The EMBO Journal Vol.2 No.5 pp.799–804, 1983

Localization of 5′ and 3′ ends of the ribosome-bound segment of template polynucleotides by immune electron microscopy

Alexandra G.Evstafieva, Ivan N.Shatsky*, Alexei A.Bogdanov, Yuri P.Semenkov[1] and Victor D.Vasiliev[2]

A.N.Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, 117234, Moscow, [1]B.P. Konstantinov Nuclear Physics Institute, USSR Academy of Sciences, 188350 Gatchina, Leningrad Region, and [2]Institute of Protein Research, USSR Academy of Sciences, 142292 Poustchino, Moscow Region, USSR

Communicated by R.Garrett
Received on 4 March 1983

Poly(U) with an average chain length of 40–70 nucleotides was modified at the 5′- or 3′-terminal residues with 2,4-dinitrophenyl derivatives. The modified poly(U) was used to form 30S·poly(U) or 70S·poly(U)·Phe-tRNA complexes. Localization of the 5′ and 3′ ends of the template polynucleotide on the 30S subunit and the 70S ribosome was performed by immune electron microscopy using antibodies against dinitrophenyl haptens. The 5′ and 3′ ends of poly(U) (putative entry and exit sites of the message) were found in the same region both on the 30S subunit and the 70S ribosome. They were located on the dorsal side of the 30S subunit between the head and the body near the groove bordering the side ledge (platform). Comparison of the size of this region with the possible length of the polynucleotide chain covered by the ribosome allowed us to suggest that the message makes a 'U-turn' (or forms a 'loop') as it passes through the ribosome.

Key words: dinitrophenyl hapten/immune electron microscopy/modification of terminal nucleotides/poly(U)/ribosome

Introduction

Determination of the three-dimensional location of components of the translational apparatus is a necessary step to elucidate the molecular mechanisms of protein synthesis. In particular, it is important to know where the message traverses the ribosome with respect to the other ribosomal components. It is known that a mRNA fragment with a length of at least 30 nucleotide residues is protected from ribonuclease action by the ribosome (Takanami and Zubay, 1964; Steitz, 1980). To get a general idea of the size of the mRNA binding site on the ribosome we have located the sites of the 5′ and 3′ ends of short poly(U) segments (40–70 nucleotides) bound to the 70S ribosome or its 30S subunit. For this, we prepared poly(U) modified at the 5′- or 3′-terminal nucleotides by 2,4-dinitrophenyl (DNP)-haptens. The modified templates were subsequently used to obtain 30S·poly(U) or 70S·poly(U)·Phe-tRNA^{Phe} complexes. Localization of the 5′ and 3′ ends of the template polynucleotide was carried out by immune electron microscopy using antibodies against DNP-derivatives (anti-DNP).

Results

Incubation of the 30S·poly(U) binary complexes (BC), where poly(U) was modified by DNP-haptens at the 5′ or 3′ ends with anti-DNP, leads to the appearance of a 'dimer fraction' in the sucrose gradient profile (Figure 1). The specificity of dimer formation has been proved as described earlier (Shatsky *et al.*, 1979; Mochalova *et al.*, 1982b). Three fractions of poly(U) with an average length of 40 (30–50), 55 (45–65) and 70 (55–85) nucleotide residues were tested. The extent of modification is similar (85–100%) both for the 5′-modified and the 3′-modified poly(U). The yield of the dimer fraction for the 5′ end does not depend on the length of poly(U) in the range 30–70 nucleotide residues. On the contrary, in the case of the 3′ end, a marked dependence on the poly(U) length is apparent. At the same 'complex'/anti-DNP ratio a considerable formation of dimers is observed only for poly(U) with an average length of 70 nucleotides. Poly(U) 40-55 nucleotides long with modified 3′ ends results only in some broadening of the 30S peak towards higher sedimentation coefficients. Analysis of the pooled sucrose gradient fractions containing 30S subunits for [^{14}C]Phe-tRNA^{Phe} binding [it has to be noted that 30S subunits do not bind Phe-tRNA^{Phe} in the absence of poly(U)] shows that, with or without antibodies in the incubation mixture, the molar proportion of bound poly(U) is similar (40%) and does

