Effects of antibody to 5 S-RNA-binding protein on protein synthesis in *Artemia salina* ribosomes

Naoya KENMOCHI,* Yoshiaki TAKAHASHI† and Norimitsu L. SATO*

*Facilities for Comparative Medicine & Animal Experimentation, and †Department of Biochemistry, Niigata University School of Medicine, Asahimachi-Dori 1, Niigata 951, Japan

The effects of an affinity-purified polyclonal antibody to *Artemia salina* ribosomal protein L5 on protein synthesis *in vitro* were examined. The antibody interacted with 60 S subunits more strongly than with 80 S ribosomes, and inhibited reassociation of ribosomal subunits to some extent at 5 mM-Mg²⁺ but not at 10 mM. Polyphenylalanine synthesis *in vitro* at 10 mM-Mg²⁺ was significantly inhibited, especially when the antibody was first preincubated with 60 S subunits prior to the assay. The incorporation of amino acid directed by globin mRNA was inhibited only when the preincubation with 60 S subunits was carried out. On the other hand, no effect was detected on elongation factor 2- and 60 S subunit-dependent uncoupled GTPase activity. These results suggest that L5 is probably located at or near the subunit interface and may play an important role in protein synthesis.

INTRODUCTION

The large ribosomal subunits of both prokaryotes and eukaryotes contain 5 S RNA, which is known to be a complex formed with one or more ribosomal proteins (for review, see ref. [1]). The structural homology of 5 S RNA is indicated in many species, suggesting the importance of this RNA. Although some hypotheses have been proposed [2–4], the role of 5 S RNA or 5 S-RNA-protein complexes in ribosomes still remains unknown.

Eukaryotic 5 S RNA forms a complex with a single ribosomal protein, and the complex is released from ribosomes by EDTA treatment [5–7] or other methods [8–10]. In rat liver, its protein moiety is identified as protein L5 [7]. One of us has shown that L5 crosslinked with poly(A)-containing mRNA in the polysome and to globin mRNA in the 80 S initiation complex following ultraviolet irradiation [11,12]. Furthermore, Uchiumi *et al.* [13] indicated that L5 crosslinked with small ribosomal subunit proteins S4 and S25 following treatment of 80 S ribosomes with 2-iminothiolane. These results suggest that rat liver L5 is probably located at the boundary between the large and small ribosomal subunits and interacts with mRNA.

Recently, we identified the 5 S-RNA-binding protein of *Artemia salina* ribosomes, and showed immunological similarity between the proteins of *Artemia* and rat liver [14]. We therefore designated the protein as *Artemia* L5 (AL5) when compared with rat liver L5. In the present work, we used the affinity-purified antibody to AL5 and examined its effects on ribosomal functions in protein synthesis *in vitro* to elucidate the role of the 5 S-RNA-protein complex in ribosomes.

MATERIALS AND METHODS

Materials

L-[U-¹⁴C]Phenylalanine (522 mCi/mmol), L-[4,5-³H]-leucine (131 Ci/mmol) and [γ -³²P]GTP (30 Ci/mmol)

were purchased from Amersham International. Affi-Gel 10 was obtained from Bio-Rad Laboratories. Poly(U) was from Sigma Chemical Co. and horseradish peroxidase-conjugated goat anti-rabbit IgG was from Kirkegaard & Perry Laboratories Inc. Pig liver elongation factor 2 (EF-2) was kindly provided by Dr. T. Uchiumi, Niigata University School of Medicine, Niigata, Japan.

Antibody purification

A polyclonal antibody to *Artemia* L5 was obtained [14] and purified by affinity chromatography according to Sommer *et al.* [15], as briefly described below. Total ribosomal 60 S proteins (35 mg) were coupled to Affi-Gel 10 in 0.1 M-Mops, pH 7.5. Immunoglobulins precipitated from antisera at 40% with (NH₄)₂SO₄ were applied on the gel and eluted with 0.2 M-glycine/HCl, pH 3.0, and 0.15 M-NaCl. Finally, 2.4 mg (5.3 pmol/ μ l) of anti-L5 antibody was obtained and its specificity was ascertained by immunoblotting [16].

