The EMBO Journal vol.10 no.8 pp.2195-2202, 1991

The path of mRNA through the *Escherichia coli* ribosome; site-directed cross-linking of mRNA analogues carrying a photo-reactive label at various points 3′ to the decoding site

Jutta Rinke-Appel, Nicole Jünke, Katrin Stade and Richard Brimacombe

Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Ihnestrasse 73, 1000-Berlin-33, FRG

Communicated by K.H.Nierhaus

mRNA analogues ~40 bases long were prepared by T7 transcription from synthetic DNA templates. Each message contained the sequence ACC-GCG (coding for threonine and alanine, respectively), together with a single thio-U residue located at a variable position on the 3′-side of these coding triplets. The thio-U residue was either substituted with 4-azidophenacyl bromide to introduce a photo-reactive group, or was left unsubstituted for direct UV cross-linking. After binding to *Escherichia coli* 70S ribosomes in the presence of tRNA-Thr or tRNA-Ala, the thio-U residue or azidophenyl group was photo-activated and the products of cross-linking (which was exclusively to the 30S subunit) were analysed. Immunological analysis of the cross-linked proteins showed that S5 and S3, together with S1, were the targets of cross-linking at positions close to the decoding site, with the cross-linking to S3 and S1 persisting at positions further away. Analysis of the 16S RNA showed cross-links to the region of bases 1390-1400 in all cases, but in one instance (with the reactive nucleotide 11 bases from the decoding site) simultaneous cross-linking was observed to the latter region and to position 532; these two RNA regions are far apart in current three-dimensional models of the 30S subunit.

Key words: reverse transcriptase/ribosomal protein antibodies/ribosome structure/mRNA analogues/site-directed cross-links

Introduction

The determination of the path followed by mRNA through the translating ribosome continues to be a central question for a more detailed understanding of the process of protein biosynthesis. It is generally accepted that the actual decoding site lies at the bottom of the cleft of the 30S subunit, as evidenced primarily by the immunoelectron microscopic localization (Gornicki *et al.*, 1984) of the base in 16S RNA (position C-1400) which can be cross-linked to the anti-codon loop of tRNA (Prince *et al.*, 1982). Several direct lines of evidence (e.g. Olson *et al.*, 1988; Stade *et al.*, 1989) indicate furthermore that the path of the 'outgoing' mRNA leads from this site upwards out of the cleft towards the anti-Shine-Dalgarno sequence, namely the extreme 3′ terminus of the 16S RNA, which is located on the platform of the 30S subunit (e.g. Lührmann *et al.*, 1981).

In contrast, the path of the 'incoming' mRNA is not at all clear, there being two distinct possibilities: either the mRNA could approach the decoding site in the cleft by wrapping around the 'front' (interface side) of the 30S subunit, or it could approach it from the 'rear' (solvent side) by simply passing through the cleft. Since it is unlikely that the phosphate backbone of the mRNA is fully extended, both these possibilities are in principle compatible with the findings that (i) ~50 nucleotides of the message are shielded by the ribosome from nuclease attack (Kang and Cantor, 1985), and (ii) that the entry and exit sites of a poly(U) mRNA analogue are located at the base of the cleft on the solvent side of the 30S subunit (Evstafieva *et al.*, 1983).

We have already published a site-directed cross-linking study, in which messenger analogues carrying a photo-reactive label at various positions on the 5′-side of the coding triplet were prepared, and the products of cross-linking to the 30S ribosomal subunit analysed (Stade *et al.*, 1989). Those experiments, as quoted above, have helped to define the path of the outgoing message, and in this paper we describe a corresponding study, in which the mRNA analogues carry a photo-reactive group at various positions on the 3′-side of the coding triplet. mRNA, in contrast to tRNA, is a flexible molecule, so it might be expected that the results of a study of this type will not be simple to interpret. Indeed, the observations that we have made are very curious. First, over a wide range of positions of the photo-label in the mRNA, a tRNA-dependent cross-linking to the 16S RNA was found within a very narrow range of positions, namely the 1390-1400 region. Secondly, the corresponding cross-linking to the ribosomal proteins was found to involve S3 and/or S5 (and also S1), although neither S3 nor S5 is close to the 1400 region (see e.g. Capel *et al.*, 1988; Brimacombe *et al.*, 1988a), which as already noted lies in the cleft of the 30S subunit. Thirdly, and most importantly, with one of the mRNA analogues two highly reproducible and tRNA-dependent cross-links to the 16S RNA were observed simultaneously, namely to the 1390-1400 region and to position 532; the latter position—like the 1400 region—has been implicated in a number of ribosomal functions (see Discussion), but in current three-dimensional models of the 16S RNA (Brimacombe *et al.*, 1988a; Stern *et al.*, 1988) the two cross-linked regions (1400 and 530) are located on opposite sides of the 30S subunit.

Results

Figure 1 shows the sequences of the mRNA analogues that were used in these experiments. As in our previous study (Stade *et al.*, 1989), the RNAs were prepared by transcription with T7 polymerase from synthetic DNA templates according to the method of Lowary *et al.* (1986), and as before each sequence contained adjacent triplets (ACC and GCG) coding for threonine and alanine, respectively, together with a single uridine residue at a specific position relative to these coding triplets. Again as before (Stade *et al.*, 1989), the sequences include tracts of A residues, to enable

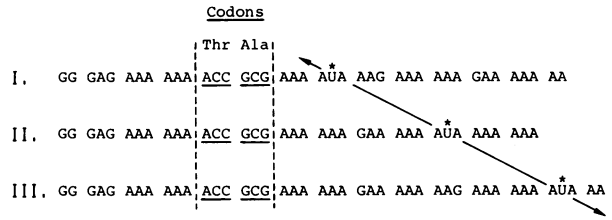

Fig. 1. The three mRNA sequences used in this study. The positions of the triplets coding for threonine and alanine, respectively, are indicated, and the position of the thio-U residue in the 3'-region of the sequence is denoted by $\overset{*}{U}$ in each case.

the cross-linked products to be isolated by affinity chromatography on oligo(dT)−cellulose. The mRNAs were radioactively labelled by incorporation of [^{32}P]ATP.