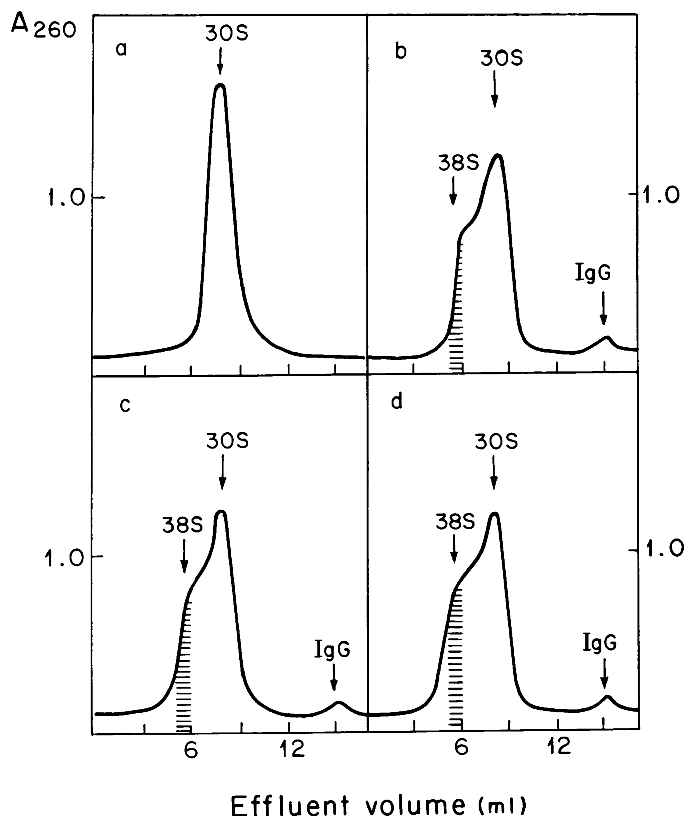

Fig. 1. Sedimentation of 30S·DNP-modified poly(U) complexes (12 A_{260} units) preincubated with anti-DNP in a 5–20% sucrose gradient. **(a)** Complexes in the absence of antibodies. **(b)** Complexes with the 5′-modified poly(U) [poly(U) length ~40 nucleotide residues] + 600 μg anti-DNP. **(c)** As in **(b)**, but the poly(U) length 70 nucleotides. **(d)** As in **(c)**, but with the 3′-modified template.

*To whom reprint requests should be sent.

© IRL Press Limited, Oxford, England.

