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The topography of the 3′-terminal region of *Escherichia coli* 16S ribosomal RNA; an intra-RNA cross-linking study

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ABSTRACT

30S ribosomal subunits, 70S ribosomes or polysomes from *E. coli* were subjected to mild ultraviolet irradiation, and the 3′-terminal region of the 16S RNA was excised by 'addressed cleavage' using ribonuclease H in the presence of suitable complementary oligodeoxynucleotides. RNA fragments from this region containing intra-RNA cross-links were separated by two-dimensional gel electrophoresis and the cross-link sites identified by our standard procedures. Five new cross-links were found in the 30S subunit, which were localized at positions 1393 – 1401 linked to 1531 – 1532, 1393 – 1401 linked to 1506, 1393 – 1401 to 1502 – 1504, 1402 – 1403 to 1498 – 1501, and 1432 to 1465 – 69, respectively. In 70S ribosomes or polysomes the first four of these were absent, but instead two cross-links between the 1400-region and tRNA were observed. These results are discussed in the context of the tertiary folding of the 3′-terminal region of the 16S RNA and its known functional significance as part of the ribosomal decoding centre.

INTRODUCTION

The functional importance of the last 150 nucleotides at the 3′-end of *E.coli* 16S ribosomal RNA has been established by a number of different methods. The Shine–Dalgarno region of mRNA is well known to interact with the extreme 3′-terminus of the 16S RNA (1), and specific sites of cross-linking to mRNA have been identified within the 3′-terminal 30 bases (2) as well as between positions 1392 and 1408 (3,4). The anticodon loop of P-site bound tRNA has been cross-linked to position C-1400 (5), and 'foot-print' sites for both P- and A-site bound tRNA have been observed in this region of the RNA structure (6). Sites of mutation conferring resistance to various antibiotics, and also foot-print sites for antibiotics, have been located in this area (7), as well as a number of nucleotides involved in the transition from the active to the inactive form of the 30S subunit (8). From a structural point of view, the majority of the modified bases in the *E.coli* 16S RNA are concentrated within the last 150 nucleotides (9), and parts of the region are located at the ribosomal subunit interface, as shown both by protection studies (e.g. 10) and by direct cross-linking to the 23S RNA (11). Several tertiary interactions between bases in single-stranded regions of this area of the 16S RNA have been postulated, on the basis of phylogenetic comparisons (12).

In an attempt to gain more insight into the overall topography of the 3′-terminal region in its different functional states, we have carried out an intra-RNA cross-linking study, using 30S subunits, 70S ribosomes or polysomes as substrates. Cross-links were introduced by mild ultraviolet irradiation, and (after isolation of 30S subunits in the latter two cases, and deproteinization) the 3′-region of the 16S RNA was examined by the 'addressed cleavage' technique with ribonuclease H. In this method, which has already been successfully applied to the analysis of intra-RNA cross-links in 23S RNA, induced either by ultraviolet irradiation (13) or bifunctional reagents (14), any desired region of the RNA can be excised by digestion with ribonuclease H in the presence of synthetic deoxyoligonucleotides complementary to the appropriate sequence(s) in the ribosomal RNA. The RNA fragments from the region of interest containing intra-RNA cross-links are then separated by gel electrophoresis, and the sites of cross-linking analysed by standard procedures (13–15). From the experimental data obtained we were able to identify several new cross-links in 30S subunits, most of which correspond closely to the tertiary interactions (12) mentioned above. However, in both 70S ribosomes and polysomes, the majority of these cross-links were suppressed, and instead cross-links to tRNA were observed.

MATERIALS AND METHODS

The preparation and ultraviolet irradiation of ^{32}P-labelled 30S ribosomal subunits from *E.coli* strain MRE 600 followed our standard protocols (15,16). For the corresponding preparation of cross-linked 70S ribosomes or polysomes, the *E. coli* cells were disrupted in a buffer containing 10 mM magnesium, and 70S and polysome fractions were separated by sucrose gradient centrifugation at 6 mM magnesium, as described in ref. 11. Here the ultraviolet irradiation (15) was carried out for 5 min directly on the appropriate sucrose gradient fractions (cf. ref. 11) with

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a solution depth of 3 mm at a concentration of 1.5 A_{260} units/ml; this irradiation dose was equivalent to that applied to the 30S subunits (cf. ref. 15). (In the case of the more dilute polysome fractions, the optical density was adjusted to this value by addition of unlabelled 70S ribosomes). After an ethanol precipitation step, the cross-linked 70S ribosomes or polysomes were re-isolated by a second sucrose gradient centrifugation at 6 mM magnesium. The 30S subunits were then isolated by a final sucrose gradient centrifugation under dissociating conditions (15).