The uridine residues in the mRNA sequences were transcribed using thio-UTP instead of UTP, and the thio-U residues thus incorporated were either substituted with *p*-azidophenacyl bromide (APAB, Hixson and Hixson, 1975) or left unsubstituted for direct UV cross-linking (Tate *et al.*, 1990; see Hajnsdorf *et al.*, 1989). In the former case, photo-activation of the azidophenyl group in the mRNA−tRNA−ribosome complexes was carried out at a wavelength >280 nm (Stade *et al.*, 1989), whereas in the latter case wavelengths >300 nm were used (Tate *et al.*, 1990). Cross-linking could be shown to take place exclusively to the APAB or thio-U residue, because in control experiments, using the same mRNA species with 'normal' U in place of thio-U, no cross-linking of mRNA to ribosomal components was observed at all under either irradiation condition (data not shown).

Hartz *et al.* (1990) have demonstrated, with their elegant 'toeprinting' assay, that mRNA bound to the ribosome is protected as far as the 15th base downstream from the first position of the coding triplet. In the mRNA sequences of Figure 1, the thio-U residue is located at positions both within and outside of this 15 base range. Our most detailed analyses were made with mRNA I, where the thio-U is at position +8 or +11 (depending on whether GCG or ACC was taken as the coding triplet, respectively), the other two mRNA species being used to gain an impression of possible contacts with the ribosome beyond the 15 base 'limit' of Hartz *et al.* (1990).

^{32}P-labelled mRNA was bound to 70S ribosomes in the presence of tRNA-Ala or tRNA-Thr (or in the absence of tRNA), at a ratio of mRNA:tRNA:70S ribosomes of ~2:5:1 (cf. Stade *et al.*, 1989, and Materials and methods). As in our previous experiments (Stade *et al.*, 1989), the mRNA−tRNA−ribosome complexes were separated from unbound tRNA and mRNA by sucrose gradient centrifugation in the presence of 10 mM magnesium, and the photo-activation of the thio-U or APAB was carried out directly on the sucrose gradient fractions containing the 70S complex; mRNA binding to this complex was stimulated 3- to 5-fold by the presence of the appropriate tRNA and represented 0.7−1.0 mol of mRNA per 70S ribosome. The cross-linked 70S complexes were then subjected to two further sucrose gradient centrifugations (Stade *et al.*, 1989). The first of these, in 0.3 mM magnesium, served to dissociate the ribosomes into subunits, and to separate non-cross-linked mRNA; in all cases, cross-linking was found to occur exclusively to the 30S subunit (data not shown). In the final

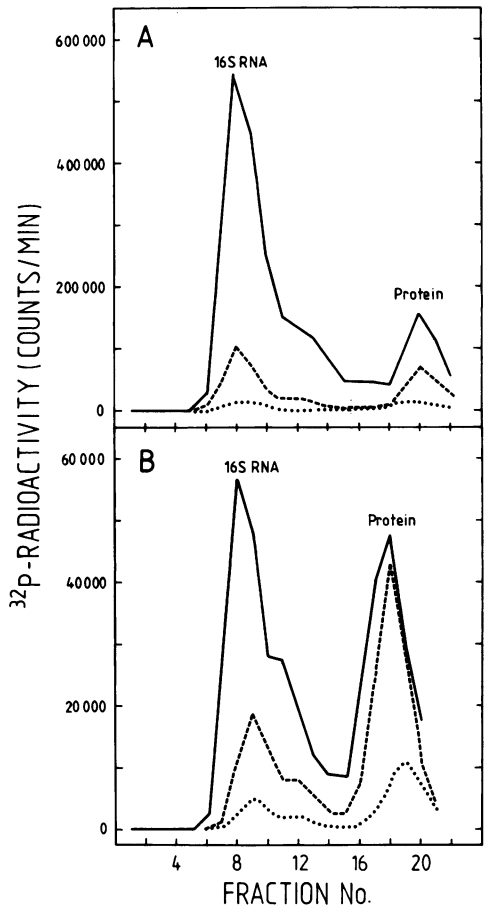

Fig. 2. Sucrose gradient profiles in SDS, showing the dissociation of 30S subunits into 16S RNA and ribosomal protein fractions cross-linked to ^{32}P-labelled mRNA I (see Figure 1). **A.** Gradients from mRNA I containing unsubstituted thio-U. **B.** Gradients from mRNA I containing thio-U substituted with APAB. In each case the solid line is the gradient from the sample prepared with tRNA-Ala, the dashed line from that prepared with tRNA-Thr, and the dotted line from the control sample without tRNA. Direction of sedimentation is from right to left. Each of the six gradients shown is derived from the same amount of initial input material.

centrifugation, SDS was included to dissociate the cross-linked 30S subunits into 16S RNA and ribosomal protein fractions; examples of these latter gradients are illustrated in Figure 2. Figure 2A shows the products of cross-linking with mRNA I (Figure 1) containing unsubstituted thio-U, whereas Figure 2B shows the corresponding products from mRNA I containing thio-U substituted with APAB.

For each of the gradients in Figure 2, the initial input of mRNA and 70S ribosomes at the beginning of the experiment was identical, and it can thus be seen that the levels of cross-linking obtained with the unsubstituted thio-U (Figure 2A) are considerably higher than those obtained with APAB (note that the scale of radioactivity in Figure 2A is ten times that of Figure 2B). The amount of cross-linking in the presence of tRNA-Ala is greater than that in the presence of tRNA-Thr, particularly to the 16S RNA fraction, which reflects in part the fact that the stimulation of mRNA binding by tRNA-Ala was normally somewhat stronger than by tRNA-Thr. To give some idea of the relative levels of cross-linking in the various cases, the reaction in the presence of tRNA-Ala with the 16S RNA fraction in Figure 2A represents a cross-linking yield of ~50% of

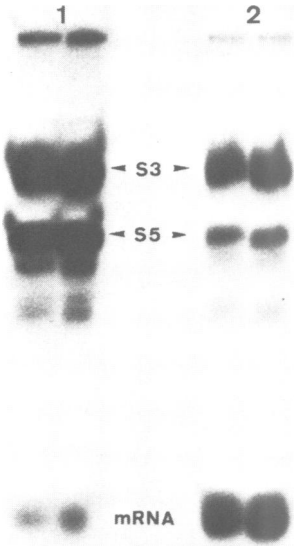

Fig. 3. Polyacrylamide gel separation of ribosomal proteins cross-linked to mRNA I. The photograph is an autoradiogram of the ^{32}P-labelled mRNA–protein complexes from an SDS–sucrose gradient such as that of Figure 2, in this case with samples prepared using tRNA-Thr. Each sample was divided between two gel slots to prevent overloading. Slots 1: from mRNA I containing unsubstituted thio-U; slots 2: from mRNA I containing thio-U substituted with APAB. After electrophoresis the complexes were extracted and the proteins identified immunologically (see Materials and methods); the bands corresponding to proteins S3, S5 or to free mRNA are indicated.