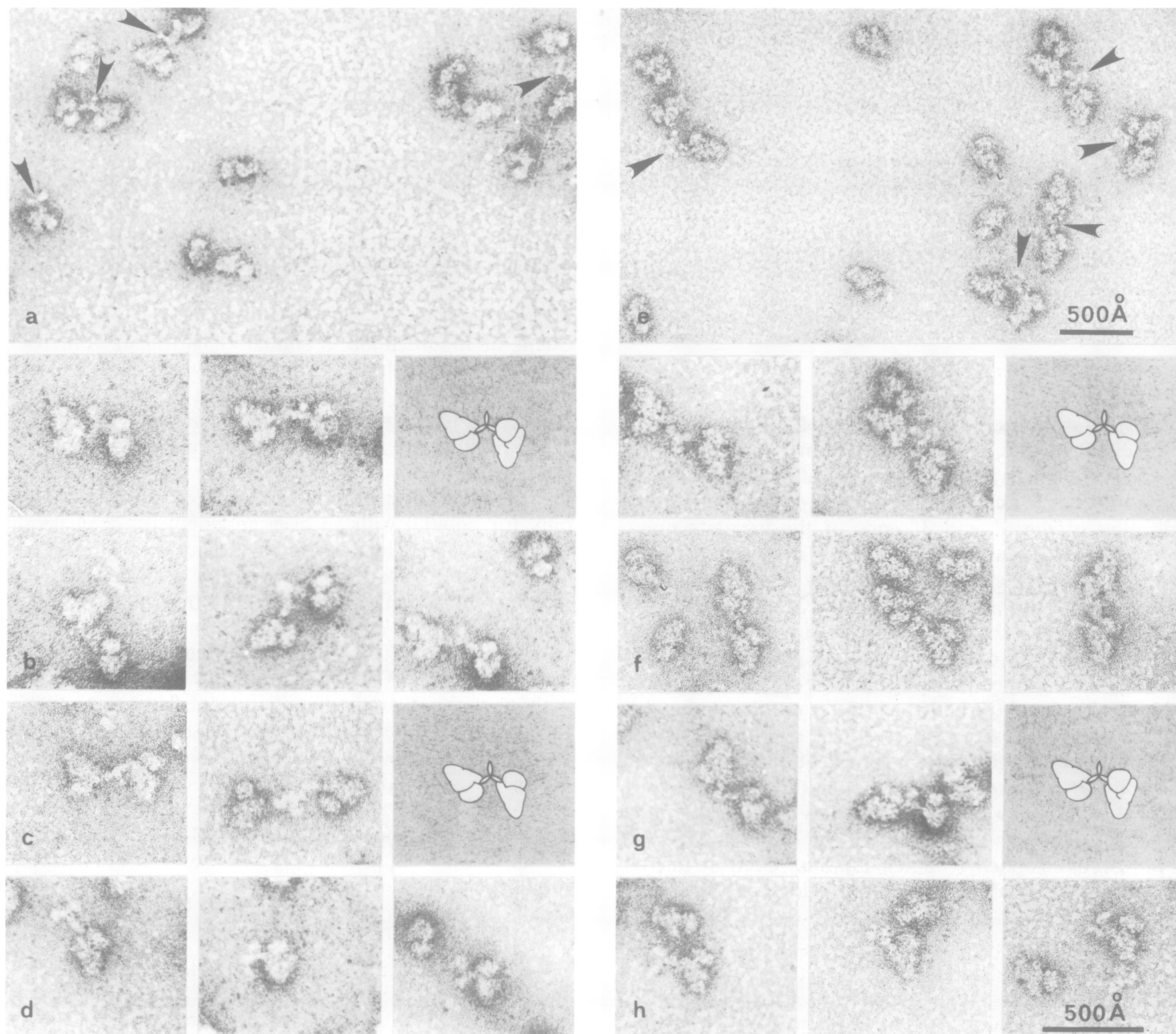

Fig. 2. Electron micrographs of 30S·DNP-modified poly(U) complexes treated with anti-DNP. General views and selected pairs of 30S subunits linked by antibody molecules. Arrows indicate antibodies in 30S·IgG·30S dimers and single 30S·IgG complexes. Interpretative drawings are given in the right-hand frames. **(a–d)** Immunocomplexes with poly(U) modified at the 5′ end. **(b)** The vertically oriented subunit of each pair is approximately in the frontal projection (Vasiliev, 1974). **(c)** The vertically oriented subunit of each pair is approximately in the intermediate or the lateral projection. **(d)** Single 30S·IgG complexes. **(e–h)** Immunocomplexes with poly(U) modified at the 3′ end. The intermediate projection of the subunit occurs more often than in the case of poly(U) modified at the 5′ end, but the antibody binding site in both the intermediate and frontal projections is the same as in **(a–d)**.

not depend on the site of modification or the length of poly(U) (data not shown). Therefore, we have concluded that the different accessibility of the poly(U) 5′ and 3′ ends for IgG binding does reflect their different locations on the 30S subunit.

