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Site-directed cross-linking of mRNA analogues to 16S ribosomal RNA; a complete scan of cross-links from all positions between '+1' and '+16' on the mRNA, downstream from the decoding site

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ABSTRACT

mRNA analogues containing 4-thiouridine residues at selected sites were used to extend our analysis of photo-induced cross-links between mRNA and 16S RNA to cover the entire downstream range between positions +1 and +16 on the mRNA (position +1 is the 5'-base of the P-site codon). No tRNA-dependent cross-links were observed from positions +1, +2, +3 or +5. Position +4 on the mRNA was cross-linked in a tRNA-dependent manner to 16S RNA at a site between nucleotides ca 1402 – 1415 (most probably to the modified residue 1402), and this was absolutely specific for the +4 position. Similarly, the previously observed cross-link to nucleotide 1052 was absolutely specific for the +6 position. The previously observed cross-links from +7 to nucleotide 1395 and from +11 to 532 were however seen to a lesser extent with certain types of mRNA sequence from neighbouring positions (+6 to +10, and +10 to +13, respectively); no tRNA-dependent cross-links to other sites on 16S RNA were found from these positions, and no cross-linking was seen from positions +14 to +16. In each case the effect of a second cognate tRNA (at the ribosomal A-site) on the level of cross-linking was studied, and the specificity of each cross-link was confirmed by translocation experiments with elongation factor G, using appropriate mRNA analogues.

INTRODUCTION

In our laboratories we have for some time been investigating the question of the path followed by mRNA through the *Escherichia coli* ribosome, using the technique of 'site-directed cross-linking' with mRNA analogues carrying 4-thiouridine ('thio-U') residues at selected positions (1–5). These analogues are bound to the ribosome in the presence of cognate tRNA molecules, the thio-U residues are then photo-activated by irradiation at wavelengths above 300 nm, and subsequently the ribosomal components involved in cross-links to thio-U are analysed. In two joint publications between the Moscow and Berlin groups (4,5) we showed that it is possible to work with mRNA analogues carrying thio-U residues at several different positions simultaneously, and that the cross-links to each individual thio-U residue can be distinguished from one another. This improvement to the methodology has the consequence that virtually any desired mRNA sequence can be applied, and in our most recent paper (5) we described a series of tRNA-dependent cross-links to 16S RNA from the 'downstream' region of the mRNA, using two different sets of mRNA analogues; these were either purely 'synthetic' sequences (3), or sequences derived from the cro-mRNA of λ-phage (4), in all cases containing Shine–Dalgarno regions and AUG initiator codons. Each of the cross-links found was independent of the type of ribosomal complex used in the assays (tight couples or 70S initiation complexes), although differences in the individual cross-linking yields were seen, depending on the class of mRNA sequence used (5). These 'universal' cross-links (i.e. cross-links found under all conditions tested) were observed from position +6 of the mRNA to nucleotide 1052 of the 16S RNA (position +1 being defined as the 5'-base of the P-site codon), from position +7 to nucleotide 1395, and from position +11 to nucleotide 532. The importance of these data is that they are not compatible either with the current models for the three-dimensional arrangement of 16S RNA (6,7), or with one model for the arrangement of the tRNA-mRNA complex in the ribosome (8,9).

In this paper, we have extended our studies with the same two types of mRNA sequence, so as to cover the whole range of mRNA positions between +1 and +16. The latter position was defined in the 'toe-printing' experiments of Hartz et al (10) as being the limit of the downstream region in the mRNA which interacts strongly with the ribosome; reverse transcriptase stops at the +16 position have also been directly demonstrated (11)

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in the case of the cro-type mRNA sequences used in our studies. While downstream mRNA-ribosome contacts may well exist outside this range (cf. 3), it seems probable that they would be highly dependent on individual mRNA sequences and secondary structures, and therefore we did not consider it worthwhile to extend our analysis beyond the +16 position at this stage.

The paper is divided into three sections. In the first we document a new 'universal' cross-link from the +4 position of mRNA to a site on the 16S RNA; this cross-link had previously been observed only with a more simple mRNA analogue which did not contain an AUG codon (2). In the second section we describe the confirmation of the cross-link data by translocation experiments with elongation factor G; we had already shown (5) that the cross-link from position +6 on the mRNA was also observed after translocation of an mRNA analogue carrying a thio-U residue at position +9, and we have now extended this approach to examine all of the 'universal' cross-links mentioned above, as well as to probe positions +1 and +3 within the P-site codon. The third section summarizes the entire data set from positions +1 to +16, including (a) an analysis of the extent to which each of the four 'universal' cross-links occurs from neighbouring positions in both types of mRNA sequence, and (b) a description of the effect of a second cognate tRNA molecule on the yield of each cross-link, again with both types of mRNA analogue.

We have already noted (2,5) that the corresponding patterns of cross-linking between the mRNA analogues and 30S ribosomal proteins are less clearcut than those to 16S RNA. This is at least in part due to the fact that it has not yet been possible to analyse the cross-link sites on the proteins at an equivalent level of resolution (i.e. at the amino acid level), and for this reason the cross-linking to ribosomal proteins is not discussed here.