Recognition of ribosomes and ribosomal subunits by anti-L5 antibody

Artemia salina ribosomes and their subunits were prepared as described previously [17]. Portions (20 pmol) of 80 S ribosomes or 60 S subunits were incubated for 10 min at 37 °C with 60 pmol of antibody in 0.1 ml of buffer containing 50 mM-Tris/HCl, pH 7.6, 125 mM-KCl and a given concentration of MgCl₂ (3–7 mM). After the mixture was cooled in an ice bath for 10 min, it was layered on top of 15–30% linear sucrose gradients in the same buffer as in the incubation, and was then centrifuged for 18 h at 14000 rev./min in a Hitachi RPS 50 rotor at 4 °C. The absorbance at 260 nm was measured and the antibody in each fraction which formed a complex with ribosomes or their subunits was analysed by standard enzyme-linked immunoadsorbent assay (e.l.i.s.a.) [18].

Association of ribosomal subunits

For ribosomal subunit reassociation measurements, 20 pmol of 60 S subunits were preincubated with

60 pmol or 120 pmol of antibody for 10 min at 37 °C in 0.1 ml of buffer containing 50 mM-Tris/HCl, pH 7.6, 125 mM-KCl and 5 mM- or 10 mM-MgCl₂. A 2-fold molar excess of 40 S subunits was added, and the mixture was incubated again for 10 min at 37 °C. Sucrose density gradient centrifugation was performed in the same manner as described above except that the centrifugal rate was 16000 rev./min, and the absorbance at 260 nm was measured.

Poly(U)-directed polyphenylalanine synthesis

Portions (20 pmol) of 80 S ribosomes or 60 S subunits were preincubated for 10 min at 37 °C with different amounts of antibody in 0.1 ml of buffer containing 50 mM-Tris/HCl, pH 7.6, 125 mM-KCl and 10 mM-MgCl₂. After preincubation, the remaining components of the assay and 60 pmol of 40 S subunits were added according to the method of Terao & Ogata [19] to give a final reaction volume of 0.3 ml in the same buffer as in the preincubation. The reaction was carried out for 30 min at 37 °C and then the radioactivity of 5% of hot trichloroacetic acid-insoluble materials was measured.

Translation of globin mRNA

Portions (10 pmol) of 80 S ribosomes or 60 S subunits were preincubated for 10 min at 37 °C with different amounts of antibody in 0.03 ml of buffer consisting of 33 mM-Hepes/KOH, pH 7.3, 83 mM-KCl, 3.3 mM-MgCl₂ and 1.7 mM-dithiothreitol. The remaining components of the assay were subsequently added [20] to give a final volume of 0.05 ml containing 20 mM-Hepes/KOH, pH 7.3, 50 mM-KCl, 2 mM-MgCl₂, 1 mM-ATP, 0.4 mM-GTP, 20 mM-creatine phosphate, 0.19 unit of creatine kinase, 19 amino acids (excluding leucine) (50 µM each), pH 5 enzymes (about 100 µg of protein and 5 µg of tRNA), initiation fractions I and II (each about 120 µg of protein), 0.05 A₂₆₀ unit of globin mRNA, 1 µCi of [³H]leucine and 40 pmol of 40 S subunits (when 60 S subunits were preincubated). The reaction was allowed to proceed for 1 h at 30 °C. The measurement of radioactivity incorporated was performed as described above.

EF-2/60 S subunit-dependent GTPase activity

Different amounts of antibody were mixed with 10 pmol of 60 S subunits in 40 µl of buffer containing 20 mM-Tris/HCl, pH 7.6, 25 mM-KCl, 5 mM-MgCl₂ and 6 mM-2-mercaptoethanol. After preincubation for 10 min at 37 °C, 10 pmol of EF-2, 3 nmol of GTP and 6.5 × 10⁴ c.p.m. of [³²P]GTP were added and the reaction was allowed to proceed for 30 min at 37 °C. The reaction was stopped by addition of 5% trichloroacetic acid, and the liberated [³²P]P_i was measured by an isobutanol/benzene method [21].

RESULTS

Reactivity of anti-L5 antibody with 60 S subunits and 80 S ribosomes

The antibody was tested for its capacity to interact with intact ribosomes and with ribosomal 60 S subunits. The results, analysed by sucrose gradient centrifugation, are shown in Fig. 1. The antibody reacts strongly with 60 S subunits but only weakly with intact ribosomes. In another experiment involving an e.l.i.s.a., the antibody bound not only to 60 S subunits but also to 80 S