Fractions from the shaded regions in Figure 1b,c and d were used for electron microscopy. General views of the preparations shown in Figure 1b and d are given in Figure 2a and e; selected pairs of 30S subunits linked by IgG molecules and single 30S·IgG complexes are presented in Figure 2b–d and f–h, respectively. The frontal and intermediate projections of the 30S subunit (Vasiliev, 1974) were analyzed to map the three-dimensional location of the antibody attachment site. The former orientation occurs more often in the case of the 5′-modified poly(U), whereas the latter is more frequent in 30S·IgG·30S pairs with the 3′-modified template. Nonetheless, in each of the projections of the 30S subunit, the ends of poly(U) are located in the same region. In the intermediate projections [45° and 225° (Vasiliev, 1974); see Figure 3] the antibody attachment site coincides with that which has been found for the 3′ end of 16S RNA (Shatsky *et al.*, 1979; Olson and Glitz, 1979). However, in the frontal projections, the IgG molecule is attached, as a rule, to the left side of the particle betwen the head and the body of the 30S subunit, rather than to the right side as in the case of the 16S 3′ end (see Shatsky *et al.*, 1979). Other antibody attachment points occur very rarely (6% of the total). It should be pointed out that the position of the antibody attachment site does not seem to depend on a poly(U) length of up to 70 nucleotide residues. Analysis of all the data allows one to map both ends of the template polynucleotide in the same site of the 30S subunit surface. It is located on the dorsal side of the 30S subunit between the head

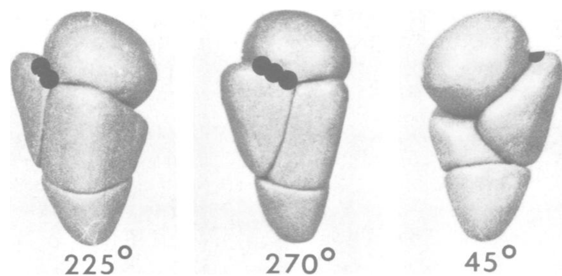

Fig. 3. Localization of the 5′ and 3′ ends of the poly(U) fragment bound to the 30S ribosomal subunit. The location site is denoted by solid circles on the two views of the 30S subunit model (Vasiliev, 1974).

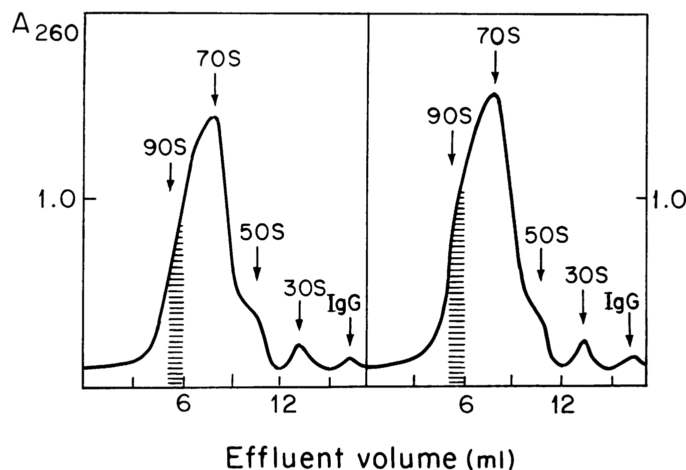

Fig. 4. Sedimentation of 70S·DNP-modified poly(U)·Phe-tRNA^{Phe} tertiary complexes after reaction with anti-DNP in a 10–30% sucrose gradient. **(a)** Tertiary complexes (13 A_{260} units) with the 5′-modified poly(U) + 215 μg anti-DNP [poly(U) length ~60–70 nucleotides]. **(b)** As in **(a)**, but for the 3′-modified template.

and the body near the groove bordering the side ledge (Figure 3).

The 30S poly(U) complex is the simplest model for studying mRNA-ribosome interaction. Therefore, we have mapped the position of the poly(U) ends in the tertiary complex 70S·poly(U)·Phe-tRNA^{Phe} (TC) which represents a system more similar to the whole translational complex. As in the case of the 30S·poly(U) complex, the yield of dimers depended on the length of the poly(U) when the template was modified at the 3′ end. Poly(U) with a length of 70 nucleotide residues has been used for mapping both ends.