In all cases, ribosomal proteins were removed from the cross-linked 30S subunits by digestion with proteinase K followed by phenol extraction (15). The isolated cross-linked 16S RNA was digested with ribonuclease H in the presence of one or two appropriate decadeoxynucleotides (13, 15, and see Results), and after a further proteinase K digestion and phenol extraction, the RNA fragments were separated by two-dimensional gel electrophoresis (16). Cross-linked RNA complexes were located on the gels by autoradiography, extracted in the usual manner (16), and subjected to total digestion with ribonuclease T_1 or A (15). The oligonucleotides released were separated by two-dimensional chromatography on polyethyleneimine cellulose plates, using the solvent system of Volckaert and Fiers (17) or our 'alternative' system (15). Secondary digestions of the isolated oligonucleotides were made in the usual way (15), and the oligonucleotide data were fitted to the 16S RNA sequence of Brosius et al. (18).

RESULTS

70S ribosomes or polysomes show a tendency to dissociate into subunits during ultraviolet irradiation, but our usual mild dose of irradiation (cf. ref. 15) does not show a serious dissociative effect. Nevertheless the irradiated sucrose gradient fractions containing the cross-linked 70S ribosomes or polysomes were subjected to a second sucrose gradient separation at 6 mM magnesium (see Materials and Methods), in order to remove any small amounts of subunits formed during the irradiation step. This ensured that the cross-links observed were indeed those present in the intact ribosomes, and were not the products of cross-linking reactions occurring in the dissociated subunits. For the experiments with isolated 30S subunits, a similar dose of irradiation was given. As a result of these mild doses, the yields of cross-links formed were low, but the pattern of products was nonetheless very reproducible.

The cross-linked 16S RNA isolated from irradiated 70S ribosomes, polysomes or 30S subunits was cleaved with ribonuclease H together with a decadeoxynucleotide complementary to bases 1382–1391 of the 16S RNA, leading to the release of an RNA fragment approximately 155 bases long (cf. refs. 13–15), comprising the 3′-terminal region of the 16S

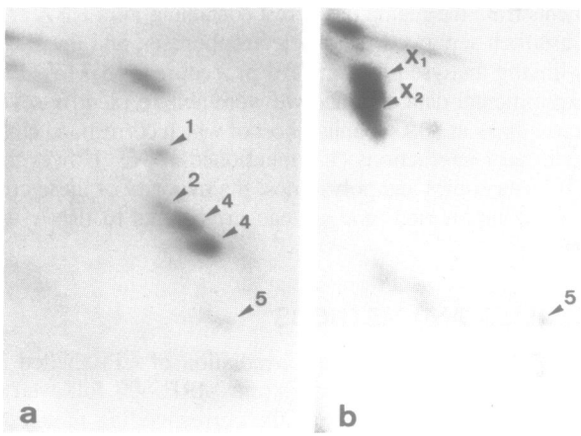

Figure 1. Two-dimensional gel electrophoretic separation of cross-linked complexes from the 3′-terminal 155 nucleotides of 16S RNA. *a*. Complexes from cross-linked 30S subunits. *b*. Complexes from cross-linked 70S ribosomes. Direction of electrophoresis is from left to right (1st dimension) and from top to bottom (2nd dimension). Only the gel region containing the intra-RNA cross-linked fragments is shown. The complexes analysed are marked 1 to 5, and X_1,X_2 and 5 in (a) and (b), respectively (see text and Table 1). Complex 5 was identical in both gels, and thus serves as a 'position marker'. Complex 4 (gel a) often split into two components, as in this case. The first dimension gels were of 5% polyacrylamide, the second dimension gels 12% (16).

