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Mapping the path of the nascent peptide chain through the 23S RNA in the 50S ribosomal subunit

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ABSTRACT

Peptides of different lengths encoded by suitable mRNA fragments were biosynthesized *in situ* on *Escherichia coli* ribosomes. The peptides carried a diazirine derivative bound to their N-terminal methionine residue, which was photoactivated whilst the peptides were still attached to the ribosome. Subsequently, the sites of photo-cross-linking to 23S RNA were analyzed by our standard procedures. The N-termini of peptides of increasing length became progressively cross-linked to nucleotide 750 (peptides of 6, 9 or 13–15 amino acids), to nucleotide 1614 and concomitantly to a second site between nucleotides 1305 and 1350 (a peptide of 25–26 amino acids), and to nucleotide 91 (a peptide of 29–33 amino acids). Previously we had shown that peptides of 1 or 2 amino acids were cross-linked to nucleotides 2062, 2506 and 2585 within the peptidyl transferase ring, whereas tri- and tetrapeptides were additionally cross-linked to nucleotides 2609 and 1781. Taken together, the data demonstrate that the path of the nascent peptide chain moves from the peptidyl transferase ring in domain V of the 23S RNA to domain IV, then to domain II, then to domain III, and finally to domain I. These cross-linking results are correlated with other types of topographical data relating to the 50S subunit.

INTRODUCTION

During the early stages of its biosynthesis on the ribosome, a nascent peptide has to traverse the large ribosomal subunit from the peptidyl transferase centre to the exit site. Whereas the peptidyl transferase centre is located at the base of the central protuberance of the 50S subunit on the side facing the 30S subunit (1,2), the exit site lies almost diametrically opposite on the solvent side of the 50S subunit (3). The distance between these sites is sufficient for ~30–40 amino acids of the growing peptide to be shielded by the ribosome from attack by proteolytic enzymes (4,5). A great deal of current interest has been focussed on the fate of newly synthesized proteins as they exit from the ribosome (6–8); however, apart from an older study in which cross-links to 50S ribosomal proteins from a set of pre-synthesized peptide affinity analogues were analyzed (9), very little information is available concerning those ribosomal components which form the environment surrounding the growing peptide chain as it passes through the large subunit. In an attempt to remedy this situation, we recently began a systematic investigation of the path of the nascent peptide, using peptide affinity analogues synthesized *in situ* on the *Escherichia coli* ribosome (10). It has been shown (11) that the peptidyl transferase centre is rich in RNA, and therefore our interest has concentrated on the analysis of contacts or neighbourhoods between the peptide and the 23S RNA; we were able to demonstrate that peptides of 1–3 amino acids in length were cross-linked to several sites within the peptidyl transferase ring in Domain V of the 23S RNA, whereas a peptide four amino acids long became cross-linked to an additional site in another secondary structural domain [domain IV, (10)]. In this paper we have extended this study to peptides of up to ~30 amino acids.

The affinity reagent we have used is again the *N*-hydroxysuccinimide ester of 4-(3-trifluoromethyl diazirino)-benzoic acid (12,13), attached to the α-NH_2 group of the N-terminal methionine residue. Our previous results (10) had shown that there was a considerable degree of overlap between the cross-link sites observed from peptides differing in length by only one amino acid, which is not surprising since the length of the diazirine reagent (~7 Å) is almost twice that of a single peptide residue (~4 Å). Accordingly, in this series of experiments we have examined peptides differing in length by several amino acids, and we describe a set of cross-links to the 23S RNA from the N-termini of peptides containing 6, 9, 13–15, 25–26 and 29–33 residues. In each case the whole 23S molecule was screened for the presence of cross-links by our standard procedure involving digestion with ribonuclease H (14). The results showed that the N-terminus of the growing peptide makes a series of dramatic leaps within the secondary structure of the 23S RNA, which not only impose important constraints on the three-dimensional folding of the RNA, but which also correlate closely with other types of topographical data. Furthermore, with each peptide some—but not all—of the cross-links observed with correspondingly shorter peptides continued to be observed. This suggests that the growing peptide is very flexible as it passes through the 'tunnel' (15–17) or 'channel' (18) which various authors have proposed for the peptide path within the 50S subunit.

MATERIALS AND METHODS

Preparation of mRNA

mRNA A (Fig. 1) was prepared by T7-transcription from a synthetic DNA template as described previously (10). For mRNA

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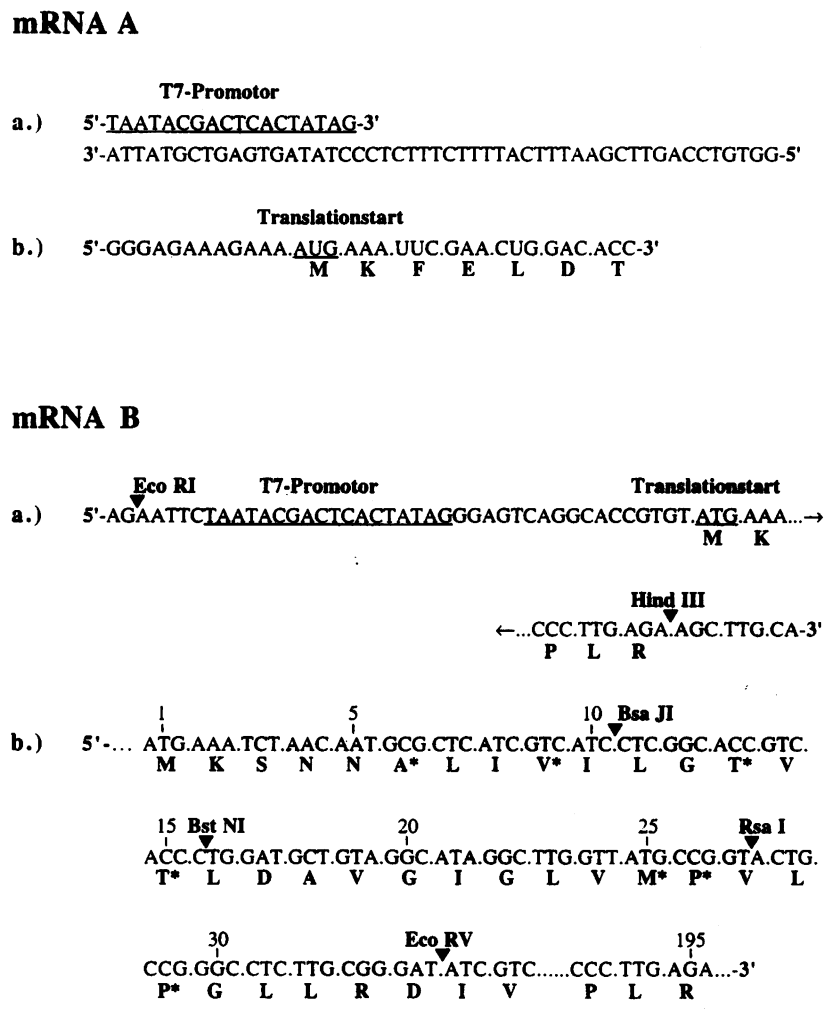