The Phe-tRNA^{Phe}/70S ratio in the purified tertiary complex was found to be 1:1. In the absence of poly(U) the Phe-tRNA^{Phe} entirely dissociates from the ribosome in the course of centrifugation (data not shown). These results suggest that most of the poly(U) molecules in the tertiary complexes are involved in codon-anticodon interactions with Phe-tRNA^{Phe} and hence occupy a correct position (or close to it) on the 70S ribosome.

Dimer formation for the tertiary complex with poly(U) modified at the 5′- or 3′-terminal nucleotides is presented in Figure 4a and b. Fractions from the shaded regions were used for electron microscopy analysis. General views, selected TC·IgG·TC dimers and single TC·IgG complexes from the preparations shown in Figure 4a and b are given in Figure 5a–d and e–h, respectively.

Two characteristic projections of the 70S ribosome are most frequently observed: an overlap projection and a non-overlap one, according to whether or not the image of the small subunit overlaps that of the large subunit (Lake, 1976, 1982). When the 70S ribosome is in the overlap projection, the 50S subunit has an asymmetric 'crown-like' view, whereas the small subunit is in its intermediate projection (see Figure 3). The two subunits are oriented in such a manner that the side ledge (platform) of the 30S subunits contacts the short side protuberance of the large subunit, the head of the 30S subunit covering a region between the short side and central protuberances of the 50S subunit. When the 70S ribosome is in the non-overlap projection the large subunit has a kidney- or a 6-like view and the small subunit is approximately in a frontal projection (see Figure 3). Such an interpretation of electron microscope images of the 70S monosome (Lake, 1976, 1982; Vasiliev *et al.*, 1983) is the only possible one to account for the results obtained in this work (see interpretative drawings in the last frames in Figure 5c and d).

In the overlap view of the 70S ribosome the antibody attachment point is located near the 3′ end of 16S RNA both for the 5′- and 3′-modified poly(U). In the non-overlap view, in both cases, the Fab part of the antibody molecule points to the partition between the head and the body of the 30S subunit. Other attachment points rarely occur (7%). In total, we have examined 100 immunocomplexes. However, frequencies of the two 70S ribosome projections in immunocomplexes strongly depend on the site of poly(U) modification. The 5′-modified tertiary complexes mainly occur in the overlap projections, whereas the 3′-modified ones are observed in the non-overlap view. Nevertheless, in all other respects, the data on mapping of the poly(U) ends on the 70S ribosome and its 30S subunit are consistent. The location site of the 5′ and 3′ ends of poly(U) on the 70S ribosome model is indicated in Figure 6.

Discussion

The validity of data on the localization of mRNA on the ribosome depends on the specificity of the complexes under investigation. As has been shown by Katunin *et al.* (1980), the 30S subunit forms a complex with poly(U) with a stoichiometry of 1:1. The complex is heterogeneous in stability and characterized by two association constants (10^6 and 10^8-10^9 M^{-1}). Stronger binding is entirely accounted for by the presence of the ribosomal protein S1. In our conditions we observed only strong complexes, i.e., those dependent on protein S1. The specificity of poly(U) binding to 70S ribosomes is supported by the presence of codon-anticodon interactions in most of the tertiary 70S·poly(U)·Phe-tRNA^{Phe} complexes used for immune electron microscopy. Furthermore, the apparent difference in accessibility of the hapten at the 5′ and 3′ ends of poly(U) for IgG binding suggests that the binding of the message is polar. All these circumstances rule out a mere non-specific sticking of the template to the ribosomal surface.

