PROTEINS AT THE mRNA BINDING SITE OF THE *ESCHERICHIA COLI* RIBOSOME

I. FISER, K. H. SCHEIT*, G. STÖFFLER** and E. KUECHLER

*Institut für Biochemie der Universität Wien, Währingerst. 17, A-1090 Wien, Austria; *Max-Planck-Institut für Biophysikalische Chemie, Göttingen, GFR; and **Max-Planck-Institut für Molekulare Genetik, Berlin, GFR*

Received 4 June 1975

1. Introduction

Factors involved in the binding of messenger RNA to ribosomes during initiation of protein synthesis have been studied extensively (for a review see [1]), but less is known about the role of the ribosome itself in these processes.

Several lines of evidence indicate that protein S1 of the 30S ribosomal subunit is involved in the binding of poly U as well as natural messenger RNA [2–9]. Recently we have described experiments which prove that protein S1 is in close contact with the messenger RNA on the ribosome [10]. For these experiments the polynucleotide poly(s^4U)[+], which can be photoactivated, was employed as messenger RNA. This polynucleotide was obtained by enzymatic polymerization of 4-thiouridine-diphosphate with *E. coli* polynucleotide phosphorylase [11]. The polymer not only resembles poly U in its physicochemical properties [11] but it can also serve as messenger RNA in an in vitro protein synthesizing system. At 20 mM Mg^{2+} concentration poly(s^4U) stimulates binding of Phe-tRNA to *E. coli* ribosomes and codes for the synthesis of poly Phe ([12], and in preparation).

The 4-thiouracil ring can be photoactivated at 330 nm to react with proteins [13]. The activity of the ribosome itself is not affected by irradiation at this wavelength [10]. [^{3}H] poly(s^4U) was bound to ribosomes in the presence of Phe-tRNA and the resulting complex was irradiated. 30S subunits were isolated, digested extensively with ribonucleases A and T1 and the labelled proteins identified by electrophoresis on polyacrylamide gels. Radioactivity was recovered in a complex which migrated more slowly en polacrylamide gels than the largest ribosomal protein. The complex could be cleaved by mild alkali treatment and protein S1 was identified as the major component [10]. In this paper we describe the identification of two more ribosomal proteins as components of this complex. The results indicate that proteins S18 and S21 are located at the messenger RNA binding site near S1.

2. Materials and methods

Ribosomes were isolated as tight couples from cold treated *E. coli* D10 according to Noll [14]. [^{3}H] Poly (s^4U) (spec. act. 5.9×10^7 cpm/mg) was synthesized by the method of Simuth et al. [11]. Antibodies against single ribosomal proteins were raised in rabbits and purified as described [15,16].

Binding of [^{3}H] poly(s^4U) to ribosomes in the presence of unlabeled Phe-tRNA was carried out in 60 mM NH_4Cl, 100 mM Tris–HCl (pH 7.2), 20 mM magnesium acetate and 4 mM dithioerythritol as described previously [10]. Samples were incubated for 20 min at 25°C. Irradiation at 300–400 nm was for 20 min at 0°C with a Philips high pressure mercury lamp SP500W using filter UG1(Schott, Mainz, GFR). 30S ribosomal subunits were isolated and digested for 2 hrs at 37°C in 3M urea, 0.1M EDTA (pH 7.4) and 10 mM dithioerythritol with 10 μg of ribonuclease

[+] *Abbreviations:* Poly(s^4U), poly(4-thiouridylic acid). *Enzymes.* Polynucleotide phosphorylase (EC 2.7.7.8); ribonuclease A (EC 3.1.4.22); ribonuclease T1 (EC 3.1.4.8).

North-Holland Publishing Company – Amsterdam

A and 1 μg of ribonuclease T1 added per ml. Total 30S protein was obtained as described previously [10].