Figure 1. mRNA sequences used for peptide synthesis. mRNA A, the DNA sequence is shown (a), together with (b) the mRNA sequence obtained by T7 transcription (10); the encoded amino acids are indicated below the sequence. mRNA B, the sequence in (a) shows the regions at the ends of the tetracycline resistance gene fragment, indicating the positions of the restriction sites. The sequence in (b) is the corresponding coding sequence, again with restriction sites and encoded amino acids indicated. Amino acids close to the C-termini of the various peptides that were radioactively labelled are marked with asterisks (see text).

B (Fig. 1), DNA from the plasmid pBR 322—which carries the tetracycline resistance gene (19,20)—was amplified by PCR using the oligodeoxynucleotides AGAATTCTAATACGACTCACTA-TAGGGAGTCAGGCACCGTGTATG and TGCAAGCTTCT-CAAGGGCATCGGTCGACG as primers; both of these carry a restriction site at their 5'-ends, and the former contains in addition the T7 promoter sequence (underlined). The isolated DNA fragment was cut with *Eco*RI and *Hin*dIII (cf. Fig. 1), and cloned into the vector pUC 19, after restricting the latter with the same two enzymes, using standard procedures (21). Following growth in DH 5α *E.coli* cells, the pUC 19 plasmid DNA was isolated and linearized with either *Bsa*JI, *Bst*NI, *Rsa*I or *Eco*RV (Fig. 1), to generate the appropriate cuts within the tetracycline resistance gene sequence. The DNA was then transcribed with T7-polymerase, and the RNA isolated as previously described (22).

Biosynthesis of peptides

Individual tRNA species (Subriden RNA, USA; Sigma Chemicals) or bulk tRNA were charged with the appropriate amino acids using tRNA-free S-150 enzymes (23). Met-tRNA^{fMet} was either formylated, or derivatized at the α-NH_2 group with the diazirine derivative as before (10). Lys-tRNA^{Lys} was derivatized at the ε-NH_2 group according to the procedure of Görlich *et al.* (24). The N-terminal methionine residue was labelled either at high or intermediate specific activity with ^{35}S, and in addition a C-terminal proximal amino acid (see text) was labelled with ^{3}H, exactly as described previously (10). In the case of the peptide with 25–26 amino acids, the labels were 'reversed', the C-terminal methionine being labelled with ^{35}S, and the diazirine-derivatized N-terminal methionine with ^{3}H. Reaction mixtures for peptide synthesis were prepared as before (10,23), using 70S tight couple ribosomes from *E.coli* together with the appropriate T7-transcribed mRNA (see above); mixtures of individual charged tRNA species were added for the synthesis of peptides up to six amino acids in length, and charged bulk tRNA for longer peptides (see text). Activation of the diazirine reagent by irradiation at 350 nm was carried out as before (13). Control samples were prepared in which the diazirine-derivatized tRNAs had been pre-irradiated before incubation with ribosomes.

Isolation of cross-linked products, and analysis of sites of cross-linking on 23S RNA

Ribosomal RNA cross-linked to peptidyl-tRNA was isolated by two sucrose gradient centrifugation steps followed by phenol extraction, as before (10). Localization of the cross-link sites was performed by ribonuclease H digestion in the presence of oligodeoxynucleotides and by primer extension analysis, using our standard procedures (25,26).

RESULTS

In this investigation we have modified the methodology that was used in our previous cross-linking studies with short peptides up to four amino acids in length (10). For the latter experiments, the peptides were encoded by an mRNA analogue transcribed with T7-polymerase from a synthetic oligodeoxynucleotide template, using the method of Lowary *et al.* (27). Peptide growth was controlled simply by supplying only those individual aminoacyl tRNA species required for the peptide concerned, and the diazirine reagent was attached either to the α-NH_2 group of the N-terminal methionine residue, or to the ε-NH_2 group of an adjacent lysine residue; in the latter case the N-terminal methionine was formylated. The N-terminal methionine was labelled with ^{35}S and in addition the C-terminal amino acid of each peptide was labelled with ^{3}H, in order to provide a positive test for the presence of the expected 'full-length' peptide in the subsequent analyses of the cross-linked tRNA–peptide–23S RNA complexes. The sequences of the DNA template used (10) and of the corresponding mRNA analogue are illustrated in Figure 1 (mRNA A).

For longer peptides this method is both cumbersome and expensive, and therefore here we adopted the following strategy (cf. ref. 28). A natural mRNA sequence together with a T7 promoter sequence was introduced into the plasmid pUC 19 as described in Materials and Methods, and the plasmid DNA was linearized with various restriction enzymes, so that—in the subsequent T7-transcription step—mRNA fragments of appropriate lengths could be generated. For this purpose a sequence was required which carried a number of potential restriction sites, and which at the same time encoded a suitably heterogeneous amino acid sequence with a lysine residue adjacent to the N-terminus, as in mRNA A (Fig. 1). The tetracycline resistance gene (19,20) fulfils these requirements, and the relevant part of its sequence—indicating the restriction sites which we exploited in this series of experiments both for the cloning and linearization procedures—is also illustrated in Figure 1 (mRNA B). After T7-transcription of the linearized plasmid DNA, the mRNA fragments were used first to generate 70S ribosomal initiation complexes as described in Materials and Methods, following which protein synthesis was allowed to take place using bulk tRNA charged with an appropriate mixture of amino acids; as before (10), an amino acid at or close to the C-terminus of the peptide encoded by each mRNA fragment was radioactively labelled as well as the N-terminal methionine. In most experiments the diazirine derivative (12) was attached to the N-terminal methionine, although in some cases—again as before—we also made use of the ε-NH_2 group of the adjacent lysine, so as to be able to generate diazirine-derivatized peptides with a natural *N*-formyl methionine terminus. Since the amino acid sequences encoded by mRNA A and mRNA B are different, one series of cross-linking experiments (see below) was made with a hexapeptide from both mRNA species, so as to 'connect' the data sets from the two mRNAs.