The results obtained in this work raise the following questions: (1) do the location sites of poly(U) ends correspond to the entry and exit sites of the message? (2) can we judge the shape which the message acquires in the ribosome? The answer to the first question is related to the presence of free ends in the template polynucleotide flanking its ribosome-bound fragment. Poly(U) 40 nucleotides long can have only short free ends because its length only slightly exceeds that of

Fig. 5. Electron micrographs of 70S·DNP-modified poly(U)·Phe-tRNA^{Phe} tertiary complexes treated with DNP-specific antibodies. General views, selected TC·IgG·TAC dimers and single TC·IgG complexes. Arrows show antibody molecules. Interpretative drawings are given in the right-hand frames. In order to show the position of the small subunit the interpretative drawings are presented as the mirror images of the correct 70S ribosome model (see Lake, 1982). **(a–d)** Immunocomplexes with poly(U) modified at the 5′ end. **(b)** The 70S ribosomes in the dimers are in the overlap projection. This projection is predominant. **(c)** The vertically oriented 70S ribosome of each pair is in the non-overlap projection. **(d)** The single TC·IgG complexes. **(e–h)** Immunocomplexes with poly(U) modified at the 3′ end. In this case the dimers with 70S ribosomes in the non-overlap projection, such as presented in the two upper rows, are predominant, but the antibody binding site in both the projections is the same as in the former case.

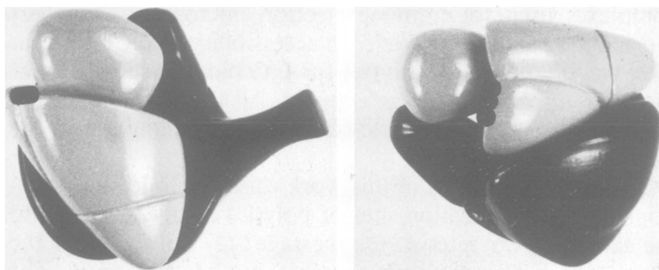

Fig. 6. Mapping of the entry and exit sites of poly(U) on the 70S ribosome model. The location site of both ends of the ribosome-bound fragment of the template is designated by solid circles.

the mRNA fragment protected by the ribosome from ribonucleases. It is clear that the presence of short free ends cannot affect significantly the precision of localization of the entry and exit sites of the message. Moreover, we have found the position of the antibody attachment point to be independent of the poly(U) length within defined limits. This can be accounted for by the following reasons. (1) For electron microscopy we selected those fractions of the 'dimer' part of the sucrose gradient that have higher sedimentation coefficients. We believe that the poly(U)·ribosome complexes from these fractions carry shorter free ends and therefore are bound into pairs more tightly and sediment more rapidly. (2) When present, the long free ends do not seem inclined to stick to the ribosomal surface. Rather, they tend to stretch between

the ribosomal particle and the Fab part of IgG. The most characteristic example is presented in the first two frames in Figure 5b. It should be mentioned, however, that such pairs occur rather rarely, probably for the reason indicated above. The low frequency of 'aberrant' antibody attachment sites (<7%) favours these two suggestions. Finally, it should be pointed out that the poly(U) segment interacting with the ribosome may be longer than the region protected from ribonucleases. Indeed, the hapten residue at the 3′ end of poly(U) with an average length of up to 55 nucleotides is not readily available for IgG binding. This is quite conceivable, if one takes into account the difference in sizes and properties between commonly used RNases and Fab parts of IgG. The fact that there is no dependence on the poly(U) length for accessibility of the 5′ end of the message seems to reflect the different organization of the respective binding sites. The latter appears to be located near the surface of the 30S subunit. Closer to the 3′ end, the polynucleotide chain may occupy a 'hollow' (such as the groove separating the head of the 30S subunit from its body). We have assumed that the 5′-terminal part of the whole binding site has a higher affinity for the polynucleotides than the 3′-terminal one. If so, the 5′ end would be always accessible for interaction with IgG [starting from a certain size of poly(U)] whereas the proportion of accessible 3′ ends would increase, as does the length of the message. All these considerations allow us to suggest that the 5′ and 3′ ends of short poly(U), as determined in this work, do reflect the real positions of its entry and exit sites.