For the immunological identification 10 μg of 30S protein (spec. act. 2.52 X 10^5 cpm per mg of protein) containing the [^{3}H] poly(s^4U) labelled complex was mixed with 1.2 mg of specific IgG antibody in 0.2 ml of a solution containing 0.5 M LiCl, 0.5 M urea, 50 mM Tris–HCl (pH 8.2), and 1 mM dithioerythritol. The mixture was layered on a 5–40% sucrose gradient in the same buffer containing 0.01% bovine serum albumin and centrifuged at 2°C as indicated in the figure legend in a Beckman SW 40 rotor. Fractions were collected and the radioactivity determined [10].

3. Results

It has been demonstrated previously [10] that alkaline hydrolysis of the [^{3}H] poly(s^4U) labelled protein complex obtained after ribonuclease degradation of 30S subunits yields several radioactively labelled proteins. Among these only S1 could be identified unambiguously by electrophoresis on polyacrylamide gels in SDS because of its large molecular weight. Most of the radioactivity was recovered in protein S1. In addition at least two further small peaks of radioactivity were found which co-elelectrophorese with stained bands on the SDS-gel. However, since serveral proteins comigrate in these bands, no definite identification was possible. Unfortunately the alkali treatment somewhat blurs the protein pattern on two-dimensional gels run according to Kaltschmidt and Wittmann [17] and electrophoretic analysis alone was not sufficient to identify all labelled proteins.

In our previous studies on affinity labelling, the identification of labelled ribosomal proteins was carried out both by gel electrophoresis and by immunological techniques employing antibodies specific for single ribosomal proteins [10,16,18]. In all cases perfect agreement between the results was obtained. For the immunological identification total protein was isolated from labelled ribosomes and mixed with an excess of a specific antibody, which results in the formation of soluble antigen-antibody complexes. Since there are presumably several antigenic determinants accessible on each protein, several antibody molecules can bind to it. This results in a considerable

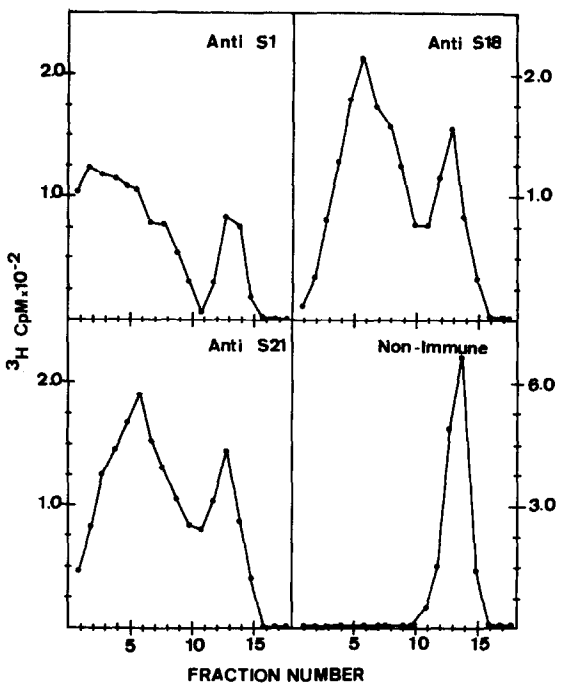

Fig.1. Immunological identification of the proteins contained in the [^{3}H] poly s^4 U labelled complex. Sucrose gradient centrifugation was carried out as described in Methods. Centrifugation time was 16 hr at 37 000 rev/min except for antibody against S1. *Top left:* Radioactivity profile obtained with IgG antibody specific for S1. The centrifugation time was 14 hr at 24 000 rev/min. 592 cpm were recovered from the bottom of the tube by resuspension in 1 ml of 5% SDS. *Top right:* IgG antibody specific for S18. *Bottom left:* IgG antibody specific for S21. *Bottem right:* Control with non-immune IgG.

increase in the sedimentation value. Consequently, upon centrifugation through sucrose gradients a large shift of the particular ribosomal protein into rapidly sedimenting fractions is observed.