After peptide synthesis, the ribosomal complexes were irradiated with UV light at 350 nm (13) to activate the diazirine group, and the 23S RNA cross-linked to peptidyl tRNA was isolated by two sucrose gradient centrifugation steps followed by phenol extraction (10,22). No cross-linking to 16S RNA was observed. Measurement of the radioactivity associated with the 70S ribosomes after the first sucrose gradient step showed that ~20% of the ribosomes had participated in peptide synthesis (cf. ref. 10). Similar measurements of the radioactivity remaining associated with the 23S RNA after phenol extraction indicated that 2–4% of the bound peptide material became cross-linked, a value which is typical for the diazirine reagents (26).

For the analysis of the cross-link sites on the 23S RNA, the cross-linked tRNA–peptide–23S RNA complexes were first subjected to digestion with ribonuclease H in the presence of pairs of oligodeoxynucleotides complementary to selected sequences in the 23S molecule (14,25). This first screening was made with a 'standard' set of oligodeoxynucleotides pairs spread across the whole 23S sequence, so that changes in the overall cross-linking pattern with progressively longer peptides could be seen at a glance in the subsequent gel electrophoresis of the ribonuclease H digested material. A typical 'family' of autoradiograms of the ribonuclease H gels for the various peptides examined is illustrated in Figure 2. As always in these experiments (10,25), each cross-linked complex appears as a doublet band, corresponding to a 23S RNA fragment covalently attached either to radioactive peptidyl-tRNA or to radioactive peptide, respectively, the ester bond to the tRNA being partially hydrolysed under the relatively harsh conditions (55°C) of the ribonuclease H digestion. The individual cross-linking patterns for each peptide will be discussed in detail below.

Regions of the 23S RNA found to contain cross-links to peptides in the first screening (Fig. 2) were examined more closely by further ribonuclease H digestions with different sets of oligodeoxynucleotides, so as to narrow down the cross-linked regions as far as possible, and examples of the gels obtained are shown in Figure 3. For each peptide, experiments were made both with a high specific activity of ^{35}S in the N-terminal methionine (so as to obtain strong autoradiograms as in Figs 2 and 3), and with a lower specific activity (so as to be able to accurately monitor the level of ^{3}H-radioactivity from the C-terminal amino acid in the individual cross-linked complexes after excision from the gels (see Materials and Methods and ref. 10). Finally, primer extension analyses were performed (25,29), in order to localize each cross-link site within the 23S RNA sequence region identified by the ribonuclease H method. Examples of these analyses are illustrated in Figure 4, and the complete set of results is summarized in Table 1. Since the intensities of the individual cross-links tended to vary somewhat from one experiment to another, the data in Table 1 are divided into three categories only, *viz.* (a) cross-links that were reproducibly observed with the radioactive C-terminal amino acid clearly present [indicating involvement of the full-length peptide (cf. ref. 10)], (b) cross-links that were reproducibly absent, and (c) cross-links that were only occasionally or very weakly present. In the latter case the radioactivity corresponding to the C-terminal amino acid was sometimes at the limit of detection, so that a distinction between the presence of full-length peptide versus shorter products could not be made with certainty.