Table 1. Location of intra-RNA cross-link sites in the 3′-region of the 16S RNA. Sequences and positions of oligonucleotides implicated as being involved in the cross-links from ribonuclease T_1 or A fingerprint data are given; missing T_1-oligonucleotides are indicated by underlining, missing A-oligonucleotides by overlining. The precise positions of the cross-linked nucleotides, insofar as they could be determined, are doubly underlined. (In some cases it cannot be excluded that the actual cross-link site is one base 'upstream' or 'downstream' from the position shown, as a result of resistance to enzymatic digestion caused by the cross-link). The substrate in which each cross-link was observed is indicated. Brief summaries of the crucial features of each cross-link site analysis are given as footnotes to the Table.

No.	Cross-linked oligonucleotides		Appearance within		
	Sequences	Positions	30S	70S	Polysomes
1	UACACACCG:GGAUCACCUCCUUA	1393-1401:1529-1542	+	-	-
2	UACACACCG:GGUAACCG	1393-1401:1504-1511	+	-	-
3	UACACACCG:mUAACAAGGU	1393-1401:1498-1506	+	-	-
4	mCmCCG:mUAACAAG	1402-1405:1498-1504	+	-	-
5	CAAAAGAAG:CUUACCACUUUG	1427-1435:1462-1473	+	+	+
X_1	UACACACCG: tRNA	1393-1401: ?	-	+	+
X_2	UACACACCG: tRNA	1393-1401: ?	-	+	+

Cross-link 1: UACACACCGp (1393–1401) and the 3′-terminal nucleotide AUCACCUCCUUA (1531–1542) were clearly absent from the ribonuclease T_1 fingerprint, whereas GGAUp (1529–1532) was missing from the corresponding ribonuclease A fingerprint. Thus the cross-link site was localized to UACACACCG (1393–1401) linked to AU (1531–1532).

Cross-link 2: UACACACCGp (1393–1401) and UAACCGp (1506–1511) were absent from the T_1-fingerprint, and GGUp (1504–1506) from the A-fingerprint. The 3′-component of the cross-link site is thus U-1506, and the 5′-component lies within UACACACCGp (1393–1401), as in cross-link 1.

Cross-link 3: This was only seen in one experiment, and is therefore assigned as tentative. Here again the 5′-component lay within positions 1393–1401, the 3′-component being defined by the absence of mUAACAAGp (1498–1504) from the T_1-fingerprint and AAGGUp (1502–1506) from the A-fingerprint; the 3′-component must therefore lie within the sequence AAG (1502–1504).

Cross-link 4: Two modified oligonucleotides were missing from the T_1-fingerprint, namely mCmCCGp (1402–1405) and mUAACAAGp (1498–1504). Secondary digestion of the cross-linked oligonucleotide showed the absence of the indigestible residues mCmC (1402–1403) and mUAAC (1498–1501), which thus defines these positions as the two components of the cross-link site.

Cross-link 5: The adjacent T_1-oligonucleotides CAAAAGp (1427–1432) and AAGp (1433–1435) were missing from a 'normal' T_1-fingerprint, thus defining the 5′-component of the cross-link site as G-1432. Using the 'alternative' fingerprint system (15), CUUACCACUUUGp (1462–1473) was also found to be absent, and from the weakness of the ACp spot in the secondary digest of the cross-linked oligonucleotide it was concluded that the 3′-component of the site lay within the sequence ACCAC (1465–1469).

Cross-links X_1 and X_2: The T_1-oligonucleotide UACACACCGp was absent in both cases, thus defining the cross-link site on the 16S RNA. The other component of the cross-link was to unidentified heterogeneous sites on tRNA (see text).

RNA. The RNA fragments from this region containing intra-RNA cross-links were separated from the rest of the RNA by two-dimensional gel electrophoresis, and typical examples of these gels are given in Figure 1. Figure 1a is the autoradiogram obtained from irradiated 30S subunits, whereas Figure 1b is that from 70S ribosomes; no differences were observed between the patterns from 70S ribosomes and polysomes. Only the gel region corresponding to the cross-linked RNA fragments is shown in Figure 1 (for examples of the 'complete' gel patterns, including the 'diagonal' of non-cross-linked RNA fragments, see refs. 13 or 14).