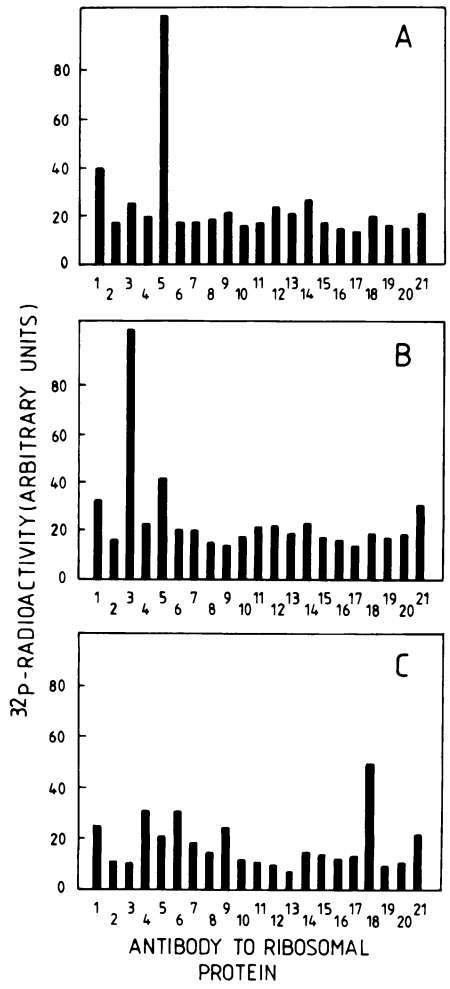

Fig. 4. Immunological analysis of 30S ribosomal proteins cross-linked to mRNA I containing thio-U substituted with APAB. The figure shows the ^{32}P radioactivity (in arbitrary units—see text) remaining bound to the agarose–antibody preparations (see Materials and methods) for each of the 21 ribosomal protein antibodies. The samples in this case were analysed directly from the SDS–sucrose gradient fractions (see Figure 2), without separation by gel electrophoresis (see Figure 3). **A**. Results from mRNA bound in the presence of tRNA-Ala. **B**. mRNA bound in the presence of tRNA-Thr. **C**. mRNA bound in the absence of tRNA.

the [^{32}P]mRNA bound to the 70S complex after the first sucrose gradient. In all cases in Figure 2, there is significant tRNA-dependent cross-linking of the mRNA to both the 16S RNA and protein fractions, whereas the samples prepared in the absence of tRNA show only low levels of cross-linking.

The ribosomal proteins cross-linked to the mRNA were identified in one of two ways. In the first method, the appropriate SDS–sucrose gradient fractions (see Figure 2) were precipitated with ethanol and the protein–mRNA complexes were separated by electrophoresis on gels containing SDS and urea, followed by immunological identification of the proteins in the individual bands (Gulle *et al.*, 1988; see Materials and methods). Alternatively, aliquots of the SDS–sucrose gradient fractions were subjected to the immunological test directly. Since the immunological assay is not quantitative with RNA–protein complexes (Osswald *et al.*, 1990), a combination of the two methods gives a better overall assessment of the relative amounts of the individual proteins involved in the cross-linking. Examples are illustrated in Figures 3 and 4.

Figure 3 shows the separation of cross-linked protein–mRNA complexes isolated from 70S samples prepared with mRNA I containing either unsubstituted thio-U or APAB-substituted thio-U, in the presence of tRNA-Thr. As expected from the corresponding results in Figure 2, the former sample (slot 1, Figure 3) shows larger amounts of the cross-linked complexes than the APAB sample (slot 2). In both cases, proteins S3 and S5 are the primary targets of cross-linking, albeit in different relative amounts, and some free mRNA is also visible in both cases.

Figure 4 shows the results of a comprehensive screening test (Stade *et al.*, 1989) with antibodies to all 21 of the ribosomal proteins from the 30S subunit, made directly from the SDS–sucrose gradient fractions. In this case the samples were from APAB-substituted mRNA I (see Figure 2B), in the presence of tRNA-Ala (Figure 4A), tRNA-Thr (Figure 4B), or in the absence of tRNA (Figure 4C). In view of the non-quantitative nature of the antibody assay just mentioned, the individual results cannot be compared with one another directly and therefore the scale of radioactivity in Figure 4 is given in arbitrary units, which in real terms normally corresponded to 200–1000 c.p.m. in the case of a positive antibody reaction. The results show a clear reaction with anti-S5 in the case of tRNA-Ala (Figure 4A), as opposed to a reaction with anti-S3 together with some anti-S5 in the case of tRNA-Thr (Figure 4B; cf. Figure 3, slot 2). The control sample without tRNA (Figure 4C) shows only low and ill-defined levels of reaction with the antibodies, anti-S18