MATERIALS AND METHODS

mRNA analogues containing thio-U were prepared by T7 transcription from suitable DNA templates as described previously (1,5). The mRNA was labelled by inclusion of [^{32}P]-UTP in the transcription reactions as before (4,5), and selected examples of the mRNA sequences used are illustrated in Figure 1. All other procedures—including formation of ribosomal complexes (either with tight couples or 70S initiation complexes) containing mRNA together with one or two cognate tRNA species, treatment with elongation factor G, irradiation of the complexes at wavelengths above 300 nm, separation of cross-linked mRNA-16S RNA fractions by successive sucrose gradient centrifugations, determination of cross-link sites on 16S RNA by treatment with ribonuclease H in the presence of selected pairs of oligodeoxynucleotides followed by reverse transcriptase analysis, and identification of cross-linked thio-U residues in the mRNA by ribonuclease T_1 fingerprinting—were also carried out as previously described (1,5).

RESULTS AND DISCUSSION

Cross-linking from the +4 position of mRNA

Tate et al (2) reported a cross-link from a thio-U residue at position +4 of an mRNA analogue to nucleotide 1407 of the 16S RNA (i.e. with a reverse transcriptase 'stop' at 1408). However, the mRNA sequence concerned did not contain an AUG codon, and the thio-U at position +4 was the first residue of a UAA stop codon; it is thus possible that the cross-link

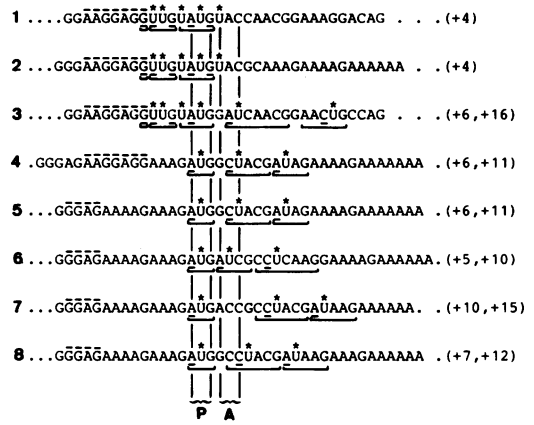

Figure 1. Examples of the mRNA sequences used in this study. In each sequence Û denotes the residues which were incorporated into the T7 transcript using a mixture of 4 thio-UTP and [^{32}P]-UTP. The underlined bases are those whose 3′-phosphate was radioactively labelled by this procedure, and the products which can be released by ribonuclease T_1 digestion as radioactive oligonucleotides are further underlined. The P-site (AUG) and A-site codons are indicated, and Shine–Dalgarno regions are indicated by the dotted lines above the sequence. The numbers in brackets to the right of each sequence give the positions of the relevant thio-U residues, numbered from the 5′-base of the P-site codon.

observed in those experiments was a special case. Cross-linking from the +4 position was accordingly re-examined, using mRNA analogue No. 1 (Figure 1). This is a 'cro-mRNA' type message (cf. 4,5), carrying an AAGGAGG Shine–Dalgarno sequence followed by the UUGUAUG initiation region characteristic of the cro-mRNA. The juxtaposed U-residue at position +4 is part of a UAC tyrosine codon. The mRNA was transcribed as usual (1,5) from the corresponding DNA template, using a mixture of 4-thio-UTP and [^{32}P]-UTP in the T7 transcription mixtures, and the ^{32}P-labelled mRNA was bound to ribosomes in the presence of either $tRNA^{fMet}$, or $tRNA^{fMet}$ plus Tyr-$tRNA^{Tyr}$, or without tRNA as a control. Cross-linking was then induced by irradiation at wavelengths above 300 nm, and the mRNA-16S RNA cross-linked complexes were isolated by successive sucrose gradient centrifugation, first at low magnesium to separate the ribosomal subunits, and then in the presence of SDS to separate ribosomal RNA and protein fractions (1). As before (5), the cross-linking was exclusively to the 30S subunit, and a preliminary localization of the cross-link sites on 16S RNA was made by the ribonuclease H method (1,5). Examples of the polyacrylamide gels obtained are illustrated in Figure 2.

Figure 2A shows the ribonuclease H digests in the presence of three different pairs of oligodeoxynucleotides made with ^{32}P-mRNA-16S RNA cross-linked complexes derived from 70S tight couples carrying mRNA No. 1 and $tRNA^{fMet}$; in each slot a strong band and a weak band can be seen, the latter corresponding to the well-established cross-link to the 3′-terminal region of the 16S RNA (1,4) from the thio-U residues at positions −3 and −4 of the mRNA. From the data of Figure 2A the stronger cross-link can be localized to the region between nucleotides 1398 and 1419 of the 16S RNA, this being the region common to all three fragments (slots 1, 2 and 3 of Fig. 2A) containing this cross-link. However, since nucleotides 1398 and 1419 correspond to the central positions of the complementary decadeoxynucleotides used in the ribonuclease H digests, the actual cross-link site most probably lies between ca nucleotides 1402 and 1415; it is our