In the gels from 30S subunits, four cross-linked complexes were reproducibly observed, marked 1, 2, 4 and 5 in Figure 1a. Complex 4 usually appeared as two spots, which gave identical results in the subsequent oligonucleotide analysis; this presumably represents a heterogeneity at the cross-link site which is below the limits of resolution of our analysis. In one experiment an additional spot (cross-link 3, see Table 1) was observed in the

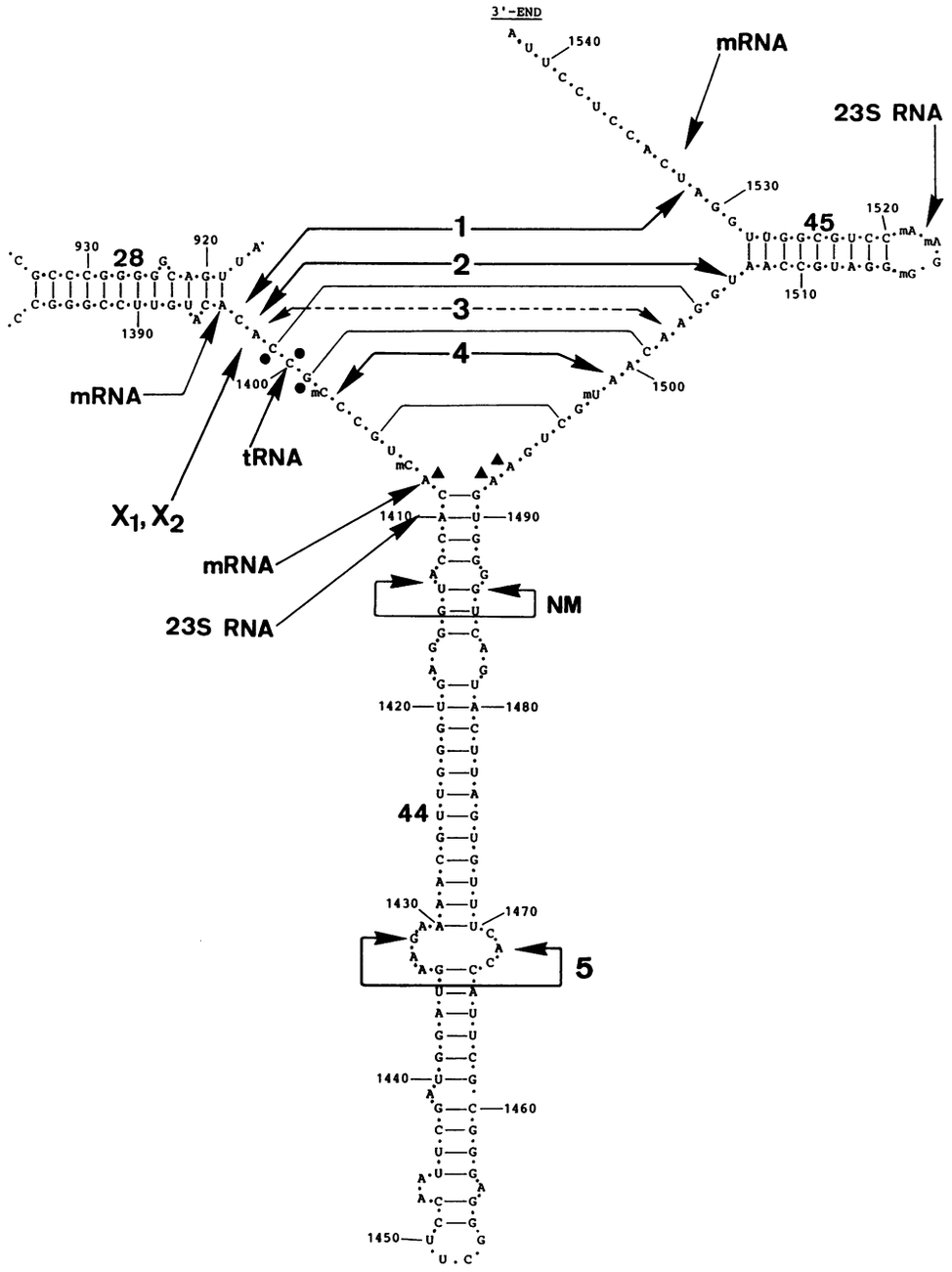

Figure 2. Secondary structure of the 3'-terminal region of 16S RNA, indicating cross-link sites and other topographical data. The helical regions are numbered as in ref. 20. The positions of cross-links 1 to 5 (Table 1) and X_1, X_2 are indicated by lines with arrowheads, the closeness of the arrowhead to the sequence region concerned depending on the precision of the cross-link site analysis (see text and Table 1). Cross-link 3 (dotted line) was only observed in one experiment. 'NM' denotes a cross-link site previously found by treatment with the bifunctional reagent 'nitrogen mustard' (21). Positions of cross-link sites to 23S RNA (11), mRNA (2–4), and the anticodon loop of tRNA (5) are indicated. Foot-print sites (6) of A-site and P-site bound tRNA are denoted by triangles and circles, respectively. Tertiary interactions in the RNA (12) are indicated by thin lines without arrowheads.

same region of the gel. The remaining radioactive spots (unmarked) which can be seen in Figure 1a correspond to fortuitous digestion products from other regions of the 16S RNA. In particular, fragments containing the previously observed high-yield ultraviolet-induced cross-link (19) between positions 497 and 545–548 of the 16S RNA often appeared as contaminants in this region of the gel; these were not considered further. In contrast to the gels from 30S subunits, the gels from 70S ribosomes or polysomes showed no trace of the radioactive complexes 1, 2, 3 or 4, but instead two new cross-linked complexes appeared, denoted X_1 and X_2 in Figure 1b. Complex 5, however, was still present in comparable amounts.

Oligonucleotide analysis showed that complexes 1–5 did indeed contain the 3′-terminal 155 nucleotides of the 16S RNA, with no other sequence regions present. On the other hand complexes X_1 and X_2 (Figure 1b) contained additional sequences, which appeared in the form of a number of extra spots in sub-molar amounts on the oligonucleotide fingerprints. Secondary digestions of these extra oligonucleotides indicated that they did not arise from either 16S, 23S or 5S ribosomal RNA, and we concluded that heterogeneous