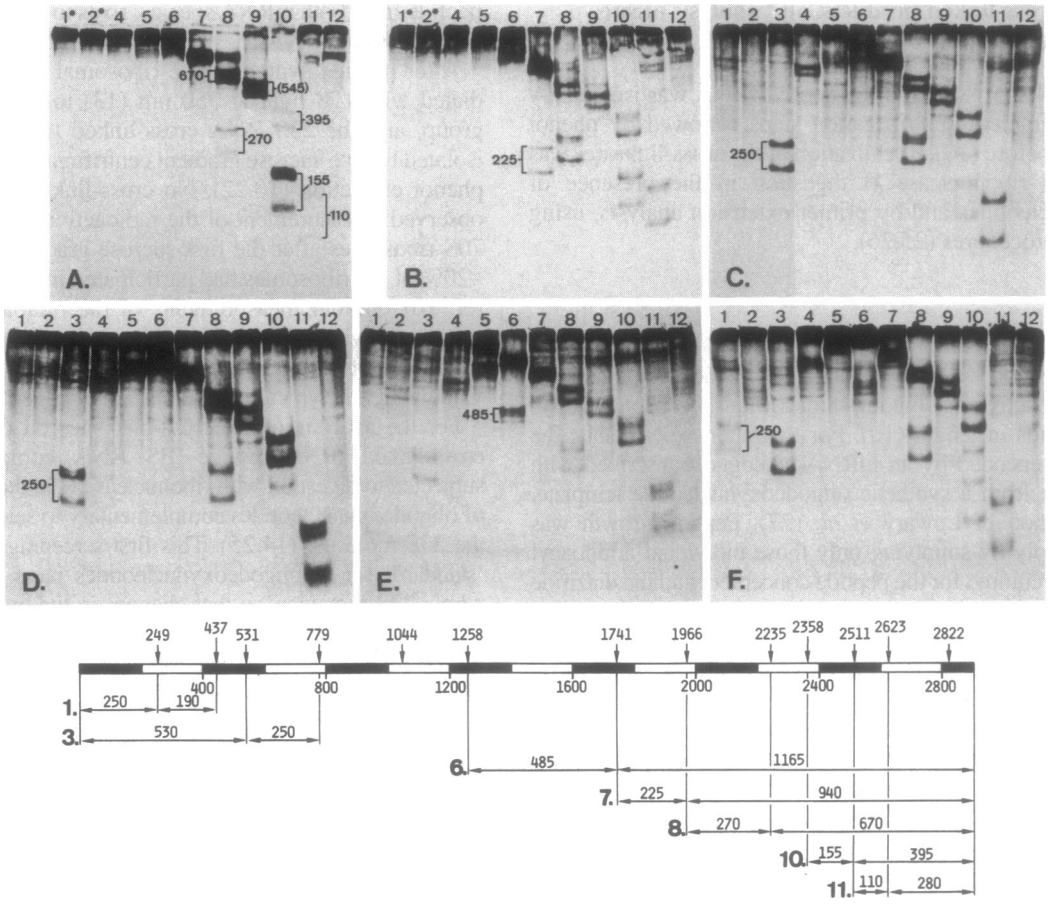

Figure 2. Ribonuclease H digests of peptidyl-tRNA–23S RNA complexes on 5% polyacrylamide gels. (**A–F**) Autoradiograms of digests derived from complexes with peptides containing 1, 4, 6, 13–15, 25–26 and 29–33 amino acids, respectively. In panels C–F the digestions were performed in the presence of oligodeoxynucleotides complementary to sequences centred on 23S RNA positions 249/437 (lane 1), 437/531 (lane 2), 531/779 (lane 3), 779/1044 (lane 4), 1044/1258 (lane 5), 1258/1741 (lane 6), 1741/1966 (lane 7), 1966/2235 (lane 8), 2235/2358 (lane 9), 2358/2511 (lane 10), 2511/2623 (lane 11) and 2623/2822 (lane 12). In panels A and B, lanes 4–12 are the same as those just listed, whereas the digests at the 5′-end of the 23S RNA used oligodeoxynucleotides centred on positions 249/494 (lane 1*) and 494/779 (lane 2*) (10). The approximate lengths of the 23S RNA fragments in each new doublet band (see text) appearing in the successive panels are indicated, and the locations of the fragments within the 23S RNA sequence are shown schematically in the lower part of the figure, arranged according to gel lane number.

Table 1. Summary of peptide-23S RNA cross-linking data

Gel lane	Localization	Cross-link	Length of peptide								
(Figure 2)	by RNAse H	site	1	2	3	4	6	9	13–15	25–26	29–33
1	60–100	91	–	–	–	–	–	–	–	–	+
6	1305–1350	?	–	–	–	–	–	–	–	+	+
	1590–1625	1614	–	–	–	–	–	–	–	+	+
3	730–760	750	–	–	–	–	+	+	+	+	+
7	1770–1810	1781	–	–	(+)	+	–	–	–	–	–
11	2605–2625	2609	–	(+)	+	+	+	+	+	+	+
	2570–2605	2585	+	+	(+)	(+)	(+)	(+)	+	+	+
10	2460–2510	2506	+	+	(+)	(+)	(+)	–	(+)	–	(+)
8	2055–2090	2062	+	+	+	+	+	+	+	+	+

The cross-links from each peptide described here and previously (10) are shown. '+' indicates that a cross-link was reproducibly observed, '(+)' that it was only weakly or occasionally present (or that it did not contain significant amounts of the labelled C-terminal amino acid), '–' that the cross-link was reproducibly absent. The Table indicates (first column) the gel lane in Figure 2 in which each cross-link first appears, (second column) the shortest RNA regions encompassing the cross-links sites identified by ribonuclease H digestion (Figs 2 and 3), and (third column) the localization of the cross-link sites by primer extension (Fig. 4). '?' indicates that no reverse transcriptase stop signal was observed.

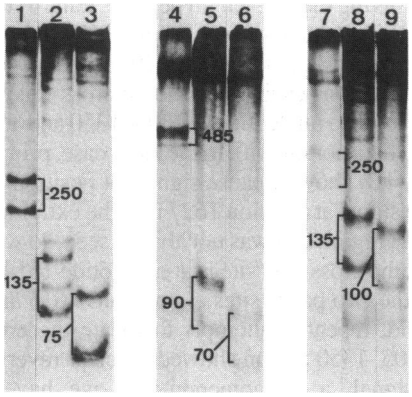

Figure 3. Autoradiograms of further ribonuclease H digestions, to narrow down the cross-linked RNA regions, on 5% gels as in Figure 2. Lanes 1–3, digestions of the complex containing the peptide with six amino acids. Lane 1, the same digest as that in Figure 2C, lane 3. Lane 2, a digest with three oligodeoxynucleotides centred on 23S RNA positions 625/761/803, giving expected fragment lengths of ~135 and 40 nt, respectively. (The weak slower-moving doublet band results from incomplete digestion by the ribonuclease H at position 761, giving a fragment of ~180 nt.) Lane 3, a digest with three oligodeoxynucleotides at positions 625/728/803, giving expected fragment lengths of ~105 and 75 nt, respectively. Lanes 4–6, digestions of the peptide complex with 25–26 amino acids. Lane 4, the same digest as that in Figure 2E, lane 6. Lanes 5 and 6, digests with oligodeoxynucleotides at positions 1258/1350 (expected fragment length ~90 nt) and 1592/1660 (~70 nt), respectively. Lanes 7–9, digestions of the peptide complex with 29–33 amino acids. Lane 7, the same digest as that in Figure 2F, lane 1. Lanes 8 and 9, digests with oligodeoxynucleotides at positions 46/183 (giving expected fragment lengths of ~135 nt between the two oligodeoxynucleotides, or 45 nt at the extreme 5′-end of the molecule) and 101/249 (~150 nt, or 100 nt at the 5′-end), respectively. (The fast-running band in lanes 8 and 9 corresponds to aminoacyl-tRNA, and is often seen moving at the gel front, as for example in Figure 2E).

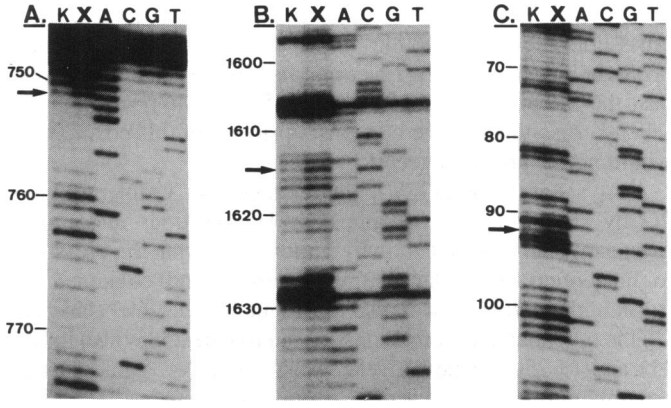

Figure 4. Primer extension analyses of 23S RNA cross-linked complexes, corresponding to those containing peptides with: (**A**) six amino acids, (**B**) 25–26 amino acids and (**C**) 29–33 amino acids. In each case lane K is a pre-irradiated control sample (see Materials and Methods), lane X is the 23S RNA cross-linked complex, [which also contains non-cross-linked 23S RNA (10)] and A, C, G and T are dideoxy sequencing lanes. Appropriate regions of the sequencing gels are shown, with the pause signals corresponding to the cross-link sites being indicated by the arrows. Oligodeoxynucleotide primers labelled with ^{32}P were used, complementary to 23S RNA positions 822–841 (A), 1704–1720 (B), and 175–192 (C).

The cross-links described in the following sections are from peptides with the diazirine derivative attached to their N-terminal methionine residue, unless otherwise specifically noted.