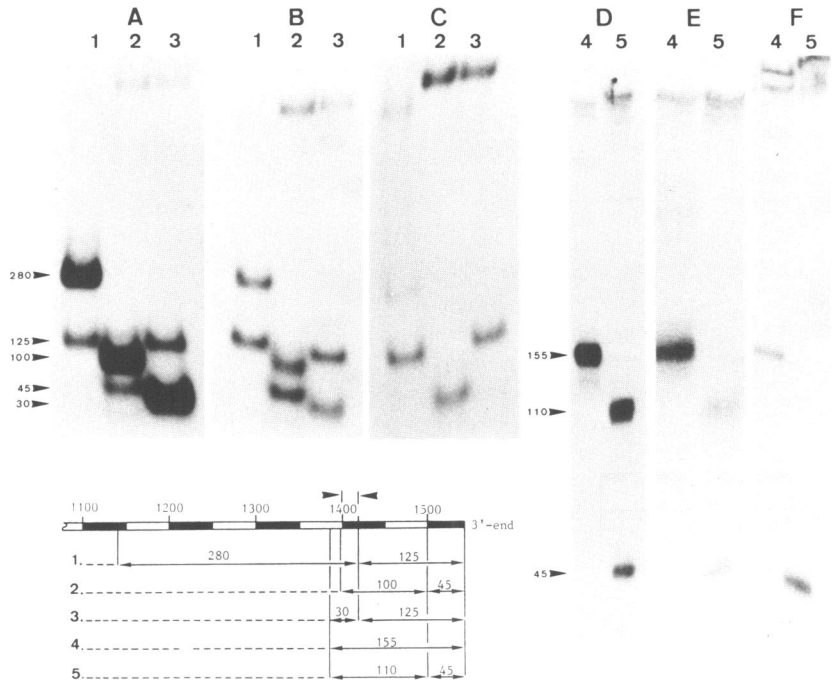

Figure 2. Ribonuclease H digests of [^{32}P]mRNA-16S complexes, with mRNA No. 1 (Fig. 1). Panels **A**, **B** and **C** are 5% polyacrylamide gels (3) (minus urea) from 70S tight couples with mRNA and (A) tRNA^{fMet}, (B) tRNA^{fMet} plus Tyr-tRNA^{Tyr}, (C) minus tRNA. Panels **D**, **E** and **F** show the corresponding results on 4% gels (plus urea) from 70S initiation complexes (4,5). The following pairs of oligodeoxynucleotides were used for the ribonuclease H digestions (listed according to the position in 16S RNA complementary to the central base of the oligonucleotide): Slot 1, positions 1140, 1419; slot 2, positions 1398, 1499; slot 3, positions 1387, 1419; slot 4, position 1387; slot 5, positions 1387, 1499. The approximate sizes of the 16S fragments (linked to the ^{32}P-mRNA) excised in each case are indicated, and the locations of these fragments within the 16S sequence are summarized in the lower part of the diagram, according to the gel slot number. The 'shortest common fragment' containing the cross-link site (positions 1398–1419) is indicated by the arrow-heads above the sequence diagram.

experience that when a cross-link site lies within the hybridizing region of a decadeoxynucleotide on the 16S RNA, then the cutting by ribonuclease H is severely inhibited, but no such inhibition was observed in this case (Fig. 2). In contrast, an example of the inhibition phenomenon can be seen in Fig. 3 (lanes 1 and 2) below.

Figure 2B shows the corresponding ribonuclease H digests made from samples derived from 70S tight couples carrying mRNA together with tRNA^{fMet} and Tyr-tRNA^{Tyr}. Here the '+4' cross-link is clearly reduced in intensity (see below for further discussion), and is now of comparable strength to the cross-link to the 3'-terminus of the 16S RNA; the cross-linked region within the 16S RNA is however the same as that in Figure 2A. Figure 2C gives the corresponding control digests in the absence of tRNA, and in this case the +4 cross-link is absent altogether. (A faint band can be seen in slot 1 at the 280-nucleotide position, but there is no corresponding band in either slot 2 or slot 3).

Similar experiments were made with mRNA No. 1 using 70S initiation complexes instead of tight couples, and the results are shown in Figures 2D–F (albeit with different pairs of oligodeoxynucleotides in the ribonuclease H digests). In each case, slot 4 (Figs. 2D–F) shows a single cross-linked fragment band, which in slot 5 resolves into two bands, comprising the +4 cross-link and the 3'-terminal cross-link, respectively. As in Figures 2A–C, the +4 cross-link is much stronger than the 3'-terminal cross-link in the presence of tRNA^{fMet} alone (Fig. 2D, slot 5), whereas in the presence of tRNA^{fMet} and Tyr-tRNA^{Tyr} (Fig. 2E, slot 5) the two cross-links are of comparable intensity, and in the absence of tRNA (Fig. 2F, slot 5) only the 3'-terminal cross-link is visible. The reduction in intensity in the presence of two tRNAs is reminiscent of the situation observed with the cross-link from position +6 of the mRNA to nucleotide 1052 of the 16S RNA (5), and will be discussed further below.