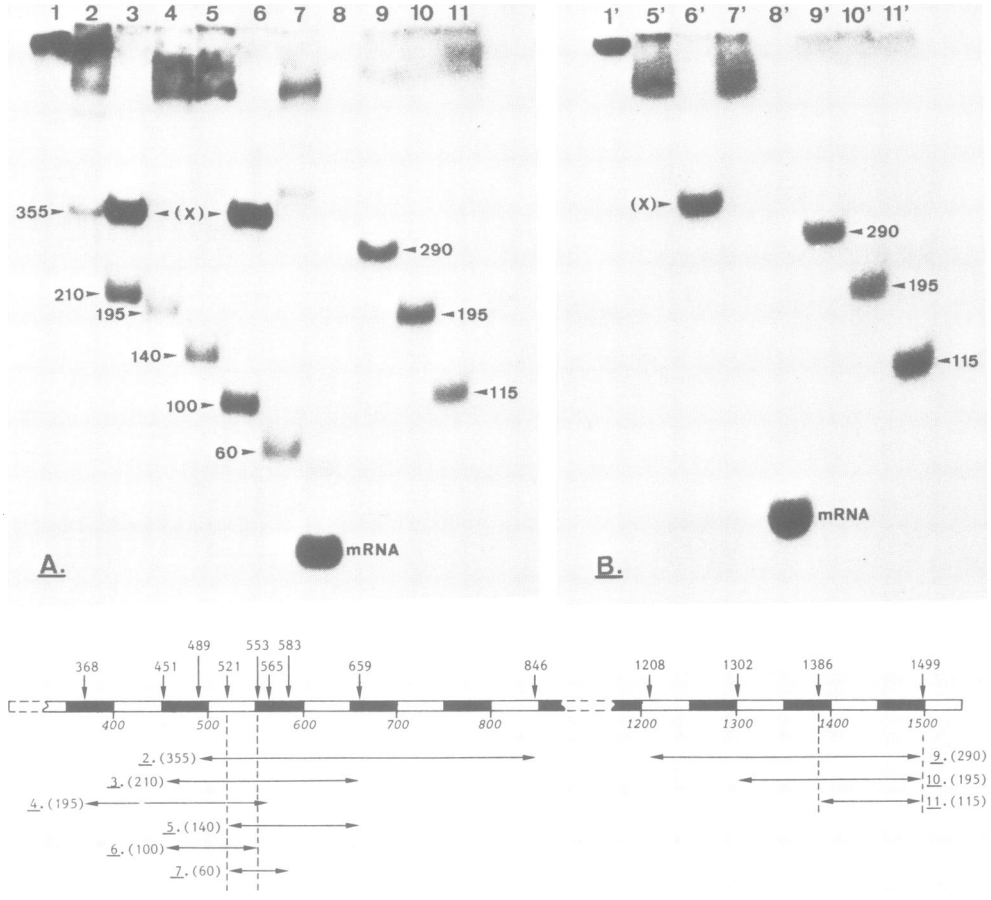

Fig. 5. Polyacrylamide gels of RNase H-digested cross-linked complexes between 16S RNA and ^{32}P-labelled mRNA I (containing unsubstituted thio-U). **A**. Sample prepared with tRNA-Thr. **B**. Control sample prepared in the absence of tRNA. Slot 1: 16S−mRNA complex incubated with RNase H in the absence of deoxyoligonucleotides. Slot 8: Free mRNA. Slots 2−7 and 8−11 contain 16S−mRNA complexes incubated with RNase H in the presence of pairs of decadeoxynucleotides as follows (the oligonucleotides are listed according to the positions in the 16S RNA complementary to the 5th base of the oligonucleotide): slot 2: oligonucleotides at positions 489, 846; slot 3: positions 451, 659; slot 4: positions 368, 565; slot 5: positions 521, 659; slot 6: positions 451, 553; slot 7: positions 521, 583; slot 9: positions 1208, 1499; slot 10: positions 1302, 1499; slot 11: positions 1386, 1499. The samples in gel B are equivalent to those in gel A with the corresponding slot numbers. The size of the 16S fragment (cross-linked to the [^{32}P]mRNA) excised in each case is indicated. ('X' is an extra band due to a cut produced by the oligonucleotide complementary to position 451, which also has a partial complementarity to 16S RNA at position ~1200). The lower part of the diagram summarizes the data within the 16S RNA sequence, and gives the slot number (gel A) together with the length and position of the excised fragment. The dotted lines indicate the 'shortest common sequence' within which each of the two cross-links must lie.

being the most prominent. (Small amounts of mRNA−protein complexes corresponding to S18 can also be seen running below the S5 band in the gel of Figure 3.) These findings were very reproducible, with the exception that anti-S1 showed a reaction which was somewhat variable (some reaction with anti-S1 can for example be seen in Figure 4A).

The results of all the various protein analyses can be summarized as follows. With mRNA I and tRNA-Ala, S5 was the principal target of cross-linking, both with APAB-substituted thio-U (Figure 4A) and with unsubstituted thio-U. With mRNA I and tRNA-Thr, S3 was the principal target, although S5 was also present (Figure 3). These cross-links to S3 and S5 were entirely tRNA-dependent (Figure 4). As just mentioned, variable amounts of S1 were also cross-linked. With mRNA II (Figure 1), the cross-linking to S5 was only very weak, whereas that to S3 and S1 was still strong, and with mRNA III, cross-links only to S3 and S1 were observed. These cross-links were seen with both tRNA species, and with both types of photo-reactive moiety. (Some cross-linking to S1 was also observed in the samples without tRNA in the case of mRNAs II and III.)

For the analysis of the cross-link sites on 16S RNA, a two-step approach was employed. First, a preliminary localization of the site was made using RNase H together with selected oligodeoxynucleotides (Brimacombe *et al.*, 1990), and the final localization was then effected with reverse transcriptase and a suitable oligodeoxynucleotide primer (see Moazed *et al.*, 1986a). Examples are shown in Figures 5 and 6 respectively.

In the RNase H method, the 16S RNA−mRNA complexes (see Figure 2) are digested with RNase H in the presence of pairs of deoxyoligonucleotides complementary to regions of the 16S RNA separated by ~50−200 nucleotides. Since only the mRNA is radioactive, a subsequent gel electrophoretic separation of the digestion products should reveal a low mol. wt radioactive complex only if the cross-link site lies between the complementary positions of the two oligonucleotides used. Once a positive 'hit' has been identified, the position of the cross-link site can be narrowed