Peptides with one to four amino acids

The cross-linking patterns from these short peptides have already been published (10), but are shown again here in Figure 2A and B as they define the pattern of cross-links to the 'peptidyl transferase ring' of the 23S RNA; these cross-links continued to appear in various combinations with all the other peptides tested (Fig. 2C–F). From the 'monopeptide' (Fig. 2A), cross-links to 23S RNA can be seen in lane 8 [the 270 nucleotide (nt) gel band corresponding to the cross-link site at nucleotide 2062], in lane 10 (the 155 nt band containing the cross-link to nucleotide 2506), and in lane 11 (the 110 nt band). The latter band was resolved in our previous experiments by further ribonuclease H digestions into two regions, one corresponding to a cross-link site at nucleotide 2585 [observed with mono- or dipeptides, but only very weakly present with tri- or tetrapeptides (Table 1)] and the other corresponding to a cross-link at nucleotide 2609 (which showed the reverse tendency in that it was weak or absent with mono- or dipeptides, but clearly present with tri- or tetrapeptides). The other gel bands visible in Figure 2A—with 670, 545 and 395 nt in lanes 8, 9 and 10, respectively—correspond in each case to the 3′-terminal region of the 23S molecule containing the cross-links further downstream (see diagram at the bottom of Figure 2).

The tetrapeptide (Fig. 2B) shows a new cross-link (the 225 nt band in lane 7) in addition to the cross-links to the peptidyl transferase ring just described; the cross-link site here was localized to nucleotide 1781 (10).

Peptides with six amino acids

A typical hexapeptide cross-linking pattern is illustrated in Figure 2C. It can be seen that the cross-link to nucleotide 1781 (Fig. 2B, lane 7) has already disappeared, and instead there is a new 250 nt band in lane 3. [With the original 'standard' set of oligodeoxynucleotides (10) as in panels A and B, the hexapeptide showed an extra spurious ribonuclease H cut in lane 2* of the gels (not shown); a cleaner pattern was obtained by using the modified set of oligodeoxynucleotides as described in the legend to Figure 2 for panels C–F.] As already noted above, hexapeptides encoded by both mRNA A and mRNA B (Fig. 1) were examined, in order to 'connect' the data sets for the two messages. In each case the technique of adding individual aminoacyl-tRNAs—rather than bulk charged tRNA (see Materials and Methods)—was used, and both peptides gave identical cross-linking patterns. The cross-linked complex in lane 3 from mRNA A shown in Figure 2C, contained ^{3}H-radioactivity from the C-terminal aspartic acid, and with mRNA B the corresponding cross-linked complex contained ^{3}H-alanine (cf. Fig. 1), indicating the presence of the full-length peptide.

Further ribonuclease H digestions of the 'new' hexapeptide cross-link are illustrated in Figure 3, lanes 1–3. Lane 1 (Fig. 3) shows the same digest as that of lane 3 in Figure 2C, whereas lanes 2 and 3 (Fig. 3) show digests each made in the presence of three oligodeoxynucleotides. Lane 2 indicates that the cross-link lies between positions ~625 and 760 (the 135 nt band), whereas lane 3 locates it between positions ~730 and 805 (the 75 nt band). The cross-link must therefore lie in the region common to both these fragments, that is to say between nucleotides ~730 and 760; the subsequent primer extension analysis (Fig. 4A) showed a

reproducible pause signal at position 751, indicative of a cross-link site at nucleotide 750 of the 23S RNA. (It should however be noted that the reverse transcriptase is not able to read past the modified base at position 745 with the result that if a second cross-link site were to exist upstream of this position, we would not be able to detect it.)

Analysis of the other hexapeptide cross-links visible in Figure 2C by similar further ribonuclease H digestions and primer extension analyses (not shown) demonstrated that the cross-link to nucleotide 2062 (lane 8) was present and contained the full-length peptide, as was also that to nucleotide 2609 (lane 11). On the other hand, the cross-links to nucleotide 2585 (also in lane 11) and to 2506 (lane 10) were very weak (cf. Table 1), and possibly only contained shorter peptide products.

Peptides with nine and 13–15 amino acids

These peptides were encoded by mRNA B, using mRNA fragments prepared from plasmids that had been cut with *Bsa*JI (for nine amino acids) or *Bst*NI (for 15 amino acids), respectively (Fig. 1); the corresponding peptides were prepared using bulk charged tRNA, either in the presence of [^{3}H]valine which occurs at position 9, or [^{3}H]threonine, which occurs at positions 13 and 15. In the latter case we cannot exclude the possibility that the cross-linked complexes containing ^{3}H-labelled peptides terminated at position 13, but—as noted in the Introduction—a difference in peptide length of two amino acids is comparable to the length of the diazirine reagent. Both of these peptides gave essentially identical cross-linking patterns to that obtained with the hexapeptide (Fig. 2C), the pattern from the longer peptide being illustrated in Figure 2D. Detailed analysis of the 250 nt fragment from lane 3 showed that the cross-link site was again to nucleotide 750 of the 23S RNA with the full-length peptide, and the only differences from the hexapeptide pattern were minor changes in the distribution of the cross-links at positions 2506 and 2585 (Table 1). (A faint band of ~250 nt is visible in Figure 2D, lane 4 but further probing of this region of the 23S RNA with ribonuclease H did not yield any results).

In the case of the peptide with 13–15 amino acids, a further series of experiments was conducted in which the diazirine label was attached to the ε-NH_2 group of the lysine residue adjacent to the N-terminal methionine (see Materials and Methods). Here, the N-terminal methionine was formylated, so that the peptide had a natural N-terminus. The results again showed cross-linking patterns identical to those of Figure 2C and D (data not shown).