The immunological approach has now been extended to identify the other proteins contained in the complex labelled with poly(s^4U). Since alkaline hydrolysis affects antigen-antibody recognition, the complex was analyzed after ribonuclease treatment and isolation of total ribosomal protein without previous exposure to alkali. We have investigated the effects of specific antibodies directed against all proteins of the 30S subunit except S17 and S20. Of all the antibodies studied, only antibodies against S18 and S21 resulted in strong shifts comparable to that obtained with antibody against S1 as seen in fig.1. The total amount of radioactivity

shifted is very similar in these three cases. As expected, non-immune IgG had no effect on the sedimentation of the radioactively labelled complex. Most other antibodies gave either no or only marginal shifts. Antibodies directed against proteins S7 and S14 resulted in partial shifts of radioactivity on the gradients; these results will be reported elsewhere (in preparation).

4. Discussion

Photoaffinity labelling of 70S ribosomes with the messenger RNA [^{3}H] poly(s^4U) results in the formation of a ribonuclease resistent complex of approx. 110 000 to 130 000 daltons containing a piece of [^{3}H] poly(s^4U). Since poly(s^4U) itself is rapidly degraded by ribonuclease A, the toal length of this piece should not exceed about 30 bases, which is the length of poly U maximally protected by the ribosome against ribonuclease digestion [19]. This size of polynucleotide corresponds to a mol. wt of about 9500. Since the molecular weight of S1 is 67 000 daltons [20], the complex is large enough to accommodate a few more ribosomal proteins of mol. wts of 10 to 20 000.

All attempts to further degrade this complex by high concentrations of ribonuclease even in the presence of various detergents, urea etc. failed. Obviously the proteins in the complex are very tightly packed thus preventing access of ribonuclease to the short piece of poly(s^4U). Degradation could only be accomplished by alkali treatment under conditions, which result in a partial hydrolysis of the polynucleotide chain. This suggests that the proteins are covalently linked via poly(s^4U). The extensive shifts obtained with antibodies to S1, S18 and S21 indicate furthermore that these three proteins occur in the complex in almost stoichiometric amounts. The immunological identification of S18 and S21 is supported by the finding on SDS-polyacrylamide gels of a discrete peak of radioactivity in the fastest moving band which contains both S18 and S21 [10]. This provides good evidence that proteins S1, S18 and S21 represent indeed part of the messenger RNA binding region of the ribosome.

The mol. wts of S18 and S21 are about 13 000 [21]. It is therefore likely that the complex contains additional proteins. Indeed we have observed that antibodies against proteins S7 and S14 alsc cause shifts on gradients (unpublished experiments), but the effects are smaller than those of antibodies against S1, S18 and S21. Nevertheless, this could indicate that S7 and S14 might occur as fractional components within the complex. This would also explain the electrophoretic heterogeneity of the complex previously observed on gels [10].

Our finding [10,12] that poly(s^4U) codes for the synthesis of poly Phe in an in vitro protein synthesizing system from *E. coli* is at variance with that of Hochberg and Keren-Zur [22] who observed an inhibitory effect of poly(s^4U) on a protein synthesizing system derived from rat liver. The poly(s^4U) which they used, was synthesized by reacting polycytidylic acid with H_2S under high pressure in aqueous pyridine for 12 days at 37°C. In contrast the poly(s^4U) used in our studies was obtained by enzymatic polymerization of 4-thiouridine-5′-diphosphate with polynucleotide phosphorylase under mild conditions [11]. Whether the discrepancies between the results are due to differences between the in vitro systems or due to differences in the methods of synthesis of poly(s^4U) remains to be established.

That protein S1 is near S21 has been suggested by the finding of a crosslink between the 3′ end of the 16S RNA and S1 [23] and by the fact that 30S subunits reconstituted with 16S RNA from colicin E3 treated ribosomes, which lacks the 3′ terminal end, do not contain S21 [24]. A deficiency in S1 of colicin E3 treated ribosomes has also been reported [9]. Furthermore, Lutter et al. [25] have demonstrated that S18 and S21 can be crosslinked by the bifunctional reagent phenylendimaleimide indicating that they are near neighbors on the 30S ribosomal subunit. Recently Pongs and Lanka [26] have identified S18 as one of the proteins labelled with a reactive AUG-derivative. All these experiments strongly support a model of the 30S ribosome, in which proteins S1, S18 and S21 contribute to the messenger RNA binding site of the *E. coli* ribosome.