cross-links to various tRNA species were involved, for the following reasons. First, the mobility of complexes X_1 and X_2 in the first-dimension gel corresponded to a chain length of ca. 230 nucleotides (thus corresponding to the 3′-terminal 155 nucleotides of 16S RNA plus tRNA). Secondly, the appearance of X_1 and X_2 as discrete spots in Figure 1b is not consistent with a cross-linking reaction with mRNA fragments, as in that case a very heterogeneous chain-length would be expected. Thirdly, some of the 'extra' oligonucleotides on the fingerprints of complexes X_1 and X_2 gave secondary digestion products which were sufficiently characteristic to be assigned to tRNA sequences.

The results of a number of oligonucleotide analyses of complexes 1 to 5 and of X_1 and X_2 are summarized in Table 1. The salient features of these analyses, with regard to the identification of the cross-link sites within the individual complexes, are given as footnotes to the Table. In the case of complexes 1 to 5, an additional series of experiments was performed, in which the 16S RNA was cleaved with a decadeoxynucleotide complementary to positions 1449–1458 of the 16S RNA as well as that complementary to positions 1382–1391; this second oligodeoxynucleotide cuts the RNA at a position midway between the 5′- and 3′-components of the cross-link sites (see Figure 2), and the gel patterns and subsequent oligonucleotide analyses obtained from these digests provided extra confirmation of the cross-links.

DISCUSSION

Figure 2 shows the positions of the intra-RNA cross-links of Table 1—together with other relevant topographical data—in the secondary structure of the 3′-terminal region of 16S RNA (20,22). It can be seen that cross-links 2 and 4 form connections between the single-stranded sequences flanking helix 44, and thus corroborate the tertiary interactions which have been proposed in this area (12). Cross-link 3, although only seen once, also fits to this pattern and is therefore tentatively included in the Figure. Cross-link 1 introduces a new constraint on the structure, by forming a connection to the single-stranded sequence on the 3′-side of helix 45. In contrast, cross-link 5—similarly to the 'nitrogen mustard' cross-link previously observed (21)—lies within helix 44. (It is noteworthy that cross-links 1 to 3 all involve the nonanucleotide UACACACCGp (positions 1393–1401, see Table 1), which—due to the three tandemly repeated AC sequences—is unfavourable for our analytical methods, and does not permit a more precise localization of the 5′-component of the cross-link sites in these cases).

