204	214-417
D	1935-2191	257	418-674
E	2132-2251	120	615-734
F	2252-2412	161	735-895
G	2413-2446	34	896-929
H	2447-2656	210	930-1139
J	2657-2724	68	1140-1207
K	2725-2901	177	1208-1384
L	2902-3241	340	1385-1542 (plus 182 bases)

(16,17), appeared to be the best potential candidates for our experiments. However, preliminary experiments with the single-stranded M13 DNA samples showed that, although a higher level of coupling could be achieved with the CNBr-Sepharose method (cf. 22), the diazobenzyloxymethyl cellulose method (16,17) was in fact much better suited to our purposes, for two reasons. First, the hybridization of ^{32}P-labelled ribosomal RNA fragments to DNA coupled to CNBr-Sepharose was very non-specific, a large amount of radioactivity being adsorbed onto the Sepharose in the absence of DNA. In contrast, hybridization to the diazotized cellulose was specific, that is to say it was dependent on the presence of a ribosomal DNA insert in the M13 DNA. Secondly, in our hands the DNA tended to "bleed off" from the CNBr-Sepharose at the hybridization temperature, whereas the DNA coupled to diazotized cellulose was stable. In consequence, the latter method was used for the immobilization of the M13 DNA in the experiments described below.

In order to develop the methodology for the selective hybridization of RNA fragments and cross-linked complexes, we chose as substrate 30S subunits that had been cross-linked by

ultraviolet irradiation in vivo (7). This cross-linking technique is the least prone to artifact, and our previous experiments had indicated that some cross-links in the 3'-half of the 16S RNA were present in the cross-linked mixtures, which we had not so far been able to identify; the cross-links concerned were masked on the two-dimensional gels (as outlined in the Introduction) by the complex series of cross-links already reported (24) from the region covering bases ca. 570 to 750 of the 16S RNA, as well as by the very predominant cross-link between positions ca 497 and 546 (5). For this reason, clones containing DNA regions complementary to the 3'-proximal half of the 16S RNA were used in this series of experiments.

Accordingly, M13 DNA samples from clones F, H, J and K (Table 1 and Fig. 1, complementary to bases 735 to 1384 of the 16S RNA) were immobilized on cellulose, and were hybridized with cobra venom digested, in vivo cross-linked ^{32}P-labelled 16S RNA fragments from 30S subunits, as described in Materials and Methods. In some experiments, sequential hybridizations were made with the same RNA sample, using two or more different M13 DNA probes in succession. After elution from the DNA, the hybridized RNA fragments were separated by two-dimensional gel electrophoresis (1), and examples of the resulting gel patterns are illustrated in Fig. 2. Fig. 2a is the autoradiogram of a control gel from an RNA sample that had not been subjected to the hybridization procedure, and thus contains the whole spectrum of cross-linked complexes resulting from the in vivo irradiation (cf. 7). Figs. 2b and c are the corresponding gels from samples after hybridization to DNA clones K and H, respectively. All the gels show the typical features of a "diagonal" of free RNA fragments, with the cross-linked complexes lying above these diagonals (1,2,5,6). It can be seen that, as expected, the patterns of fragments and complexes from the hybridized samples (Figs. 2b, c) are significantly simpler than that of the control (Fig. 2a).

A number of cross-linked complexes were extracted from gels such as those shown in Figs. 2b and c, and were subjected to oligonucleotide analysis by our usual methodology (1,15). The cross-link sites identified in the complete series of experiments are summarized in Table 2, which also lists the salient features