The peptide with 25 (or 26) amino acids

This peptide was encoded by a fragment of mRNA terminating at the *Rsa*I restriction site (Fig. 1). Since there is a methionine at position 25 of the amino acid sequence, the radioactive labels were interchanged, so that the N-terminal methionine carrying the diazirine derivative was labelled with ^{3}H, and the bulk charged tRNA contained [^{35}S]methionine. Thus, the detection of ^{35}S-radioactivity in the cross-linked complexes was indicative of the presence of a peptide with 25 amino acids, although from the position of the *Rsa*I restriction site the proline residue at position 26 may also have been encoded. The corresponding ribonuclease H gel is shown in Figure 2E, and here the 'new' cross-link can be seen in lane 6 (the 485 nt band). Further ribonuclease H digestions (Fig. 3, lanes 4–6) revealed that two cross-linked regions were in fact present in this case, both found to contain ^{35}S-radioactivity and hence involving the full-length peptide. Lane 4 (Fig. 3) shows the same digestion as that of lane 6 (Fig. 2E), and lanes 5 and 6 show digests giving a 90 and a 70 nt band, respectively. Subsequent digestions enabled these two cross-linked regions to be narrowed down to nucleotides ~1305–1350 and 1590–1625 of the 23S RNA (data not shown). In the latter case, primer extension analysis (Fig. 4B) showed pause signals at positions 1615, 1617 and 1627; the signal at position 1627 is at the extreme limit of the ribonuclease H region and was not always seen, so we tentatively conclude that the cross-link site is at nucleotide 1614, adjacent to the upper of the two pause sites at positions 1615 and 1617. On the other hand, repeated attempts to locate the cross-link site within the 1305–1350 region showed no clear reverse transcriptase pause signal, a phenomenon that we have previously observed in other cross-link site analyses (30).

The remaining cross-link sites from the 25-amino acid peptide (Fig. 2E) included those at nucleotides 750, 2609, 2585 and 2062, but not those at nucleotides 1781 or 2506 (Table 1).

The peptide with 29–33 amino acids

The final peptide was encoded by a fragment of mRNA B terminating at the *Eco*RV restriction site (Fig. 1). Here the choice of a suitable ^{3}H-labelled amino acid occurring for the first time near to the C-terminus was limited to arginine (at position 33) or proline (at positions 26 and 29). Proline was chosen, as it is commercially available at a higher specific activity, and by comparison with the results from the shorter peptides (above) we concluded that the presence of proline in the cross-linked products was indicative of a peptide containing at least 29—and more probably 33—amino acids. With this peptide a new cross-link was observed close to the 5′-terminus of the 23S RNA in lane 1 of the ribonuclease H gel (Fig. 2F). Although the mobility of the 250 nt band in the gel was somewhat anomalous, the further ribonuclease H digestions (Fig. 3, lanes 7–9) confirmed that there was indeed a cross-link within this region. Lane 7 (Fig. 3) shows the same digest as that of lane 1 (Fig. 2F), and the digests in lanes 8 and 9 (Fig. 3) narrow the cross-linked region down to positions ~46–183 of the 23S RNA (the 135 nt band in lane 8) and to positions 1–101 (the 100 nt band in lane 9). The common region (positions ~46–101) was more closely localized by other digestions (not shown) to positions 60–100, and the primer extension analysis (Fig. 4C) showed pause signals at positions 92 and 94, from which we conclude that the cross-link site lies at nucleotide 91 of the 23S RNA. The remaining cross-links from this peptide (Fig. 2F) were essentially identical to those observed with the 25–26 amino acid peptide (Table 1).

DISCUSSION

The principal feature of the data which is presented here (Table 1) is that the N-termini of progressively longer peptides are—as would be expected—able to form cross-links to a succession of sites on the 23S RNA. The locations of these sites in the corresponding secondary structural regions of the 23S RNA are illustrated in Figure 5, and Figure 6 shows these regions in the context of the whole 23S molecule. The cross-links from the shortest peptides have already been described (10), and these lie within the peptidyl transferase ring (which connects helices 73, 74, 89, 90 and 93) at sites identical with, or close to, positions that had previously been identified in studies using photo-affinity analogues of aminoacyl-tRNA (25,32 and see Fig. 5). Sites of foot-printing to tRNA at the ribosomal A and P sites have also been