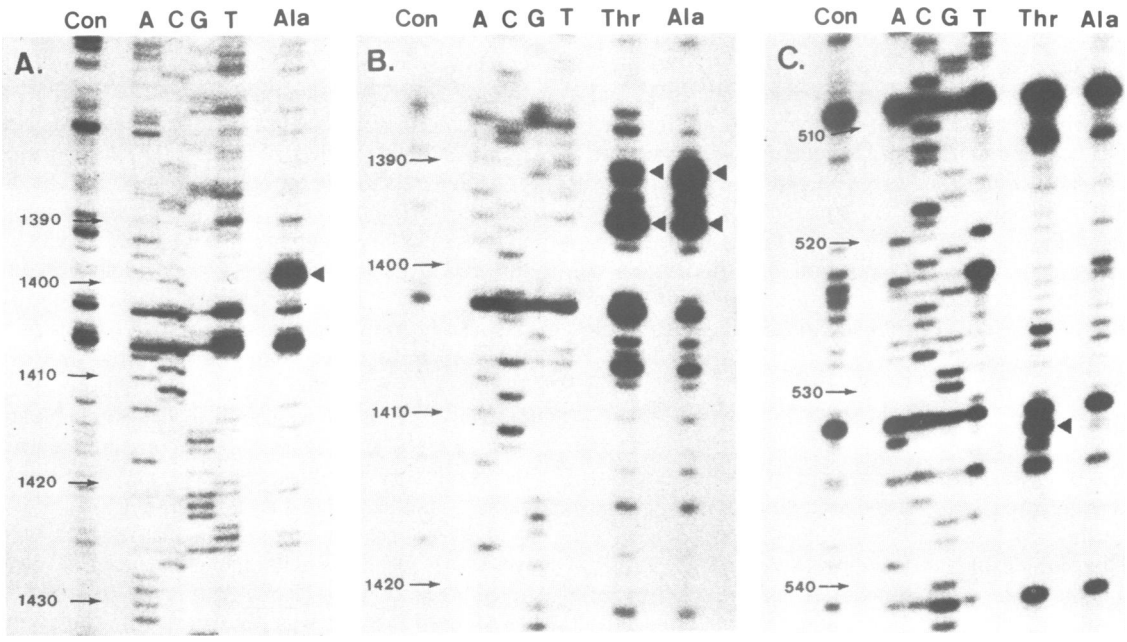

Fig. 6. Reverse transcriptase analysis of cross-link sites in the 16S RNA. In each panel the first (control) slot represents the transcript of free 16S RNA separated from the cross-linked complex by affinity chromatography on oligo(dT)−cellulose (see text). The next slots are A, C, G and T sequencing lanes, respectively, and the slots marked Ala or Thr are the transcripts obtained from the cross-linked samples (using tRNA-Ala or tRNA-Thr as appropriate), after isolation by chromatography on oligo(dT)−cellulose. The positions within the 16S sequence are numbered, major transcription stops in the cross-linked samples being marked with the filled triangles. **A**. mRNA I, containing thio-U substituted with APAB, transcribed with an oligodeoxynucleotide primer complementary to positions 1490−1507 of the 16S RNA. **B** and **C**. mRNA I, containing unsubstituted thio-U, transcribed with oligodeoxynucleotides complementary to positions 1443−1459 and 557−573, respectively. [The sequence of 16S RNA in the regions around the cross-links reads: UGUACACACC GmCCCGUmCACA (1391−1410), GCAGCCmGCGG UAAUACGGAG (521−540).]

down by using different combinations of oligonucleotides; Figure 5 shows the results of such an experiment. In Figure 5A, the sample was prepared in the presence of tRNA-Thr, using mRNA I with unsubstituted thio-U. Figure 5B is the corresponding control without tRNA. It can be seen (Figure 5A) that in the tRNA-Thr sample, two cross-link sites are simultaneously present in the 16S RNA, one in the region of bases ~520−550, and the other nearer to the 3′ terminus. In the sample without tRNA (Figure 5B), only the second of these cross-links is present, and in the corresponding sample using tRNA-Ala (not shown) again only the second cross-link was observed. Further RNase H digestions (not shown) indicated that the latter cross-link was very close to position 1400; in particular a deoxynucleotide centred on position 1398 (which normally cut the 16S RNA very efficiently in the presence of RNase H) was unable to cut the 16S RNA−mRNA complex.

Cross-linking to the 1400 region was observed with all three mRNA species and in all samples, that is to say with tRNA-Ala, tRNA-Thr, or without tRNA, and with APAB-substituted or unsubstituted thio-U, although the level of cross-linking was very low in the case of the unsubstituted thio-U samples with mRNAs II and III. In contrast, only the sample with tRNA-Thr and mRNA I (containing either unsubstituted thio-U or APAB-substituted thio-U) showed the cross-link to the 520−550 region (Figure 5A), and this result was entirely reproducible. (A weak cross-linking was also observed with mRNAs II and III in the presence of tRNA-Ala in the region of bases 330−490 of the 16S RNA, but this site has not been localized further.)

Although the cross-link to the 1400 region of the 16S RNA appeared to be the same for all samples, as analysed by the RNase H method, the corresponding reverse transcriptase analysis revealed some subtle differences. For this assay, the cross-linked mRNA complexes were first subjected to affinity chromatography on oligo(dT)−cellulose, which separates the cross-linked complex from free 16S RNA by making use of the oligo(A) tracts in the mRNA sequences (Figure 1; see Stade *et al.*, 1989). The free 16S RNA, which did not bind to the oligo(dT)−cellulose, was used as a control in the reverse transcriptase assays. The reverse transcription (see Materials and methods) was carried out using oligodeoxynucleotides 17 bases long (Moazed *et al.*, 1986a) complementary to positions ~50−100 bases downstream of the cross-link site, as defined by the RNase H data. The cross-link site itself should be revealed as a strong 'stop' in the reverse transcription at the base immediately preceding the cross-linked nucleotide, and this position should of course be consistent with the RNase H results. (This is not a trivial point, as quite reproducible artefacts are often seen in the reverse transcriptase gels, probably arising from nicks in the RNA, which are unavoidable after a long experimental procedure of this type.)

The reverse transcription assay was only made with cross-linked samples derived from mRNA I, and examples of the sequencing gels obtained are shown in Figure 6. In Figure 6A, a strong stop can be seen at position 1399 (indicating a cross-link site at position 1398), the sample here being mRNA I, APAB-substituted, in the presence of tRNA-Ala. The same result, albeit weaker, was found with the corresponding tRNA-Thr sample. (The two stops just below this position in Figure 6A are due to the methylated

bases at positions 1402 and 1407.) Figure 6B shows the results obtained with both tRNA-Ala and tRNA-Thr, using mRNA I containing unsubstituted thio-U. Here the most prominent transcription stops are at positions 1392 and 1396, and there are noticeable (and reproducible) differences in intensity between the stops in the alanine and threonine samples. Finally, Figure 6C gives an example of the other cross-link site found with tRNA-Thr and mRNA I (using unsubstituted thio-U; see Figure 5A). Here a clear transcription stop can be seen at position 533 (indicating a cross-link at position 532), which is not present in either the tRNA-Ala or the control samples. Again, this result was entirely reproducible.