Fig. 1. Recognition of 80 S ribosomes and 60 S subunits by anti-L5 antibody

Portions (20 pmol) of 80 S ribosomes (*b, c, d*) or 60 S subunits (*a*) were incubated for 10 min at 37 °C with 60 pmol of anti-L5 antibody. After the mixture was cooled on ice, it was centrifuged through 15–30% linear sucrose gradients for 18 h at 14000 rev./min in a Hitachi RPS 50 rotor at 4 °C. The incubation and the subsequent centrifugation were carried out at 3 mM-Mg²⁺ (*a, b*), 5 mM-Mg²⁺ (*c*) or 7 mM-Mg²⁺ (*d*). The arrows indicate the positions of 80 S ribosomes or 60 S subunits. ●, Absorbance at 260 nm; ○, antibody binding to 80 S ribosomes or 60 S subunits, which was determined by e.l.i.s.a. Data are from typical experiments which were replicated twice.

ribosomes, to a lesser but still appreciable extent (results not shown). The binding of antibody to 80 S ribosomes is considerably weaker than to 60 S subunits, therefore the antibody is probably detached from 80 S ribosomes during the centrifugation. The effect of the antibody on the dissociation of 80 S couples was also examined at different Mg²⁺ concentrations. Some effects on the dissociation of 80 S ribosomes were observed at 3 mM-Mg²⁺, but not at concentrations of 5 mM or 7 mM (Figs. 1*b–d*). These results indicate that L5 is located on the surface of 60 S subunits and is probably at or near the subunit interface.

Effect of anti-L5 antibody on subunit reassociation

We tested the effect of the L5 antibody on the formation of 80 S ribosomes from 40 S subunits and 60 S subunits that had been preincubated with antibody. As shown in Fig. 2, the association of ribosomal subunits is inhibited at 5 mM-Mg²⁺, and the extent of the inhibition is dependent on the amount of the antibody which was preincubated with 60 S subunits, although there is no inhibition of 80 S formation at 10 mM-Mg²⁺ (Fig. 2*d*).

Fig. 2. Effect of anti-L5 antibody on association of ribosomal subunits

Portions (20 pmol) of 60 S subunits were preincubated with nonimmune or anti-L5 IgG for 10 min at 37 °C. After a 2-fold molar excess of 40 S subunits was added, the mixture was incubated again for 10 min and then centrifuged for 18 h at 16000 rev./min as described in the Materials and methods section. The Mg^{2+} concentration used was 5 mM (a-c) or 10 mM (d). The arrows indicate the positions of 80 S ribosomes or 60 S subunits. Preincubation was with (a) 120 pmol of nonimmune rabbit IgG; (b) 60 pmol of anti-L5 IgG; (c), (d) 120 pmol of anti-L5 IgG. Data are from typical experiments replicated three times.

Effect of anti-L5 antibody on polyphenylalanine synthesis

The antibody was tested for its effect on poly(U)-directed polyphenylalanine synthesis *in vitro*. The synthesis was strongly inhibited by the antibody when 60 S subunits were preincubated with an excess molar ratio of the antibody prior to the synthesis assay (Fig. 3). In the present assay, the synthesis was carried out at 10 mM- Mg^{2+} . At this concentration, there was no inhibition of 80 S formation by the antibody-treated 60 S subunits (Fig. 2d). When intact 80 S ribosomes instead of 60 S subunits were preincubated with the antibody, there was also inhibition of polyphenylalanine synthesis. However, the extent of inhibition was less than that observed when 60 S subunits were preincubated with the antibody. Nonimmune rabbit IgG had no significant effect on polyphenylalanine synthesis.

Effect of anti-L5 antibody on translation of globin mRNA

To examine the effect of the antibody on the translation of natural mRNA, the incorporation of [3H]leucine directed by globin mRNA was assayed. As indicated in Fig. 4, the translation of globin mRNA was inhibited when 60 S subunits were preincubated with the antibody, but the extent of the inhibition was less than that observed

Fig. 3. Effect of anti-L5 antibody on polyphenylalanine synthesis

Anti-L5 antibody was incubated with 20 pmol of either 80 S ribosomes (●) or 60 S subunits (○) with molar ratios as indicated. The preincubated ribosomes or subunits were then tested for polyphenylalanine synthesis activity using [^{14}C]phenylalanine as described in the Materials and methods section. The amounts of phenylalanine polymerized in the absence of antibody were taken as 100%, these values were about 260 pmol for 80 S ribosomes and 70 pmol for 60 S subunits. □, Nonimmune rabbit IgG. Results represent the means for two separate determinations.

with polyphenylalanine synthesis (Fig. 3). When 80 S ribosomes were preincubated with the antibody, the translation of the globin mRNA was not appreciably inhibited even when a 6-fold excess (molar ratio) of the antibody was allowed to react with 80 S ribosomes (Fig. 4).