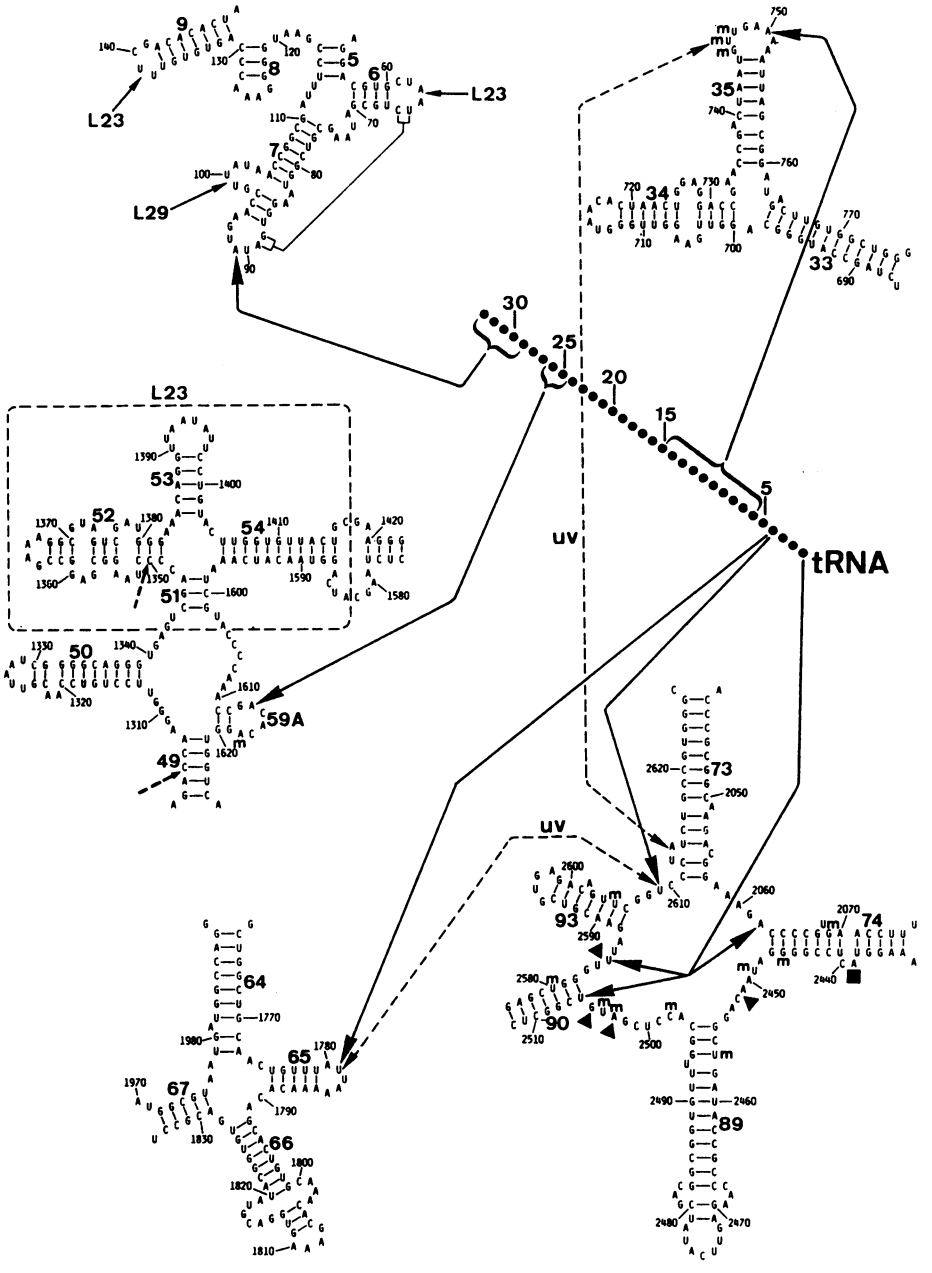

Figure 5. Locations of the cross-link sites within elements of the secondary structure of 23S RNA. The peptides are represented by a 'string of beads' attached to tRNA, and the cross-links to the various 23S RNA regions (with helices numbered as in ref. 31) are indicated by arrows. The cross-links observed by Steiner *et al.* (32) and by Mitchell *et al.* (25) from affinity-labelled aminoacyl tRNA are indicated by filled triangles and a filled square, respectively. 'm' denotes a modified nucleotide (33–35). Sites of intra-RNA cross-linking (36) are joined by broken lines and marked 'UV', the binding site of protein L23 (37) is boxed in with a broken line, and cross-link sites to proteins L23 and L29 (38) are indicated by arrows. The broken arrows at positions 1305 and 350 demarcate the ribonuclease H fragment encompassing the cross-link site to the peptide with 25–26 amino acids for which no reverse transcriptase stop signal was observed (Table 1).

reported in this area at positions 2439, 2451, 2505–06, 2584–85 and 2602 (39,40). Peptides containing three or four amino acids are cross-linked to nucleotides 1781 and 2609 (10); although these two positions are widely separated in both the primary and secondary structure of the 23S RNA, their juxtaposition is supported by an older observation in which the same positions were cross-linked to one another by direct UV-irradiation of 50S subunits (36), as discussed previously (ref. 10; Fig. 5).

The next 'leap' made by the growing peptide within the RNA structure is to nucleotide 750 in helix 35 (Table 1; Fig. 5), and again the proximity of this position to the peptidyl transferase ring is corroborated by an older direct UV-cross-link between positions 746 and ~2614 (36,41; Fig. 5). It is also noteworthy that a footprint site from the antibiotic vernamycin B to position 752 has been reported (42). Thus the peptide appears to move out from the peptidyl transferase ring in the direction of helix 73. The

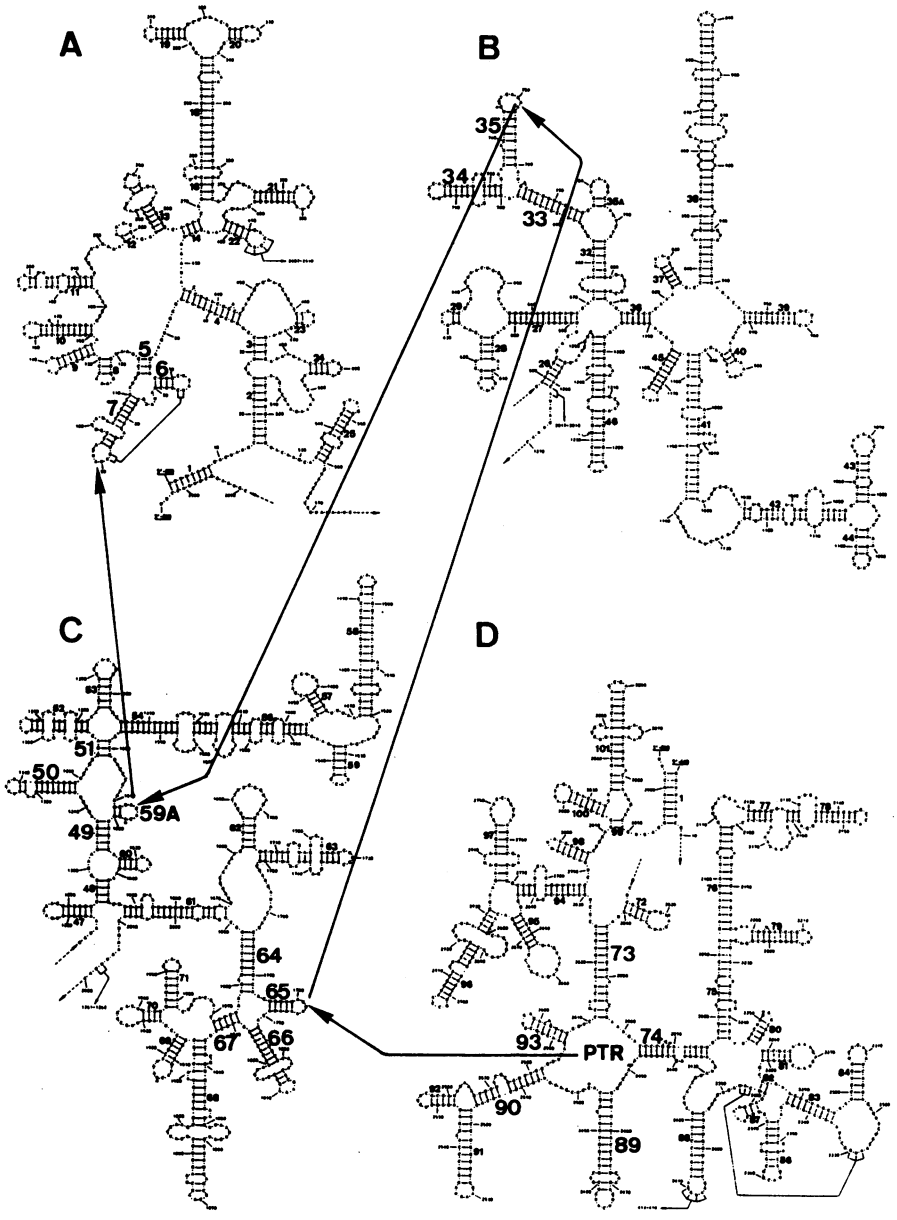

Figure 6. Locations of the cross-link sites in the complete 23S RNA secondary structure. The regions shown in detail in Figure 5 are picked out with larger helix numbers, and the path of the growing peptide is indicated by the arrows. 'PTR' stands for peptidyl transferase ring.

cross-link to nucleotide 750 was first seen with a hexa-peptide, and no new cross-link was found either with the nona-peptide or the peptide containing 13–15 amino acids (Table 1). With the latter peptide, the same cross-link at position 750 was also observed when the N-terminal methionine residue was formylated and the diazirine label was attached to the adjacent lysine (Fig. 1); thus, a peptide with the natural N-terminus appears to be following the same path as peptides carrying an N-terminal diazirine group, at least at the level of resolution of our experiments.