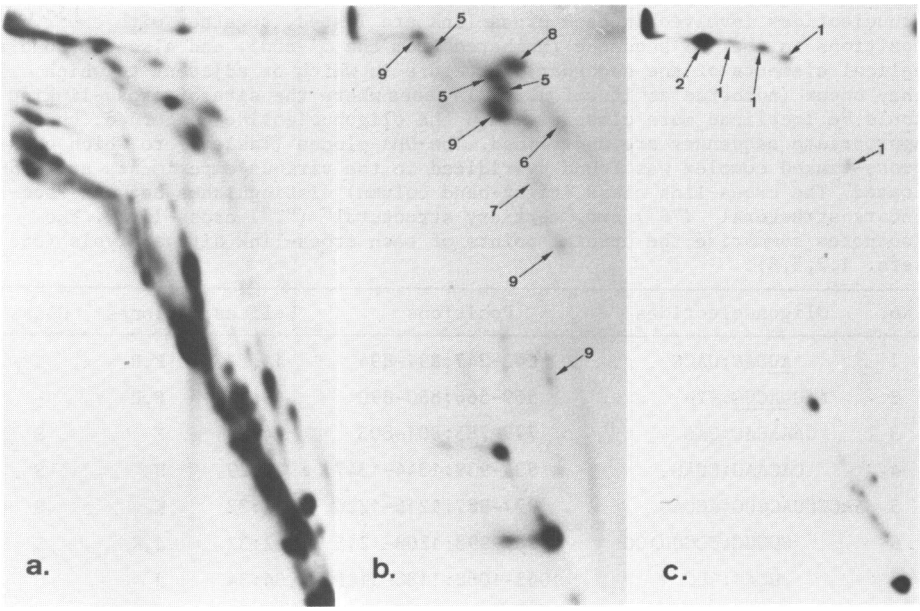

Figure 2: Autoradiograms of two-dimensional gel separations of partially digested cross-linked RNA complexes. (a) Control gel of "total" RNA, not subjected to the hybridization procedure. (b) Gel of fragments and cross-linked complexes isolated after hybridization with immobilized DNA from clone "K" (Table 1). (c) Corresponding gel from clone "H". The numbered spots correspond to the cross-linked complexes listed in Table 2. In each gel the first dimension ran from left to right, and the second from top to bottom.

of each cross-link site analysis. The cross-links are numbered 1 to 9 in Table 2, and the cross-linked spots in Figs. 2b and c are marked correspondingly. (Spots corresponding to cross-links 3 and 4 are not visible on either of the two gel examples shown in Fig. 2). Several of the cross-linked complexes (such as number 9 in Fig. 2b and number 1 in Fig. 2c) appeared in a number of different positions on the gels, according to the length of the RNA fragments involved, and some of the longer complexes were able to hybridize to two or more adjacent DNA clones (see Table 2), depending on the precise conditions of the partial nuclease digestion procedure in the individual experiments. As usual (cf. 1,2,5,6) the cross-links found belonged to two different classes, those that were "secondary structural" (i.e. those occurring

Table 2: Locations of cross-link sites in 16S RNA. The ribonuclease T_1 oligonucleotides involved in each cross-link are listed, together with their positions in the 16S sequence (numbered from the 5'-end), and also the double-helical elements of the secondary structure in which or adjacent to which they occur (numbered as in ref. 23). In cases where the site of cross-linking could be localized more closely within the oligonucleotide concerned, the appropriate sequences are underlined. The DNA clones (Table 1) to which each cross-linked complex was found hybridized in the various experiments are indicated. The cross-link class (right-hand column) distinguishes between "secondary structural" ("S") and "tertiary structural" ("T") cross-links. The footnotes summarize the crucial points of each cross-link site analysis (cf. refs. 1,2,5,6).