Effect of anti-L5 antibody on EF-2- and 60 S-subunit-dependent GTPase activity

To investigate further the inhibitory effect of the antibody in the polyphenylalanine synthesis system, the EF-2/60 S subunit-dependent uncoupled GTPase activity was assayed. The uncoupled GTPase activity, which was dependent on EF-2 and 60 S subunits is shown in Fig. 5a. As shown in Fig. 5b, there was no significant inhibition of the activity when 60 S subunits were preincubated with the antibody prior to the assay.

DISCUSSION

It was shown that the antibody to *Artemia* L5 bound to ribosomal 60 S subunits (Fig. 1a) and that it inhibited the subunit reassociation to some extent at 5 mM- Mg^{2+} (Fig. 2). Antibody binding to 80 S ribosomes was also observed when analysed by e.l.i.s.a., but the binding was scarcely seen in the sucrose gradient analysis (Fig. 1).

Fig. 4. Effect of anti-L5 antibody on translation of globin mRNA

Portions (10 pmol) of either 80 S ribosomes (●) or 60 S subunits (○) were preincubated with anti-L5 antibody at different molar ratios as indicated, and translation activity of globin mRNA was then assayed using [^3H]leucine as described in the Materials and methods section. The amounts of leucine incorporated in the absence of antibody were taken as 100%, these values were about 15 pmol for 80 S ribosomes and 9 pmol for 60 S subunits. □, Nonimmune rabbit IgG. Results represent the means for two separate determinations.

Fig. 5. Effect of anti-L5 antibody on EF-2- and 60 S-subunit-dependent GTPase activity

(a) 60 S-subunit-dependent GTPase activity was measured in the presence (○) or in the absence (△) of 10 pmol of EF-2 with different amounts of 60 S subunits as indicated. (b) 60 S subunits (10 pmol) were preincubated with different molar ratios of anti-L5 antibody as indicated, and the GTPase activity in the presence of 10 pmol of EF-2 was then tested with [^{32}P]GTP as described in the Materials and methods section. □, Nonimmune rabbit IgG. Each point represents the average of duplicate samples.

This indicates that the antibody binds better to 60 S subunits than to 80 S ribosomes. Furthermore, some effects of the antibody on dissociation of 80 S couples were observed at 3 mM- Mg^{2+} (Fig. 1b). These results suggest that *Artemia* L5 is probably located at or near the subunit interface, and that this epitope may be partially hidden in the intact ribosome. *Escherichia coli* 5 S-RNA-binding proteins (L5, L18 and L25) were seen to be located on the subunit interface by means of immune electron microscopy [22,23]. Rat liver L5 was found to be involved in the interface on the basis of its cross-linking with 40 S proteins [13] and mRNAs [11,12]. In general, 5 S-RNA-binding protein may be located at the boundary between the large and the small subunits.

The antibody was tested for its effect on ribosome function. It strongly inhibited poly(U)-directed polyphenylalanine synthesis when it was allowed to interact with 60 S subunits prior to the addition of 40 S subunits (Fig. 3). The polyphenylalanine synthesis was carried out at 10 mM- Mg^{2+} . There was no inhibition of the subunit reassociation at this Mg^{2+} concentration (Fig. 2d), and hence the polyphenylalanine synthesis is considered to be interfered with by other effects of the antibody. Translation of globin mRNA was done to confirm the inhibitory effect of the antibody with a more complete assay system which involved both the initiating and the elongating processes in protein synthesis. The translation was inhibited when 60 S subunits were preincubated with the antibody prior to the assay, but not when 80 S ribosomes were preincubated (Fig. 4). The binding of antibody to 80 S ribosomes is considered to be possible but rather difficult. Therefore, the antibody which bound to 80 S ribosomes may have been displaced by factors involved in protein synthesis or by 40 S subunits during the translation processes. No inhibition of the formation of the 80 S initiation complex with a 5' fragment of tobacco mosaic virus RNA in reticulocyte lysate was observed (results not shown). Accordingly, the inhibition of the translation may result from the interference with the elongation cycle.