The location of the entry and exit sites makes it possible to conceive how the message passes through the ribosome. Indeed, if the bound fragment of the template chain is supposed to be stretched in a 'straight line' then its ends would be separated by at least 90 Å, even assuming the chain length to be ~30 nucleotide residues (Takanami and Zubay, 1964; Steitz, 1980) and the displacement per base to be the shortest one [3 Å for duplex poly(U)·poly(A) (Sasisekharan and Sigler, 1965)]. However, we see that the entry and exit sites of poly(U) are close together in all electron microscopy images. Thus, the entire binding site of poly(U) on the ribosome is organized in such a way that the nucleotide sequences proximal to its entry and exit points are not separated by a great distance. Their proximity allows one to suggest that the ribosome-bound fragment of the template polynucleotide makes a 'U-turn' or a 'loop' as it passes through the ribosome. Of course, the poly(U)·ribosome·Phe-tRNA^{Phe} complex is a model of the ribosome at the elongation step rather than the initiation step. Moreover, the macromolecular structures of natural mRNA and poly(U) are different. Nevertheless, we believe that the 'loop' may be formed from the very beginning of translation.

Though an idea of the template loop is rather speculative and should be supported by independent and more precise methods, as well as by the use of natural mRNA fragments, it is attractive for the following reason: in the course of initiation it is not required that the ends of the mRNA fragment interacting with the ribosome be separated greatly from each other. This is particularly essential for the internal coding regions of polycystronic mRNAs. In this case, separation of the ends would require not only melting of the secondary structure within the mRNA initiation site itself, but would involve strong conformational changes in other parts of the mRNA macromolecular structure.

Materials and methods

Preparation of poly(U) fragments and their modification by DNP-haptens

Poly(U) (Reanal) was digested with cobra venom ribonuclease into fragments 20–150 nucleotides in length (Boldyreva *et al.*, 1978). The fragments were then separated into fractions with a narrow chain length distribution on a Sephadex G-100 (Pharmacia) column. The chain length was estimated by gel electrophoresis under the conditions described in Maxam and Gilbert (1977), using 5S rRNA and its 41- and 79-nucleotide-long fragments as markers (provided by M.Saarma). Modification of poly(U) at the 5′-phosphate group by DNP-ethylene-diamine was carried out as described earlier (Mochalova *et al.*, 1982a, 1982b). Poly(U) modification at the 3′-terminal ribose by N-(γ-2,4-dinitrophenyl)aminobutyric acid hydrazide was carried out as reported in Stöffler-Meilicke *et al.* (1981). The extent of poly(U) modification was estimated from the ratio A_{260}/A_{367}, assuming the molar extinction coefficients of the DNP-group (367 nm) and U residue in poly(U) (260 nm) to be 16 000 and 8800, respectively (Mochalova *et al.* 1982a; Inners and Felsenfeld, 1970).

Preparation of 30S·poly(U) and 70S·poly(U)·[^{14}C]Phe-tRNA^{Phe}

Ribosomes and ribosomal subunits were isolated from *Escherichia coli* MRE 600 as described by Kirillov *et al.* (1980). 30S subunits were mixed in buffer I (100 mM NH_4Cl/10 mM $MgCl_2$/10 mM Tris·HCl, pH 7.2) with a 1.3-fold molar excess of modified poly(U) and the mixture was then incubated for 1 h at 0°C. 30S·poly(U) complex was separated from unbound poly(U) by short low speed centrifugation through Biogel P-100 (Bio-Rad) equilibrated with the same buffer. To prepare 70S tertiary complexes, the 30S subunits were mixed with a 2-fold molar excess of modified poly(U), an equimolar amount of 50S subunits and a 3-fold molar excess of [^{14}C]Phe-tRNA^{Phe} (1440 pmol/A_{260}, 350 Ci/mol , [^{14}C]Phe-tRNA^{Phe} was prepared as described in Semenkov *et al.* (1976). The mixture was incubated for 1 h at 0°C. The tertiary complex was purified by sucrose gradient centrifugation (10–30%) in buffer II (200 mM NH_4Cl/12 mM $MgCl_2$/10 mM Tris HCl, pH 7.2) using a Beckman SW41 rotor (21 000 r.p.m., 12 h, 2°C), dialyzed for 4 h against the same buffer and concentrated by 20% polyethylene glycol (mol. wt. 20 000–40 000, Fluka).