No.	Oligonucleotides	Positions	Helices	Clones	Class
1	AUUAG:UACG	243-247:891-894	11:27	F,H	T
2	AAUUACUG:-?-	559-566:850-890	19:-?	F,H	-
3	CAAACAG:UAG	779-785:801-803	24:24	F	S
4	CACAAG:CUAG	934-939:1344-1347	29:29	H	S
5	AACCUUACCUG:ACCAG	977-987:1216-1220	32:32	K	S
6	UCUUG:CCCUUACG	989-993:1208-1215	32:32	J,K	S
7	UCAG:AUG	1065-1068:1188-1190	34:34	J	S
8	UUAAG:CCAG	1090-1094:1161-1164	37:40	H,J,K	T
9	UUG:ACCUCAUAAAG	1125-1127:1280-1290	39:41	H,J,K	T

Footnotes: 1: AUUAGp and UACGp both clearly missing from fingerprints. Secondary digestion of cross-linked spot on fingerprints showed AGp and ACp, but no AUp. 2: AAUUACUGp missing from fingerprints, and AAUp present in secondary digest of cross-linked spot. 3'-component of cross-link site (in the 850-890 region) could not be localized. 3: UAGp missing from fingerprints. CAAACAGp is a tentative assignment as the 5'-component of the cross-link site, as this oligonucleotide was also the 5'-terminus of the RNA fragments in each case. 4: CACAAGp and CUAGp both missing from fingerprint. 5,6: Two closely neighboured cross-links, both in helix 32. In each case the indicated pair of oligonucleotides was missing from the fingerprints. Cross-link 6 is identical to that previously reported (24). 7: UCAGp and AUGp both missing from fingerprints. A neighbouring but not identical cross-link in helix 34 was reported previously (24). 8,9: Both cross-links identical to those previously reported (5).

within or at the end of a single double-helical element of the 16S RNA secondary structure (23,25,26)) or "tertiary structural" (i.e. those connecting two separate secondary structural elements). The cross-links are classified accordingly in Table 2.

The most important cross-link found in this study was cross-link 1 (Table 2), which is a new tertiary structural cross-link between positions 243-247 and 891-894 of the 16S RNA, connecting helices 11 and 27. The absence of AUp in the secondary

digests with ribonuclease A of the cross-linked oligonucleotide from the ribonuclease T_1 fingerprints (cf. 1,2,5,6) enabled the 5'-component of the site to be further narrowed down to the corresponding AU sequence at positions 243-244 (see Table 2). The significance of this cross-link site for the three-dimensional structure of the E. coli 16S RNA (cf. 23) will be discussed below.

Cross-link 1 was very reproducibly observed, and it usually appeared in several positions with RNA fragments of different lengths on the two-dimensional gels, such as that shown in Fig. 2c. The strongest spot on the latter gel, however, corresponded to cross-link 2, for which we were only able to identify the 5'-component of the cross-link site (Table 2). The 3'-component of the site lies within the sequence region 850-890, but could not be localized further, although several versions of this cross-linked complex were found in the various experiments. Since the analysis of an intra-RNA cross-link site involves the simultaneous identification of two separate RNA regions, this type of situation is not uncommon, particularly if one or other component of the cross-link site lies in an unfavourable position in the sequence. Cross-links 3, 4, 5, 6 and 7 are all secondary structural cross-links, the first two (in helices 24 and 29 (23,25), respectively) not having been previously observed. Cross-links 5 and 6 were both within helix 32, cross-link 5 being "new", whereas cross-link 6 is identical to one previously reported (24). Similarly, cross-link 7 in helix 34 was neighboured to but not identical with another cross-link previously observed (24) in this latter helix (Table 2). Cross-links 8 and 9 were identical to the two tertiary cross-links described by Atmadja et al (5), with cross-link 9 appearing at several positions on the gels (Fig. 2b), as already mentioned.