To investigate further the inhibitory effect of the antibody on the polyphenylalanine synthesis system, the EF-2- and 60 S-subunit-dependent GTPase activity, which is considered to be involved in the protein synthesis-elongation cycle, was assayed (Fig. 5). There was no inhibitory effect on the GTPase activity, and hence this suggests that the antibody did not inhibit the translocation of peptidyl-tRNA in the elongation cycle [24]. Nag *et al.* [25] indicated that a monoclonal antibody to *Escherichia coli* 5 S-RNA-binding protein L5 inhibited the peptidyltransferase activity. Similarly, the inhibition of polyphenylalanine synthesis observed in the present work may be due to interference with the peptidyltransferase centre by the antibody. Actually, rat liver L5 has been shown to be crosslinked to globin mRNA [12] and to be located at the peptidyl (P) site [26] in the 80 S initiation complex. In addition, it was cross-linked to the heptanucleotide (ACAAUGC) including an initiator AUG when the 80 S initiation complex was formed with a 5' fragment of tobacco mosaic virus RNA in reticulocyte lysate (Y. Takahashi, S. Hidaka, T. Tanaka & K. Ogata, unpublished work). Consequently, *Artemia* L5 is probably located at or near the interface between the two subunits, and may be at the peptidyltransferase centre and play a significant role in protein synthesis.

We are grateful to Dr. Toshio Uchiumi for providing pig liver EF-2 and for his helpful suggestions.

REFERENCES

1. Erdmann, V. A. (1976) *Prog. Nucleic Acid Res. Mol. Biol.* **18**, 45–90
2. Erdmann, V. A., Sprinzl, M. & Pongs, O. (1973) *Biochem. Biophys. Res. Commun.* **54**, 942–947
3. Azad, A. A. & Lane, B. G. (1973) *Can. J. Biochem.* **51**, 1669–1672
4. Garret, R. A., Douthwaite, S. & Noller, H. F. (1981) *Trends Biochem. Sci.* **6**, 137–139
5. Blobel, G. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1881–1885
6. Nazar, R. N., Yaguchi, M., Willick, G. E., Rollin, C. F. & Roy, C. (1979) *Eur. J. Biochem.* **102**, 573–582
7. Terao, K., Takahashi, Y. & Ogata, K. (1975) *Biochim. Biophys. Acta* **402**, 230–237
8. Petermann, M. L., Hamilton, M. G. & Pavlovec, A. (1972) *Biochemistry* **11**, 2323–2326
9. Isoda, N., Tanaka, T. & Ishikawa, K. (1981) *J. Biochem. (Tokyo)* **90**, 551–554
10. Marion, M.-J. & Reboud, J.-P. (1981) *Biochim. Biophys. Acta* **652**, 193–203
11. Takahashi, Y. & Ogata, K. (1981) *J. Biochem. (Tokyo)* **90**, 1549–1552
12. Takahashi, Y. & Ogata, K. (1985) *Eur. J. Biochem.* **152**, 279–286
13. Uchiumi, T., Kikuchi, M. & Ogata, K. (1986) *J. Biol. Chem.* **261**, 9663–9667
14. Kenmochi, N. & Ogata, K. (1989) *Zool. Sci.* **6**, in the press
15. Sommer, A., Etchison, J. R., Gavino, G., Zecherle, N., Casiano, C. & Traut, R. R. (1985) *J. Biol. Chem.* **260**, 6522–6527
16. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
17. Kenmochi, N., Tsurugi, K. & Ogata, K. (1981) *J. Biochem. (Tokyo)* **89**, 1293–1308
18. Engvall, E. (1980) *Methods Enzymol.* **70**, 419–439
19. Terao, K. & Ogata, K. (1971) *Biochim. Biophys. Acta* **254**, 278–295
20. Takahashi, Y. & Ogata, K. (1982) *Biochim. Biophys. Acta* **697**, 101–112
21. Conway, T. W. & Lipmann, F. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **52**, 1462–1469
22. Wittmann, H. G. (1983) *Annu. Rev. Biochem.* **52**, 35–65
23. Stöffler-Meilicke, M., Noah, M. & Stöffler, G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6780–6784
24. Bielka, H. (1982) in *The Eukaryotic Ribosome* (Bielka, H., ed.), pp. 201–252, Springer-Verlag, Berlin, Heidelberg and New York
25. Nag, B., Tewari, D. S., Sommer, A., Olson, H. M., Glitz, D. G. & Traut, R. R. (1987) *J. Biol. Chem.* **262**, 9681–9687
26. Fabijanski, S. & Pellegrini, M. (1981) *MGG, Mol. Gen. Genet.* **184**, 551–556

Received 11 October 1988/5 December 1988; accepted 12 December 1988