Dimer formation and electron microscopy

Preparation of anti-DNP and selection of optimal antibody: ribosome ratio were performed as described in Mochalova *et al.* (1982b) and Shatsky *et al.* (1979), respectively. Incubation of 30S binary complexes (BC) or 70S tertiary complexes (TC) with anti-DNP was carried out for 1 h at 0°C. Isolation of BC·IgG·BC dimers and their preparations for electron microscopy were performed according to Shatsky *et al.* (1979) using buffer I. Tc·IgG·TC dimers were isolated in buffer II under conditions indicated above for TC purification. Electron microscopy was performed as described in Shatsky *et al.* (1979).

Acknowledgements

We would like to express our gratitude to Professor A.S.Spirin for valuable discussions. We also thank Drs.O.M.Selivanova and S.N.Ryazantsev for help in electron microscopy experiments and Drs.I.V.Boni, R.Cedergren and R.Zimmermann for critical reading of the manuscript.

References

Boldyreva,L.G., Boutorin,A.A. and Vassilenko,S.K. (1978) *Biokhimiya*, **43**, 1659-1664.

Inners,L.D. and Felsenfeld,G. (1970) *J. Mol. Biol.*, **50**, 373-389.

Katunin,V.I., Semenkov,Yu.P., Makhno,V.I. and Kirillov,S.V. (1980) *Nucleic Acids Res.*, **8**, 403-421.

Kirillov,S., Makhno,V. and Semenkov,Y. (1980) *Nucleic Acids Res.*, **8**, 183-196.

Lake,J.A. (1976) *J. Mol. Biol.*, **105**, 131-159.

Lake,J.A. (1982) *J. Mol. Biol.*, **161**, 89-106.

Maxam,A.M. and Gilbert,W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 560-564.

Mochalova,L.V., Shatsky,I.M. and Bogdanov,A.A. (1982a) *Bioorg. Khim.*, **8**, 239-242.

Mochalova,L.V., Shatsky,I.N., Bogdanov,A.A. and Vasiliev,V.D. (1982b) *J. Mol. Biol.*, **159**, 637-650.

Olson,H. and Glitz,D. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3769-3773.

Sasisekharan,V. and Sigler,P.B. (1965) *J. Mol. Biol.*, **12**, 296-298.

Semenekov,Yu.P., Makhno,V. and Kirillov,S. (1976) *Mol. Biol.*, **10**, 754-763.

Shatsky,I.N., Mochalova,L.V., Kojouharova,M.S., Bogdanov,A.A. and Vasiliev,V.D. (1979) *J. Mol. Biol.*, **133**, 501-515.

Steitz,J.A. (1980) in Chambliss,G., Craven,G.R., Davies,J., Davis,K., Kahan,L. and Nomura,M. (eds.), *Ribosomes. Structure, Function and Genetics,* University Park Press, Baltimore, MD, pp. 479-495.
Stöffler-Meilicke,M., Stöffler,G., Odom,O.W., Zinn,A., Kramer,G. and Hardesty,B. (1981) *Proc. Natl. Acad. Sci. USA,* **78**, 5538-5542.
Takanami,M. and Zubay,G. (1964) *Proc. Natl. Acad. Sci. USA,* **51**, 834-839.
Vasiliev,V.D. (1974) *Acta Biol. Med. Germ.,* **33**, 779-793.
Vasiliev,V.D., Selivanova,O.M., Baranov,V.N. and Spirin,A.S. (1983) *FEBS Lett.*, in press.