DISCUSSION

The results described here demonstrate that the use of the M13 clones does indeed lead to simpler electrophoretic patterns of cross-linked products and hence to a simplified analysis of the individual cross-linked complexes. The method should prove particularly useful with the 50S subunit (cf. 1,2), or with

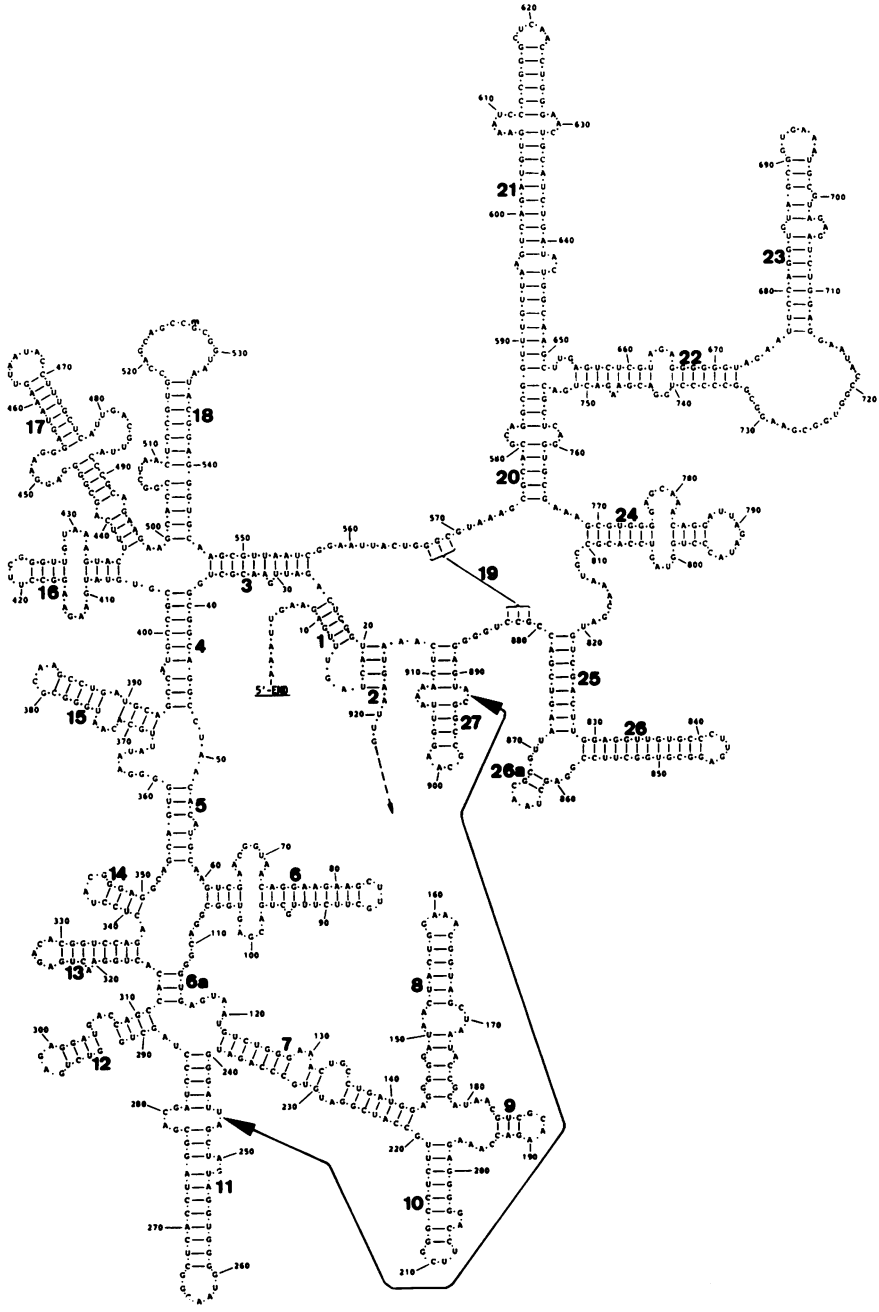

Figure 3: The 5'-half of the secondary structure of E. coli 16S RNA (23,25), showing the location of cross-link 1 (Table 2).