In the experiments of Tate et al (2), the cross-link from the +4 position of the mRNA was further localized with the help of a reverse transcriptase analysis. In those experiments (2) the 'stop' signal in the reverse transcriptase gel coincided with the corresponding stop in the control lanes arising from the methylated nucleotide at position 1407, but the stop signal at this position was reproducibly stronger in the case of the cross-linked samples. However, in the series of experiments we report here a slightly different result was found, in that now the reverse transcriptase stop corresponding to the nearby methylated nucleotide at position 1402 of the 16S RNA was reproducibly stronger in the cross-linked samples than that at position 1407 (data not shown). Similar reverse transcriptase patterns were seen both when a ribonuclease H excised 16S RNA fragment linked to mRNA was taken as substrate for the reverse transcription (5), and when a cross-linked mRNA-16S RNA complex purified by affinity chromatography on oligo-(dT) cellulose was used (3). (For the latter method an mRNA analogue carrying an A-rich 3'-region (mRNA No. 2, Fig. 1) was substituted for mRNA No. 1.)

Thus, it appears that the precise nature of the cross-link is somewhat different in the two sets of experiments (those reported here and those of Tate et al (2)), although it is clear from the ribonuclease H data that the region of 16S RNA concerned is

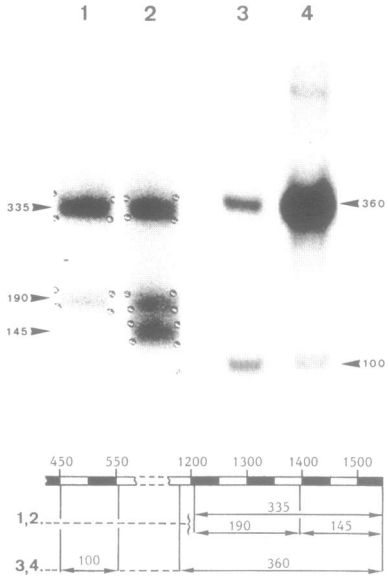

Figure 3. Ribonuclease H digests of [^{32}P]mRNA-16S RNA complexes derived from 70S tight couples, on 5% gels minus urea (3), with and without treatment with elongation factor G. Lane 1: Gel from a complex made with mRNA No. 8 together with tRNA^{fMet} and Ala-tRNA^{Ala}, without EF-G treatment. Lane 2: the same, but with EF-G treatment. Lane 3: Gel from complex made with mRNA No. 7 (Fig. 1) together with tRNA^{fMet} and Thr-tRNA^{Thr}, without EF-G treatment. Lane 4: the same, but with EF-G treatment. The gel bands are marked as in Fig. 2, and the data are summarized in a sequence diagram also as in Fig. 2. The oligonucleotide pairs (cf. Fig. 2) used for the digestions were: Slots 1 & 2, positions 1208, 1398; slots 3 & 4, positions 451, 553 (the former oligonucleotide also being complementary to a 16S site centred on position 1180).