Further constraints on this region of the 16S RNA are provided by the interface cross-links of Mitchell et al. (11), which identified positions 1408–1411 and the loop-end of helix 45 as being connected to the same oligonucleotide of 23S RNA; these two sites in the 16S RNA must therefore lie close together on the interface side of the 30S subunit. The interface cross-links (11) correspond well with the 'bridge' between the 30S and 50S subunits observed by Frank et al (23) in their recent three-dimensional reconstruction from electron micrographs of 70S ribosomes in amorphous ice. However, a location of these 16S RNA regions at the interface is in contradiction to the observations by DNA hybridization microscopy of Oakes et al. (24), who placed both the '1400' and '1500' sites on the opposite (i.e. solvent) side of the 30S subunit.

It is interesting that cross-links 1 to 4 were only seen in 30S subunits. These cross-links were entirely absent in both 70S ribosomes and polysomes, and were 'replaced' in each case by the cross-links X_1 and X_2 to tRNA (Figure 1, Table 1). It is often assumed that 70S ribosome preparations represent 'vacant couples', but this is clearly not the case here, as tRNA is obviously present and no difference was seen between the cross-linking patterns in 70S ribosomes and polysomes. (This is consistent with the observations of Remme et al (25), who measured the tRNA content of ^{32}P-labelled 70S ribosomes and polysomes, and found ca. 0.6 and 2.0 mols of tRNA, respectively.) More important is the fact that this is the first time that we have observed a significant difference in the cross-linking patterns of ribosomes or subunits in different functional states. Cross-links X_1 and X_2 most probably correspond to the C-1400 P-site cross-link of Prince et al. (5), but—as with cross-links 1 to 3—the 'unfavourable' oligonucleotide at positions 1393–1401 is involved, which prevented us from making a more precise localization of the site on the 16S RNA. It could be argued that the disappearance of cross-links 1 to 4 in 70S ribosomes or polysomes is a simple 'shielding' effect of the 50S subunit. However, this seems unlikely, because helix 44 is located at the subunit interface (see the above discussion), and cross-link 5—which lies within this helix—was found in comparable amounts in all cases (compare Figures 1a and 1b). More plausible is the possibility that cross-links 1 to 4 are prevented either by shielding from the tRNA itself, or by local re-arrangements of the 16S RNA caused by tRNA binding. As already mentioned in the Introduction, a number of bases in this area of the RNA are involved in the transition from the active to the inactive state of the 30S subunit (8). On the other hand, the phylogenetically conserved tertiary interactions (12)—which cross-links 1 to 4 corroborate—are unlikely to merely represent a functionally inactive configuration, and we believe that the different patterns of cross-linking reported here represent two subtly different but both functionally important states of the ribosome. Such a situation would be consistent with (for example) the 'two-state' model proposed by Kössel et al. (26), although—as just mentioned—a simple shielding effect cannot be entirely excluded.

Holbrook et al. (27) have recently shown that unpaired bases within an RNA helix preserve a loosely helical structure, and it is plausible to propose that the single-stranded regions flanking helix 44 (bases 1397–1408 and 1492–1506) are organized into

such a type of loose helical array. If this is the case, then the data from 'site-directed cross-linking' experiments with mRNA (2–4) can also be incorporated logically into the structure. Such a cross-link has been observed from position '+4' of the mRNA (i.e. the 5'-base of the A-site codon) to position 1408 of the 16S RNA (3), which fits well with the A-site tRNA foot-print (6) at positions 1408 and 1492–1493 (cf. Figure 2). The corresponding P-site foot-print (6) is at positions 1399–1401, in agreement with the C-1400 cross-link to the anticodon loop of P-site bound tRNA (5). On the other hand an mRNA cross-link to position 1396 of 16S RNA was observed from position '+7' of the mRNA (3, and cf. 4), which—since this is 'downstream' on the mRNA from the '+4' site just mentioned—would appear at first sight to be contradictory. The same would appear to be true for the cross-links observed from positions on the 5'-side of the P-site codon of the mRNA (2), which were identified as lying within the 20 to 30 nucleotides at the extreme 3'-terminus of the 16S RNA (Figure 2). However, in a helical arrangement as suggested above, it does in fact seem possible to satisfy all these cross-links in a single structure. A revised three-dimensional model of the 16S RNA (cf. refs. 22, 28), which incorporates all these considerations, is currently being constructed in our laboratory and will be published in due course.