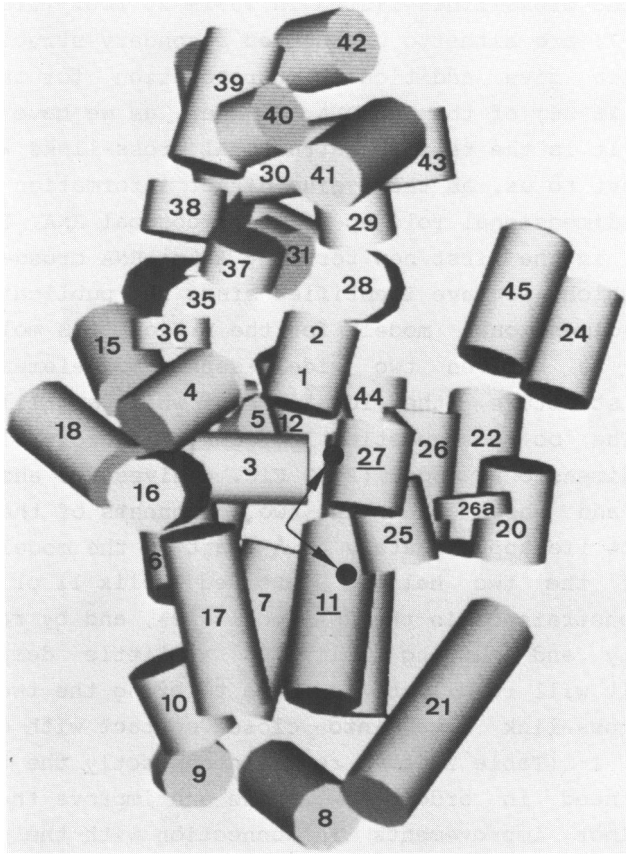

Figure 4: The three-dimensional model of E. coli 16S RNA (23), showing the location of cross-link 1 (Table 2). The black circles denote the positions of the cross-linked nucleotides, the two positions being 40 Å apart from one another in three dimensions. The orientation of the model is 315° (cf. ref.23).

mixtures of complexes obtained by cross-linking with bi-functional chemical reagents (cf. 1,6), where the electrophoretic patterns are correspondingly more complicated than those from ultraviolet irradiated 30S subunits (Fig. 2), and where we have so far only been able to identify a few of the many cross-link sites involved. Experiments with such systems are currently in progress, using the M13 clones described here together with a similar set of clones complementary to the 23S RNA, and their results will be reported in due course.

Among the cross-links listed in Table 2, four (cross-links 3, 4, 5 and 7) are hitherto unobserved secondary structural cross-links, which give additional corroboration for the secondary structure (25,26) of the 16S RNA. However, as we have often noted (1,2,5,6), it is the tertiary structural cross-links which are of most interest to us, as these give direct information relating to the three-dimensional folding of the ribosomal RNA. Cross-link 1 (Table 2) is the first new tertiary intra-RNA cross-link in the 16S RNA which we have identified since the publication (23) of our three-dimensional model for the E. coli 16S molecule. This cross-link is between two widely-separated elements of the secondary structure, the locations of which are illustrated in Fig. 3. The obvious question is: does this cross-link fit with the three-dimensional model (23)? Fig. 4 gives the answer to this question, and shows that the two components of the cross-link site in fact lie approximately 40 Å apart in the model structure. Neither of the two helices involved (helix 11 or 27) is very tightly constrained in the RNA model (23), and by raising helix 11 slightly and setting helix 27 a little deeper into the structure it will readily be possible to bring the two components of the cross-link site into close contact with one another. Cross-link 1 (Table 2) thus represents exactly the type of data which we need in order to refine and improve the 16S model. Similar minor improvements in connection with the placement of some RNA-protein cross-link sites have been proposed elsewhere (27), and we intend to publish a refined set of coordinates for the RNA helices in the model, as soon as this is justified by the accumulation of a sufficient number of such modifications based on new data.

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+To whom correspondence should be addressed

*Present address: Department of Endocrinology, Queen Elizabeth Hospital, Birmingham, UK

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