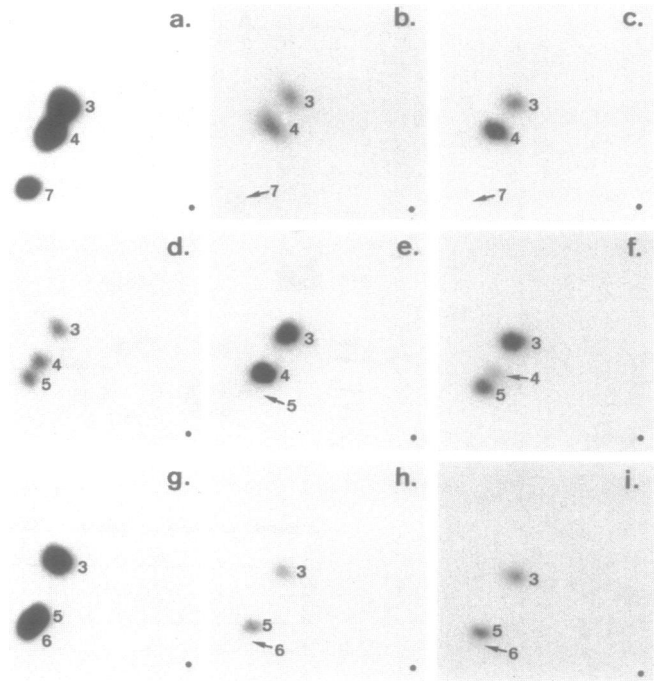

Figure 4. Ribonuclease T_1 fingerprints (4,5) of isolated cross-linked complexes containing ^{32}P-labelled mRNA. Direction of the first dimension is from right to left, that of the second from bottom to top, and the sample application point is indicated by a dot in each case. Panels a, b, c are from mRNA No. 6 (Fig. 1), panels d, e, f from mRNA No. 4, and panels g, h, i from mRNA No. 8. In each case the first of the three panels (a, d, g) is a fingerprint of the non-cross-linked mRNA, with the spots being marked according to their chainlength, *viz.* (panel a) spot 3 AŮGp, spot 4 AŮCGp, spot 7 CCŮCAAGp; (panel d) spot 3 AŮGp, spot 4 AŮAGp, spot 5 CŮACGp; (panel g) spot 3 AŮGp, spot 5 AŮAAGp, spot 6 CCŮACGp (cf. Fig. 1). Panel b: Fingerprint of mRNA No. 6 cross-linked to 110-nucleotide fragment (16S positions 1390–1500) before EF-G treatment (missing spot 7 has Ů at +10 position in mRNA). Panel c: Fingerprint from same complex after EF-G treatment (missing spot 7 has Ů translocated to +7 position in mRNA). Panel e: Fingerprint of mRNA No. 4 linked to 340-nucleotide fragment (1200–1542) before EF-G treatment (missing spot 5 has Ů at mRNA position +6). Panel f: The same, after EF-G treatment (missing spot 4 has Ů at mRNA position +8). Panel h: Fingerprint of mRNA No. 8 linked to 190-nucleotide fragment (1210–1400) before EF-G treatment (missing spot 6 has Ů at mRNA position +7). Panel i: mRNA No. 8 linked to the new 145-nucleotide fragment (1400–1542) appearing after EF-G treatment (missing spot 6 now has Ů at mRNA position +4).

the same in both cases, and the specificity for the +4 position on the mRNA is also the same. (A similar type of heterogeneity at the cross-link site was previously observed (3) in the reverse transcriptase gels from the cross-link to the 1395 region of 16S RNA.) The two modified bases at positions 1402 and 1407 are in any event likely to be close neighbours in three dimensions—at least intermittently—in this complex and flexible region of the 16S RNA (see e.g. 12). We therefore regard this as one of the 'universal' cross-links, and refer to it for convenience as the '1402/7' cross-link. Since both the modified nucleotides give rise to strong stops in the reverse transcriptase gels (cf. ref. 3), it is unfortunate that the method does not provide conclusive evidence in this case; under more rigorous conditions, where the stops from the modified nucleotides were eliminated, no difference could be observed between the reverse transcriptase patterns from the control and cross-linked samples. In consequence, the ribonuclease H result—defining the region of the cross-link as being between nucleotides ca 1402–1415—remains the proven experimental fact. The same cross-linked region was identified when a synthetic-type mRNA sequence (such as mRNA's No. 4–8, Fig. 1) was used, carrying a thio-U at the +4 position (data not shown), and with both classes of mRNA the cross-link was entirely specific for the +4 position.

It is also worth noting here that if Watson–Crick base-pairing is not impaired by the particular chemistry of a cross-link site on the 16S RNA, then there is no *a priori* reason for the reverse transcriptase to stop or pause at that cross-link site; in such cases even a strong cross-link such as this one (as revealed by the ribonuclease H analysis, Figs. 2A,D) would be missed altogether if the cross-links are assayed by the reverse transcriptase method alone, as in the experimental procedures used by other authors (e.g. 13).

The formal proof that the cross-link is to the +4 position of the mRNA, and not to one of the other thio-U residues, would normally be made by ribonuclease T_1 fingerprinting of an mRNA-16S RNA fragment, isolated from one of the ribonuclease H digestion mixtures (cf. 4,5). However, in the case of the +4 position this cannot be done directly, since the preceding nucleotide is the G-residue of the AUG start codon, and the ribonuclease T_1 oligonucleotide containing the +4 residue (ŮACCAACGp in mRNA No. 1) is therefore not labelled (see Fig. 1). This problem was overcome by using an mRNA analogue carrying a thio-U residue at position +7 (mRNA No. 8, Fig. 1), and translocating this into the +4 position with elongation factor G. This, together with other similar translocation experiments, is described in the following section.

Cross-linking before and after translocation with EF-G

Translocation of the mRNA with EF-G prior to cross-linking, combined with subsequent ribonuclease H digestion of the cross-linked mRNA-16S RNA complexes and ribonuclease T_1 fingerprinting of the isolated ribonuclease H digestion products, provides a useful method both for confirming established cross-links and also for probing 'awkward' positions in the mRNA such as the +4 position just discussed, or the +1 and +3 positions within the P-site codon; the translocation reaction should lead to the appearance of a new band in the ribonuclease H digest and/or to a change in the T_1-fingerprint, depending on the individual situation. We have already demonstrated (5) that the cross-link from position +6 of the mRNA to nucleotide 1052 of the 16S RNA can be observed after translocation of an mRNA carrying a thio-U residue at position +9, and further examples of pre- and post-translocational ribonuclease H digests and ribonuclease T_1 fingerprints are presented in Figures 3 and 4, respectively.