The peptide with 25–26 amino acids showed cross-links to two sites in a completely different area of the 23S RNA (Fig. 5). The RNA regions concerned were unambiguously narrowed down by ribonuclease H digestions to positions ~1305–1350 and 1590–1625 (Table 1), respectively, but only in the latter case we were able to identify a cross-link site (to nucleotide 1614) by primer extension. We have often pointed out (10,26,43) that the use of the primer extension method in cross-link analyses can lead to artefacts—that is to say the identification of 'false' cross-link sites—and for that reason we always localize the cross-linked regions as far as possible with the ribonuclease H method; however, the cross-link in the 1305–1350 region is a case of the reverse phenomenon, where the primer extension method misses a cross-link site altogether (cf. ref. 30). This could be due either to the cross-link site coinciding with a strong natural reverse transcriptase pause signal, or to the cross-link being to an atom within (for example) the ribose moiety, so that no pausing of the reverse transcriptase occurs. Nonetheless, it is clear that the two

cross-linked regions, although separated by 300 nt in the primary sequence, lie close together in the secondary structure of the 23S RNA, in the region of helices 49–51 and 59A (Fig. 5). It is noteworthy that the cross-link site at nucleotide 1614 is close to a modified base at position 1618. We have previously pointed out (44) that the modified nucleotides in *E.coli* 16S and 23S RNA (see e.g. Fig. 5) lie clustered at or close to sites implicated by cross-linking and foot-printing studies as being neighboured to the mRNA–tRNA–peptide functional complex, and in fact the base at position 1618 was the only modification which up to now could not be assigned to this cluster.

A further point of interest is that the cross-link sites to the peptide with 25–26 amino acids are close to the 23S RNA region that has been identified as the binding site for protein L23 (37; Fig. 5). On the other hand, the corresponding cross-link site from the peptide with 29–33 amino acids to nucleotide 91 in helix 7 (Fig. 5) is very near to a cross-link site found for this protein at position ~63 (38), by virtue of the tertiary interaction (45) joining bases 64–65 with bases 88–89. A cross-link site to protein L29 at position ~100, as well as a further cross-link site to L23 at position ~138 (38), have also been identified in this area of the 23S RNA. From the peptide protection studies quoted in the Introduction (4,5), a peptide with 29–33 amino acids should be approaching the exit site on the 50S subunit. Immuno electron microscopic studies have located this site at the 'rear' of the 50S subunit (3), close to positions where epitopes for both proteins L23 and L29 have similarly been identified (1). However, L23 has also been identified as the target of cross-linking in a study with a photo-affinity analogue of puromycin (46), and on this basis should be close to the peptidyl transferase centre. Thus, there is circumstantial evidence for an involvement (or at least a proximity) of L23 at various points along the path of the growing peptide, although it should be noted that L23 was not among those proteins identified in the earlier study of (9) using affinity labelled peptide analogues. Experiments are in progress in our laboratory to make corresponding analyses of the proteins that are cross-linked by the diazirine-derivatized peptides.

In the context of the whole 23S RNA molecule (Fig. 6), our results show that the growing peptide makes contact with a remarkably widely-separated set of points along the RNA chain. From the peptidyl transferase ring in domain V of the 23S RNA, the peptide moves to domain IV (helix 65), then to domain II (helix 35), then to domain III (helix 59A) and finally to domain I (helix 7). Expressed in the converse way, these sites on the 23S RNA must be arranged in three dimensions so as to be compatible with the differences between the lengths of the various peptides involved. This imposes severe folding constraints, which will be important for model-building studies with the 23S RNA (cf. ref. 36). It is of course very likely that we have missed some significant points along the peptide path, such as in the 'gap' (Table 1; Fig. 5) between peptides with 15 and 25 amino acids, and further experiments with different peptide sequences or different cross-linking reagents clearly need to be made.

The physical nature of the peptide path is still under discussion. Some authors (15–17) favour the concept of a 'tunnel' through the 50S subunit, whereas others (18) prefer the idea of a deep 'channel' along the subunit surface, or a combination of tunnel and channel (47). In fact, in terms of molecular structure these two concepts need not be very different from one another; the 50S subunit (or 23S RNA) structure only needs to 'open up' slightly for a tunnel to become a cleft or channel, and vice versa. Of more interest to us is the question of the dimensions of the channel or tunnel, and whether significant peptide folding can take place within the ribosome. Fluorescence measurements (17) have shown that the peptide pathway is accessible to iodide ions, but is not large enough to be accessible to Fab antibody fragments (48). Homopeptides of different compositions become accessible to Fab or IgG binding at different chain lengths (49), suggesting that the peptide pathway can accommodate different types of peptide secondary structure (50), and Hardesty *et al.* (48) go further by proposing that a significant part of the protein folding process does indeed take place within the 50S subunit.

Our cross-linking data provide circumstantial support for this latter view, because the progressively longer peptides still become cross-linked to some—but not all—of the sites found with the shorter peptides (Table 1). Thus, the cross-links to nucleotides 2506 and 2585 essentially disappear with peptides of three amino acids or more (although the 2585 site seems to re-appear with the longest peptides), and the 1781 cross-link was only observed with tri- or tetrapeptides. On the other hand, after making their initial appearance, the cross-links to nucleotides 2062, 2609, 750, 1614 and 1305–1350 all remained visible with the longer peptides. The presence in each case of radioactivity from the labelled C-terminal amino acid of the peptide in the individual peptide–23S RNA cross-linked complexes (Figs 2 and 3) precludes the trivial possibility that this observation is merely due to the occurrence of shorter abortive peptide products in the reaction mixtures. This indicates that the peptide channel or tunnel must be wide enough for the peptide to fold back on itself, so as to be able to reach a sub-set of the 'earlier' cross-links, and at the same time suggests that the configuration of the nascent peptide is still very fluid at this stage. Here again, further experiments with different peptide sequences and different cross-linking reagents will need to be made in order to shed more light on this question.

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