Acknowledgement

We want to thank Dr H. Tuppy for discussions, Dr A. Stütz for his help in the synthesis of [^{3}H] poly

(s^4U). This work was supported by a grant from the Fonds zur Förderung der wissenschaftlichen Forschung.

References

[1] Haselkorn, R. and Rothmann-Denes, L. B. (1973) Ann. Rev. Biochem. 42, 397–438.
[2] Van Duin, J. and Kurland, C. G. (1970) Mol. Gen. Genet. 109, 169–176.
[3] Noller, H. F., Chang, C., Thomas, G. and Aldrige, J. (1971) J. Mol. Biol. 61, 669–679.
[4] Tal, M., Aviram, M., Kanarek, A. and Weiss, A. (1972) Biochim. Biophys. Acta 281, 381–392.
[5] Acharya, A. S. and Moore, P. B. (1973) J. Mol. Biol. 76, 207–221.
[6] Rummel, D. P. and Noller, H. F. (1973) Nature New Biol. 245, 72–75.
[7] Van Duin, J. and Van Knippenberg, P. M. (1974) J. Mol. Biol. 84, 185–196.
[8] Szer, W. and Leffler, S. (1974) Proc. Natl. Acad. Sci. USA 71, 3611–3615.
[9] Dahlberg, A. E. (1974) J. Biol. Chem. 249, 7673–7678.
[10] Fiser, I., Scheit, K. H., Stöffler, G. and Kuechler, E. (1974) Biochem. Biophys. Res. Comm. 60, 1112–1118.
[11] Simuth, J., Scheit, K. H. and Gottschalk, E. M. (1970) Biochim. Biophys. Acta 204, 371–380.
[12] Bähr, W., Faerber, P. and Scheit, K. H. (1973) Eur. J. Biochem. 33, 535–544.
[13] Frischauf, A. M. and Scheit, K. H. (1973) Biochem. Biophys. Res. Comm. 53, 1227–1233.
[14] Noll, M. (1972) Doctoral Thesis, Northwestern University, Evanston, Illinois.
[15] Stöffler, G. and Wittmann, H. G. (1971) Proc. Natl. Acad. Sci. USA 68, 2283–2287.
[16] Czernilofsky, A. P., Collatz, E. E., Stöffler, G. and Kuechler, E. (1974) Proc. Natl. Acad. Sci. USA 71, 230–234.
[17] Kaltschmidt, E. and Wittmann, H. G. (1970) Proc. Natl. Acad. Sci. USA 67, 1276–1282.
[18] Hauptmann, R., Czernilofsky, A. P., Voorma, H. O., Stöffler, G. and Kuechler, E. (1974) Biochem. Biophys. Res. Comm. 56, 331–337.
[19] Takanami, M. and Zubay, G. (1964) Proc. Natl. Acad. Sci. USA 51, 834–839.
[20] Inouye, H., Pollack, Y. and Petre, J. (1974) Eur. J. Biochem. 45, 109–117.
[21] Dzionara, M., Kaltschmidt, E. and Wittmann, H. G. (1970) Proc. Natl. Acad. Sci. USA 67, 1909–1913.
[22] Hochberg, A. A. and Keren-Zur, M. (1974) Nucleic Acids Res. 1, 1619–1630.
[23] Kenner, R. A. (1973) Biochem. Biophys. Res. Comm. 51, 932–938.
[24] Bowman, C. M., Dahlberg, J. E., Ikemura, T., Konisky, J. and Nomura, M. (1971) Proc. Natl. Acad. Sci. USA 68, 964–968.
[25] Lutter, L. C., Zeichhardt, H., Kurland, C. G. and Stöffler, G. (1972) Mol. Gen. Genet. 119, 357–366.
[26] Pongs, O. and Lanka, E. (1975) Proc. Natl. Acad. Sci. USA, in press.