Figure 3, lanes 1 and 2, shows ribonuclease H digests of mRNA No. 8 (Figure 1), bound to 70S tight couples in the presence of $tRNA^{fMet}$ and Ala-$tRNA^{Ala}$; lane 1 is the sample without EF-G treatment, lane 2 the sample with EF-G treatment. The mRNA here is a purely 'synthetic' sequence (cf. 3) carrying the thio-U residues at positions +7 and +12 (in addition to the thio-U in the AUG codon), and the radioactive bands in Figure 3, lane 1 (335 and 190 nucleotides) both correspond to the cross-link from the +7 position on the mRNA to nucleotide 1395 of the 16S RNA (5); the 190-nucleotide band is weak, because one of the oligodeoxynucleotides used to generate this ribonuclease H fragment (centred on position 1398) overlaps the cross-link site, and consequently most of the mRNA remains in the 335-nucleotide band, although this effect is somewhat variable (cf. lanes 1 and 2). The corresponding fingerprint (Fig. 4h) shows the absence of the oligonucleotide CCŮACGp (cf. Fig. 1), confirming that the cross-link is to the +7 position. After translocation (Fig. 3, lane 2), a new band of 145 nucleotides appears corresponding to the '1402/7' cross-link (cf. Fig. 2). However, the fingerprint from this 145-nucleotide band (Fig. 4i) also shows the absence of CCŮACGp, demonstrating that the same thio-U residue—now in the +4 position after EF-G treatment—is responsible for the cross-link.

A further example of this type of experiment is illustrated in Figure 3, lanes 3 and 4, the translocation reaction in this case being particularly (and reproducibly) efficient. Here the mRNA is No. 7 (Fig. 1), carrying thio-U residues at positions +10 and +15. Prior to translocation (Fig. 3, lane 3) a weak band of 360 bases corresponding to the cross-link to nucleotide 1395 of the 16S RNA can be seen, as well as the 100-nucleotide band of comparable intensity corresponding to the cross-link to nucleotide 532 of the 16S RNA which was previously observed from the +11 position of the mRNA (cf. ref. 5, and see below for further discussion); the fingerprint data (not shown, but see ref. 5) indicated that the cross-link in the first of these two bands was rather non-specific (containing a 'minus tRNA' contribution—see next Section), whereas the cross-link in the 100-nucleotide band was from the +10 position of the mRNA. After translocation (Fig. 3, lane 4) there is a dramatic increase in the intensity of the 360-nucleotide fragment, now representing the strong cross-link from position +7 of the mRNA to nucleotide 1395 (5), whereas the 100-nucleotide fragment is weaker than before; the fingerprint from the latter band (not shown) showed a distribution of the cross-link between the CCŮACGp and AŮAAGp oligonucleotides (Fig. 1), indicating a mixture of the pre-translocation situation (where the cross-link is to the +10 position), and the post-translocational state (where a weak cross-link to the thio-U residue now at the +12 position occurs). A similar situation was observed with mRNA No. 6 (Fig. 1), where the thio-U residues are at positions +5 and +10. In this case the cross-link from position +10 to nucleotide 532 disappeared completely after translocation (not shown), whereas the cross-link to nucleotide 1395 again increased dramatically (as in Fig. 3, lane 4). The fingerprints corresponding to the latter cross-link are shown in Figures 4b and c; before translocation the fingerprint (Fig. 4b) of the weak band from the ribonuclease H digestion (cf. Fig. 3, lane 3) shows an almost complete absence of the oligonucleotide CCŮCAAGp (indicating predominant cross-linking to the +10 position—see next Section), whereas after translocation (Fig. 4c) the now intense ribonuclease H band (cf. Fig. 3, lane 4) showed a total absence of this oligonucleotide (indicating specific cross-linking to the same thio-U residue, now at the +7 position).

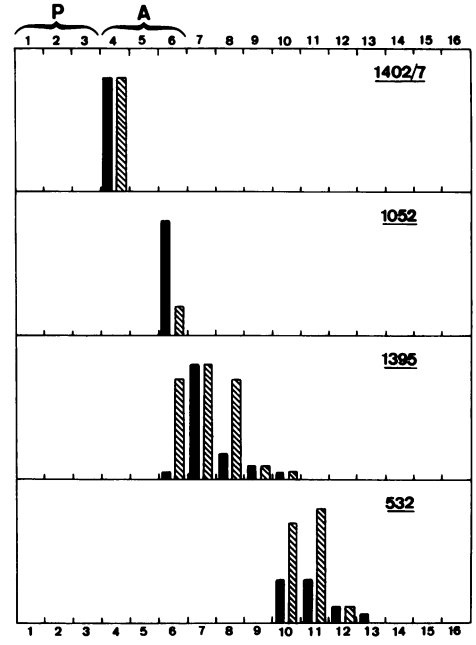

Figure 5. Summary of cross-linking data from downstream positions +1 to +16 of mRNA. Black bars represent data from cro-type mRNA sequences (e.g. mRNAs 1 to 3, Fig. 1), cross-hatched bars data from synthetic sequences (e.g. mRNAs 4 to 8). Positions of the P- and A-site codons are marked. No scale of intensity is given, to emphasize the only semi-quantitative nature of the diagram. Each panel is marked with the 16S RNA sequence position involved in the cross-link (cf. 5), the order of relative cross-linking intensity being 1395 ≥ '1402/7' > 1052 > 532 (cro-type mRNA), and 1395 ≥ 1402/7 > 532 > 1052 (synthetic sequences).