REFERENCES

1. Shine, J. and Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. USA 71*, 1342–1346.
2. Stade, K., Rinke-Appel, J. and Brimacombe, R. (1989) *Nucleic Acids Res. 17*, 9889–9908.
3. Tate, W., Greuer, B. and Brimacombe, R. (1990) *Nucleic Acids Res. 18*, 6537–6544.
4. Rinke-Appel, J., Jünke, N., Stade, K. and Brimacombe, R. (1991) *EMBO J. 10*, 2195–2202.
5. Prince, J.B., Taylor, B.H., Thurlow, D.L., Ofengand, J. and Zimmermann, R.A. (1982) *Proc. Natl. Acad. Sci. USA 79*, 5450–5454.
6. Moazed, D. and Noller, H.F. (1990) *J. Mol. Biol. 211*, 135–145.
7. Moazed, D. and Noller, H.F. (1987) *Nature 327*, 389–394.
8. Moazed, D., Van Stolk, B., Douthwaite, S. and Noller, H.F. (1986) *J. Mol. Biol. 191*, 483–493.
9. Carbon, P., Ehresmann, C., Ehresmann, B. and Ebel, J.P. (1979) *Eur. J. Biochem. 100*, 399–410.
10. Gornicki, P., Baudin, F., Romby, P., Wiewiorowski, M., Kryzosiak, W., Ebel, J.P., Ehresmann, C. and Ehresmann, B. (1989) *J. Biomol. Struct. Dynam. 6*, 971–984.
11. Mitchell, P., Oßwald, M. and Brimacombe, R. (1992) *Biochemistry*, in press.
12. Haselman, T., Camp, D.G. and Fox, G.E. (1989) *Nucleic Acids Res. 17*, 2215–2221.
13. Mitchell, P., Oßwald, M., Schüler, D. and Brimacombe, R. (1990) *Nucleic Acids Res. 18*, 4325–4333.
14. Döring, T., Greuer, B. and Brimacombe, R. (1991) *Nucleic Acids Res. 19*, 3517–3524.
15. Brimacombe, R., Greuer, B., Gulle, H., Kosack, M., Mitchell, P., Oßwald, M., Stade, K. and Stiege, W. (1990) In Spedding, G. (ed.), *Ribosomes and Protein Synthesis: A Practical Approach*, IRL Press, Oxford, pp. 131–159.
16. Brimacombe, R., Stiege, W., Kyriatsoulis, A. and Maly, P. (1988) *Methods Enzymol. 164*, 287–309.
17. Volckaert, G. and Fiers, W. (1977) *Analyt. Biochem. 83*, 228–239.
18. Brosius, J., Palmer, M.L., Kennedy, P.J. and Noller, H.F. (1978) *Proc. Natl. Acad. Sci. USA 75*, 4801–4805.
19. Atmadja, J., Brimacombe, R., Blöcker, H. and Frank, R. (1985) *Nucleic Acids Res. 13*, 6919–6936.
20. Brimacombe, R. (1991) *Biochimie 73*, 927–936.
21. Atmadja, J., Stiege, W., Zobawa, M., Greuer, B., Oßwald, M. and Brimacombe, R. (1986) *Nucleic Acids Res. 14*, 659–673.
22. Stern, S., Weiser, B. and Noller, H.F. (1988) *J. Mol. Biol. 204*, 447–481.
23. Frank, J., Penczek, P., Grassucci, R. and Srivastava, S. (1991) *J. Cell Biol. 115*, 597–605.
24. Oakes, M.I., Kahan, L. and Lake, J.A. (1990) *J. Mol. Biol. 211*, 907–918.
25. Remme, J., Margus, T., Villems, R. and Nierhaus, K.H. (1989) *Eur. J. Biochem. 183*, 281–284.
26. Kössel, H., Hoch, B. and Zeltz, P. (1990) *Nucleic Acids Res. 18*, 4083–4088.
27. Holbrook, S.R., Cheong, C., Tinoco, I. and Kim, S.H. (1991) *Nature 353*, 579–581.
28. Brimacombe, R., Atmadja, J., Stiege, W. and Schüler, D. (1988) *J. Mol. Biol. 199*, 115–136.