Discussion

The results described in this paper are in notable contrast to the corresponding set of site-directed cross-linking data obtained using mRNA analogues with the photo-reactive label on the 5′-side of the decoding triplets (Stade *et al.*, 1989). In the latter experiments a simple pattern of cross-linking was observed, involving protein S7 and the extreme 3′ terminus of the 16S RNA, whereas here the situation is more complex and difficult to explain. For this reason the experiments were repeated many times, and showed a very high degree of reproducibility. Depending on whether tRNA-Ala or tRNA-Thr was used, the position of the photo-reactive uridine residue (see Figure 1) was at position +8 or +11 (mRNA I), +17 or +20 (mRNA II) or +26 or +29 (mRNA III) in relation to the first base of the coding triplet. At position +8, tRNA-dependent cross-linking to protein S5 was found (Figure 4), at position +11 cross-linking was to S3 and S5 (Figure 3), whereas at the more remote positions the cross-linking was to S3 and S1. The corresponding cross-linking to the 16S RNA involved the 1390–1400 region at all positions of the photo-reactive group, and this was also observed in the absence of tRNA (albeit very weakly). The additional very specific tRNA-dependent cross-link to position 532 occurred only when the photo-label was at position +11 (using mRNA I with tRNA-Thr; Figures 5 and 6).

The positions of the photo-reactive group in mRNAs II and III lie outside the 15-nucleotide limit found in the 'toeprinting' experiments of Hartz *et al.* (1990) already described above. Since cross-linking to S1, S3 and the 1390–1400 region was found with both these mRNA species (which have essentially no potential for secondary structure formation) it seems likely that the 3′-region of the mRNA must coil back onto the 30S subunit. The same pattern of cross-linking (although at a much lower level; cf. Figure 1) was also observed in the absence of tRNA. The consistent cross-linking to S1 and S3 could be explained on the grounds that both are large proteins. Indeed S1 is so large that cross-links to it are not very informative from a detailed topographical point of view, although it is worth noting that both neutron scattering (Capel *et al.*, 1988) and immunoelectron microscopic data (Walleczek *et al.*, 1990) place this protein at the 'rear' (i.e. solvent side) of the 30S subunit. Why, however, should there be concomitant cross-linking almost exclusively to a single short region—the 1390–1400 region—of the 16S RNA? It would be tempting to dismiss this finding as a 'hot-spot' artefact, but this possibility can be excluded. The cross-linking to the 1390–1400 region was observed with mRNAs containing both unsubstituted thio-U and APAB-substituted thio-U (with different irradiation conditions in each case), and the precise site of the cross-linking varied between nucleotides 1392 and 1398 (Figure 6). Furthermore, cross-linking to this region has already been found in a wide variety of other situations and experimental conditions, including cross-links to tRNA in both the A- and P-sites (Ofengand *et al.*, 1986), cross-links to a simple poly(A) mRNA analogue (Stiege *et al.*, 1988), and site-directed cross-links to mRNA at positions very close to the 3′-side of the coding triplet (Tate *et al.*, 1990). The region has also been implicated in many other types of functional study, including footprinting of tRNA (Moazed and Noller, 1986), 30S–50S subunit association (Herr *et al.*, 1979), or the active–inactive transition of the 30S subunit (Moazed *et al.*, 1986b). Clearly, this highly conserved sequence region has some very peculiar properties, and seems at least to be rather flexible within the 30S subunit.

Our interest has focused on the results obtained with mRNA I. Whereas with the photo-reactive group in the '+8' position (using tRNA-Ala) cross-linking to S5 and the 1390–1400 region was observed, with the photo-reactive group in the '+11' position (using tRNA-Thr) four cross-links were found, *viz.* to proteins S3 and S5, and to positions 532 and 1390–1400 of the 16S RNA. It should be noted that this result is in precise agreement with a previous study (Towbin and Elson, 1978) using short oligonucleotide mRNA analogues carrying an affinity label at the 3′ terminus. In those experiments, S3 and S5 were also the targets of cross-linking, as well as two (albeit unidentified) sites on the 16S RNA. In a more recent study with similar oligonucleotide mRNA analogues, Vladimirov *et al.* (1990) identified a number of cross-linked proteins, including some from the 50S subunit; S3 and S5 were among the proteins found. Current models for the three-dimensional organization of the 16S RNA (Brimacombe *et al.*, 1988a; Stern *et al.*, 1988) place the 1390–1400 region at the base of the cleft of the 30S subunit, with position 532—as well as the mass centres of proteins S3 and S5 (see Capel *et al.*, 1988) —being located on the opposite side of the 'head' of the subunit. S3 and S5 have been identified as a cross-linked protein pair (Traut *et al.*, 1980), and both have been implicated in the binding of dihydrostreptomycin to the ribosome (Schreiner and Nierhaus, 1973). It is conceivable that, despite the somewhat distant position of their mass centres from the cleft, these proteins could extend towards the 1390–1400 region sufficiently to explain the simultaneous cross-linking that was observed. In this context it is also of interest that protein S5 has been cross-linked to the D-loop of tRNA (Abdurashidova *et al.*, 1989).

The simultaneous cross-linking to positions 532 and the 1390–1400 region of the 16S RNA is, however, very hard to account for. It is possible that the mRNA has two distinct binding sites, or that the 3′-region of the mRNA can move about freely within the subunit. It must also be remembered that the use of a single tRNA molecule to bind the mRNA may not accurately reflect the situation in the 'elongating ribosome'. Whatever the reason, then again the result cannot be dismissed as a trivial artefact, because—like the 1390–1400 region—the '530 loop' of the 16S RNA has also been implicated in many functional studies. These include the footprinting of tRNA (Moazed and Noller, 1986) or antibiotics (Moazed and Noller, 1987), and sites of mutation

causing resistance to streptomycin (Melançon *et al.*, 1988, and cf. Schreiner and Nierhaus, 1973, as mentioned above), suppression of an ochre mutation (Shen and Fox, 1989), or lethality (Powers and Noller, 1990). Interestingly, both the 530 loop and the 1390–1400 region have been proposed by Trifonov (1987) to be involved in mRNA binding to the 30S subunit, on the basis of mRNA sequence analyses.