As a final example, Figures 4e and f show fingerprints corresponding to the same '1395' cross-link, but here from an mRNA analogue carrying thio-U residues at positions +6 and +11 (mRNA No. 4, Fig. 1). Before translocation (Fig. 4e), the fingerprint shows a reduction in intensity of the oligonucleotide CŮACGp (containing the thio-U at position +6), whereas after translocation (Fig. 4f) the cross-link has largely 'flipped' to the oligonucleotide AŮAGp (with the thio-U residue now at position +8).

A large number of experiments (with various different ribonuclease H digestions in each case) were performed in this

manner, so as to examine the cross-linking to 16S RNA from every position on the mRNA from +1 to +16, with translocation 'into' or 'out of' each position. The results, combined with other data (including that already published (2–5)), have led to a clear overall picture of the cross-links formed from the downstream region of the RNA, which is summarized in the next and final Section.

Summary of the cross-linking data

Figure 5 summarizes the cross-linking data to 16S RNA from the mRNA between positions +1 and +16. This Figure compiles data from a large number of experiments with different mRNA sequences and different ribosomal binding conditions, as well with different ribonuclease H digestions, etc. The mRNA analogues furthermore each contain several thio-U residues, and hence more than one cross-link is usually present in each case. Consequently, the relative levels of the individual cross-links from a single mRNA analogue can be compared fairly accurately (cf. Fig. 2), but, on the other hand, the quantification of the relative levels of cross-linking from one set of messengers to another is inevitably only approximate. For this reason no scale of cross-linking intensity is given in Figure 5, the important parameter being the positional specificity along the mRNA.

From translocation experiments similar to those described in the previous Section it was established that there are no tRNA-dependent cross-links from positions +1 or +3 of the mRNA; the translocation approach is of course necessary to probe these positions since they normally contain the A- and G-residues of the AUG initiator codon. The U-residue at position +2 also showed no tRNA-dependent cross-linking, either with or without translocation, although some cross-linking to this position was observed in 'minus tRNA' control samples (see also below).

The first tRNA-dependent cross-link in the downstream region of the mRNA is that from the +4 position to nucleotides '1402/7' of the 16S RNA, as described above (Fig. 2). This cross-link was observed at comparable intensity with both the cro-mRNA type of mRNA sequence (mRNAs No. 1 and 2, Fig. 1) and the synthetic type of sequence (similar to mRNAs No. 4 to 8, Fig. 1), and can thus be added to the list of 'universal' cross-links (5), as already discussed. The cross-link was absolutely specific for the +4 position, and no cross-linking at all to 16S RNA was observed from the neighbouring +5 position.

The cross-link from position +6 to nucleotide 1052 of the 16S RNA has been documented in detail (5). As with the +4 cross-link, the +6 cross-link is also tRNA-dependent, entirely specific for the +6 position, and formed with both classes of mRNA. However, as previously noted (5), the cross-linking is much less intense with the synthetic type of mRNA sequence. We had deduced (5) that the reason for this difference must be connected with the length and/or nucleotide composition of the spacer region between the Shine–Dalgarno sequence and the AUG codon, and this question was investigated further using mRNAs No. 3, 4 and 5 (Fig. 1), all of which carry a thio-U at the +6 position. mRNA No. 3 is a cro-type sequence, which showed a strong '+6/1052' cross-link (5, and Fig. 5), whereas mRNA No. 5 is a synthetic sequence showing a weak cross-link. In mRNA No. 3 the spacer region is four nucleotides long (UUGU), and in mRNA No. 5 it is nine (AAAAGAAAG). mRNA No. 4 is a 'hybrid' sequence, with the same Shine–Dalgarno sequence as mRNA No. 2, but now with the spacer AAAG instead of UUGU; this mRNA showed only the weak +6/1052 cross-link (data not shown), which suggests that the U-rich spacer is the critical factor involved, although sequence context effects in the immediate downstream region cannot be excluded. Further investigation of this question, as well as a study of the cross-linking to 16S RNA from the spacer region itself is currently in progress.

In the case of the synthetic mRNA sequences, cross-linking from the +6 position was also observed to nucleotide 1395 of the 16S RNA (5). The latter cross-link was found with this type of mRNA sequence from positions +7 and +8 as well, although the peak was clearly at position +7. With the cro-mRNA sequences, only traces of cross-linking to position 1395 were found from position +6, and the cross-linking from position +8 was also relatively weak, so that the peak cross-linking from position +7 is thus considerably more pronounced than with the synthetic sequences (Fig. 5). At positions +9 and +10, weak cross-linking to nucleotide 1395 from both types of mRNA sequence was observed (cf. Fig. 3, lane 3), but this was also seen in the absence of tRNA. Analysis by reverse transcriptase of the cross-link from positions +6, +7 and +8 showed that the site of cross-linking to 16S RNA (nucleotide 1395 (3,5)) was identical in each case, indicating that there is a flexibility of either the mRNA or the ribosomal RNA in this area, rather than a shift of the cross-link to a neighboured position on the 16S RNA from these neighbouring positions in the mRNA. In the minus tRNA control samples, traces of the 1395 cross-link were sometimes seen at positions outside those indicated in Figure 5.

The cross-link to nucleotide 532 of the 16S RNA showed a similar type of positional variation. With the synthetic mRNA sequences, this cross-link was observed from positions +10, +11 (most strongly) and +12 (very weakly) of the mRNA, and reverse transcriptase analysis demonstrated that the cross-link site on the 16S RNA was identical from both the +10 and +11 positions. In this case the cross-linking yields were lower with the cro-type mRNA sequences, but the positional specificity was the same, although in some experiments a weak cross-link was also observed with the cro-mRNAs from the +13 position, either directly or after translocation from position +16. No further cross-links were found with either type of sequence up to and including position +16 of the mRNA.

With the cro-mRNA sequences the relative intensities of the four 'universal' cross-links at their respective peak positions on the mRNA (Fig. 5) were 1395 ≥ '1402/7' > 1502 > 532, and the situation was the same with the synthetic sequences, except that the order of the last two was reversed, the 532 cross-link being stronger than the 1052 cross-link. These values refer to the levels of cross-linking found with ribosomal complexes containing $tRNA^{fMet}$ at the P-site. However, in the presence of a second cognate tRNA at the ribosomal A-site, significant effects were observed on the cross-linking intensities from mRNA positions in the immediate vicinity of the decoding region. As shown in Figure 2, the '1402/7' cross-link from the +4 position of the mRNA (Fig. 5) is strongly reduced by the binding of a second tRNA. A similar reduction has already been reported (5) for the +6/1052 cross-link, again with both types of mRNA, and we have previously suggested (5) that the low amounts of the +6/1052 cross-link which remain may be ascribed to that fraction of the complexes which only carried a P-site bound tRNA; the same argument can be applied to the case of the '1402/7' cross-link (Fig. 2).

With the synthetic mRNA sequences a strong reduction of the cross-linking from position +6 to nucleotide 1395 of the 16S

RNA (Fig. 5) was also observed when a second tRNA was bound, and the corresponding cross-link from position +7 was reduced with both classes of mRNA. In contrast, at positions farther out from the decoding site, the binding of a second tRNA caused either no effect or even an increase in the level of cross-linking. Thus, with both types of mRNA sequence the cross-linking to nucleotide 1395 from position +8 of the mRNA was unaffected, whereas with the cro-type sequences the corresponding weak cross-linking to nucleotide 1395 from positions +9 and +10 (Fig. 5) was enhanced. In the case of the synthetic sequences, we had previously reported that the +11/532 cross-link was slightly increased by a second tRNA, but further analyses have led us to conclude that this effect was not significant; cross-linking to nucleotide 532 from positions +10, +11 or +12 in both types of mRNA (Fig. 5) is in general not affected, although with the cro-sequences a weak cross-link to this nucleotide from position +9 has been observed in the presence of a second tRNA.

The strongly inhibitory effect of the second tRNA on the levels of cross-linking within the A-site (positions +4 and +6) could be due to shielding by the codon-anticodon interaction, and a corresponding shielding by the P-site bound tRNA could explain the absence of any tRNA-dependent cross-linking from positions +1 to +3 of the mRNA. However, since the cross-linking from position +7 (but not that from +8) is also reduced in the presence of the second tRNA as just noted, it may be that this reflects a reduction in the flexibility of the mRNA-tRNA-ribosome complex in the immediate vicinity of the decoding site when the second tRNA is bound. This latter explanation is supported by the observation (14) that the previously reported (4) rather non-specific cross-linking of proteins S18 and S21 to the seven-nucleotide UUGUAUG initiator region of the cro-type mRNA sequences (cf. Fig. 1) is abolished by the binding of a second tRNA, whereas the corresponding cross-linking of S7 specifically to the UUG sequence (which is thus somewhat farther away from the actual decoding site) remains unaffected.

We have already commented in detail (5) on the fact that the mRNA cross-linking data are not compatible with current models for the three-dimensional arrangement of the 16S RNA (6,7). We have also noted the fact (3) that the cross-links to nucleotides 532 and 1395 (positions which are far apart in these models) coincide with regions of the 16S RNA proposed by Trifonov (15) to be involved in mRNA binding to the ribosome, on the basis of mRNA sequence analysis. However, the short distance between the two mRNA positions concerned (+7 and +11) precludes the possibility of any extensive base-pairing of the mRNA simultaneously to these regions, and the flexibility of the cross-linking to these two sites (Fig. 5) as discussed above suggests rather that the Trifonov sequences may be involved in some kind of transient dynamic interaction with the mRNA, possibly related to the translocation process.

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