So far, it has been assumed that the functional involvement of the 530 loop—at least in the case of tRNA binding (Moazed and Noller, 1986)—is of an allosteric nature, as this loop is at least 65 Å distant from the 1400 position. This distance estimate is based primarily on the electron microscopic evidence locating the m7G-527 (Trempe *et al.*, 1982) and C-1400 residues (Gornicki *et al.*, 1984). However, a closer examination of the data reveals that there is in fact a discrepancy here. DNA hybridization microscopy (Oakes and Lake, 1990) places positions 1392–1407 and 1942–1505 deep in the cleft of the 30S subunit, in accordance with the location of position C-1400 (Gornicki *et al.*, 1984). The congruent location of the two regions is supported by the proposal of phylogenetically conserved tertiary interactions between bases 1401 and 1501, and 1405 and 1496 (Haselman *et al.*, 1989). On the other hand, the mapping by immuno-electron microscopy of N6-monomethyl adenosine, which occurs in eukaryotes at a highly conserved sequence position corresponding to position 1500 in *Escherichia coli*, gives a location indistinguishable from that of the m7-guanosine residue at position 527 in *E.coli* (Montesano and Glitz, 1988), which as already noted lies on the opposite side of the 30S subunit (Trempe *et al.*, 1982). Similarly, the m7-guanosine residue in eukaryotes also maps at the identical location to the m7-guanosine from *E.coli* (Montesano and Glitz, 1988), although in eukaryotes this residue is in a sequence position corresponding to position 1338 of the *E.coli* sequence; the models for the three-dimensional arrangement of the 16S RNA (Brimacombe *et al.*, 1988a; Stern *et al.*, 1988) place this latter residue in or near to the cleft, rather than on the opposite side of the subunit where the 530 loop is located.

The most likely explanation at the moment for these contradictions is that the 'neck' of the 30S subunit may be narrower than current models suggest. This factor, coupled with a flexibility of the 1400 region as suggested above, could enable the 530 and 1400 regions to come into close proximity to one another. It should, however, be noted that a structural alteration of this nature would involve a radical re-organization of the three-dimensional models of the 16S RNA. Clearly, much work remains to be done before the question of the precise path followed by the mRNA can be finally answered, and the further application of the site-directed cross-linking method remains a promising approach to the problem. Up to now the principal disadvantage of the method has been that we have been obliged to work with mRNA analogues containing only a single substituted uridine residue, which imposes severe restrictions on the sequences that can be used. Tate *et al.* (1990) showed, and we have confirmed here, that the use of unsubstituted thio-U as a photo-reactive group leads to cross-linking yields that are in general higher than those obtained with the more bulky aryl azide group (APAB). This in turn raises the possibility that the versatility of the method could be improved by using more 'natural' mRNA analogues, in which several uridine or thio-U residues could simultaneously be present. We have now been able to demonstrate (O.Dontsova, A.Kopylov and R.Brimacombe, manuscript submitted) that this is indeed possible, and that the products of cross-linking to several individual thio-U residues within the mRNA sequence can be distinguished from one another.

Materials and methods

Preparation of ^{32}P-labelled mRNA analogues

The synthesis of DNA templates, preparation of 4-thio-UTP, transcription with T7 polymerase in the presence of [^{32}P]ATP, and purification of the RNA transcripts, were all carried out exactly as described by Stade *et al.* (1989). The nucleotide sequences of the isolated RNA products were confirmed by 3′-end labelling of an aliquot with [^{32}P]pCp (England and Uhlenbeck, 1978), followed by enzymatic sequence analysis according to the method of Donis-Keller *et al.* (1977). Substitution of the thio-U residue in the mRNA by APAB was performed under the conditions of Stade *et al.* (1989).

Preparation and cross-linking of mRNA – 70S ribosome complexes

70S ribosomes from *E.coli* MRE 600 were incubated together with ^{32}P-labelled mRNA and tRNA-Ala or tRNA-Thr under the conditions of Stade *et al.* (1989), with the exceptions that the ratio of 70S:mRNA:tRNA was ~1:2:5, and mRNA was used containing either unsubstituted thio-U or APAB-substituted thio-U. The normal amount of starting material was 720 pmol of 70S ribosomes per sample. Control samples without tRNA were run in parallel. The 70S–mRNA–tRNA complexes were isolated as before (Stade *et al.*, 1989) on sucrose gradients, and then subjected to photo-activation. In the case of mRNA containing APAB-substituted thio-U, the irradiation (at wavelengths >280 nm) was carried out as described by Stade *et al.* (1989), whereas with unsubstituted thio-U the irradiation conditions of Tate *et al.* (1990) were used (with wavelengths >300 nm). The cross-linked products were separated by two further sucrose gradient centrifugation steps, in 0.3 mM magnesium and in SDS, respectively (see Results and Stade *et al.*, 1989).

Analysis of cross-linked ribosomal proteins

Pooled fractions from the top of the SDS–sucrose gradients (above) containing ribosomal proteins cross-linked to ^{32}P-labelled mRNA were concentrated by precipitation with ethanol (5 vol) and then applied to 10% polyacrylamide gels in SDS and urea (Brimacombe *et al.*, 1988b). The gel bands were visualized by autoradiography, and the cross-linked complexes extracted from the gels. The proteins in the extracted fractions were identified by the agarose antibody affinity chromatography method of Gulle *et al.* (1988), using antibodies to the individual 30S ribosomal proteins (a generous gift from Dr G.Stöffler). Alternatively, aliquots (~2000 c.p.m.) of the SDS–sucrose gradient fractions were submitted directly to this identification procedure, omitting the gel separation step (see Stade *et al.*, 1989).

Analysis of cross-link sites in the 16S RNA

SDS–sucrose gradient fractions (above) containing [^{32}P]mRNA cross-linked to 16S RNA were concentrated by precipitation with ethanol (2 vol), and aliquots (~5000 c.p.m.) were digested with RNase H in the presence of selected pairs of decadeoxynucleotides according to the procedure of Brimacombe *et al.* (1990), with the minor modifications of Mitchell *et al.* (1990). The digestion products were separated on 5% polyacrylamide gels (Stade *et al.*, 1989) and visualized by autoradiography. After a preliminary localization of the cross-link site(s) on 16S RNA by this procedure (see Results), the remainder of the cross-linked mRNA–16S RNA fraction was separated from non-cross-linked 16S RNA by affinity chromatography on oligo(dT)–cellulose as described by Stiege *et al.* (1988). Traces of oligo(dT) (which interfere with the subsequent reverse transcriptase analysis) were removed by adsorption to oligo(dA)–cellulose, and aliquots of the samples were then submitted to reverse transcriptase analysis in the presence of a suitable oligodeoxynucleotide primer (17 bases long) using the method of Moazed *et al.* (1986a), as modified by Tate *et al.* (1990). Aliquots of non-cross-linked 16S RNA from the oligo(dT)–cellulose fractionation were used as control samples.

References

Abdurashidova,G.G., Tsvetkova,E.A. and Budowsky,E.I. (1989) *FEBS Lett.*, **243**, 299–302.

Brimacombe,R., Atmadja,J., Stiege,W. and Schüler,D. (1988a) *J. Mol. Biol.*, **199**, 115–136.

Brimacombe,R., Stiege,W., Kyriatsoulis,A. and Maly,P. (1988b) *Methods Enzymol.*, **164**, 287–309.
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.* Oxford University Press, Oxford, pp. 131–159.
Capel,M.S., Kjeldgaard,M., Engelman,D.M. and Moore,P.B. (1988) *J. Mol. Biol.*, **200**, 65–87.
Donis-Keller,H., Maxam,A.M. and Gilbert,W. (1977) *Nucleic Acids Res.*, **4**, 2527–2538.
England,T.E. and Uhlenbeck,O.C. (1978) *Nature*, **275**, 560–561.
Evstafieva,A., Shatsky,I.N., Bogdanov,A.A., Semenkov,Y. and Vasiliev,V.D. (1983) *EMBO J.*, **2**, 799–804.
Gornicki,P., Nurse,K., Hellmann,W., Boublik,M. and Ofengand,J. (1984) *J. Biol. Chem.*, **259**, 10493–10498.
Gulle,H., Hoppe,E., Osswald,M., Greuer,B., Brimacombe,R. and Stöffler,G. (1988) *Nucleic Acids Res.*, **16**, 815–832.
Hajnsdorf,E., Favre,A. and Expert-Bezançon,A. (1989) *Nucleic Acids Res.*, **17**, 1475–1491.
Hartz,D., McPheeters,D.S. and Gold,L. (1990) In Hill,W.E., Dahlberg,A., Garrett,R.A., Moore,P.B., Schlessinger,D. and Warner,J.R. (eds), *The Ribosome; Structure, Function and Evolution.* ASM Press, Washington, DC, pp. 275–280.
Haselman,T., Camp,D.G. and Fox,G.E. (1989) *Nucleic Acids Res.*, **17**, 2215–2221.
Herr,W., Chapman,N.M. and Noller,H.F. (1979) *J. Mol. Biol.*, **130**, 433–449.
Hixson,S.H. and Hixson,S.S. (1975) *Biochemistry*, **14**, 4251–4254.
Kang,C. and Cantor,C.R. (1985) *J. Mol. Biol.*, **181**, 241–251.
Lowary,P., Sampson,J., Milligan,J., Groebe,D. and Uhlenbeck,O.C. (1986) In Van Knippenberg,P.H. and Hilbers,C.W. (eds), *Structure and Dynamics of RNA.* NATO ASI Series Vol. 110, Plenum Press, New York, pp. 69–76.
Lührmann,R., Stöffler-Meilicke,M. and Stöffler,G. (1981) *Mol. Gen. Genet.*, **182**, 369–376.
Melançon,P., Lemieux,C. and Brakier-Gingras,L. (1988) *Nucleic Acids Res.*, **16**, 9631–9639.
Mitchell,P., Osswald,M., Schüler,D. and Brimacombe,R. (1990) *Nucleic Acids Res.*, **18**, 4325–4333.
Moazed,D. and Noller,H.F. (1986) *Cell*, **47**, 985–994.
Moazed,D. and Noller,H.F. (1987) *Nature*, **327**, 389–394.
Moazed,D., Stern,S. and Noller,H.F. (1986a) *J. Mol. Biol.*, **187**, 399–416.
Moazed,D., Van Stolk,B.J., Douthwaite,S. and Noller,H.F. (1986b) *J. Mol. Biol.*, **191**, 483–493.
Montesano,L. and Glitz,D.G. (1988) *J. Biol. Chem.*, **263**, 4939–4944.
Oakes,M.I. and Lake,J.A. (1990) *J. Mol. Biol.*, **211**, 897–906.
Ofengand,J., Ciesiolka,J., Denman,R. and Nurse,K. (1986) In Hardesty,B. and Kramer,G. (eds), *Structure, Function and Genetics of Ribosomes.* Springer-Verlag, New York, pp. 473–494.
Olson,H.M., Lasater,L.S., Cann,P.A. and Glitz,D.G. (1988) *J. Biol. Chem.*, **263**, 15196–15204.
Osswald,M., Greuer,B. and Brimacombe,R. (1990) *Nucleic Acids Res.*, **18**, 6755–6760.
Powers,T. and Noller,H.F. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1042–1046.
Prince,J.B., Taylor,B.H., Thurlow,D.L., Ofengand,J. and Zimmermann, R.A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5450–5454.
Schreiner,G. and Nierhaus,K.H. (1973) *J. Mol. Biol.*, **81**, 71–82.
Shen,Z. and Fox,T.D. (1989) *Nucleic Acids Res.*, **17**, 4535–4539.
Stade,K., Rinke-Appel,J. and Brimacombe,R. (1989) *Nucleic Acids Res.*, **17**, 9889–9908.
Stern,S., Weiser,B. and Noller,H.F. (1988) *J. Mol. Biol.*, **204**, 447–481.
Stiege,W., Stade,K., Schüler,D. and Brimacombe,R. (1988) *Nucleic Acids Res.*, **16**, 2369–2388.
Tate,W., Greuer,B. and Brimacombe,R. (1990) *Nucleic Acids Res.*, **18**, 6537–6544.
Towbin,H. and Elson,D. (1978) *Nucleic Acids Res.*, **5**, 3389–3407.
Traut,R.R., Lambert,J.M., Boileau,G. and Kenny,J.W. (1980) In Chambliss,G., Craven,G.R., Davies,J., Davis,K., Kahan,L. and Nomura,M. (eds), *Ribosomes; Structure, Function and Genetics.* Univerestiy Park Press, Baltimore, MD., pp. 89–110.
Trempe,M.R., Oghi,K. and Glitz,D.G. (1982) *J. Biol. Chem.*, **257**, 9822–9829.
Trifonov,E.N. (1987) *J. Mol. Biol.*, **194**, 643–652.
Vladimirov,S.N., Babkina,G.T., Venijaminova,A.G., Gimautdinova,O.I., Zenkova,M.A. and Karpova,G.G. (1990) *Biochim. Biophys. Acta*, **1048**, 245–256.
Walleczek,J., Albrecht-Ehrlich,R., Stöffler,G. and Stöffler-Meilicke,M. (1990) *J. Biol. Chem.*, **265**, 11338–11344.

Received on February 21, 1991