PROTEIN TIGHTLY BOUND TO GLOBIN mRNA

Günter Blobel

The Rockefeller University, New York, N.Y. 10021

Received February 28, 1972

SUMMARY

mRNA released from reticulocyte ribosomes by the puromycin-induced dissociation in high concentrations of salt contains two proteins (or polypeptide chains) of a molecular weight of 78,000 ± 1,000 and 52,000 ± 1,000.

A frequently followed procedure for the isolation of mRNA from reticulocyte ribosomes involves in a first step dissociation of ribosomes by magnesium-chelating agents, such as EDTA; this permits the separation of a distinctly sedimenting mRNA-protein complex from the bulk of the ribosomal RNA which is retained in the faster sedimenting ribosomal subunits. In a second step mRNA is isolated after deproteinization of the complex by detergents such as SDS (1).

Recently an alternative procedure for the isolation of mRNA was described (2). It grew out of an effort to disassemble the eukaryotic polysome into biologically functional components [dissociation by EDTA causes inactivation of both ribosomal subunits, except under carefully controlled conditions and in the presence of urea (3)]. With reticulocyte ribosomes serving as a model system, it was found that treatment with puromycin-salt induced disassembly of polysomes into biologically functional ribosomal subunits and led to the concomitant release of mRNA. Buoyant density analysis of the released mRNA led to the tentative conclusion that the mRNA was protein-free (2). Thus, it appeared that the two proteins, which were found to be attached to globin mRNA after EDTA-induced dissociation of ribosomes in low concentrations of salt (4, 5) were removed by the high salt concentrations

of the pellet into a clear solution of dissociated ribosomes. Sucrose gradient analysis of this solution, using a SB283 rotor of the IEC centrifuge, was as described previously (2).

Electrophoresis of proteins in acrylamide-sodium dodecyl sulfate gels: Electrophoresis was performed in a vertical electrophoresis cell (E-C Apparatus Corp., Philadelphia, Pa.) in slabs 3 mm thick, each provided with eight slots, by the procedure described by Maizel (6). The ratio of acrylamide to bisacrylamide was 30:0.8.

RESULTS

In order to eliminate any of the large number of proteins which are

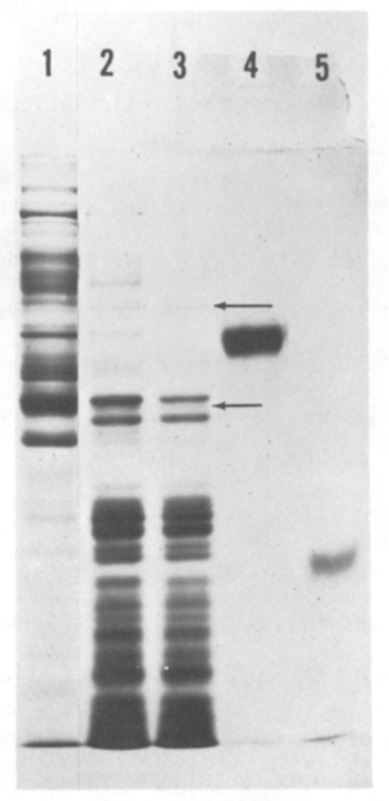

Fig. 1. Electropherogram in a 10% acrylamide gel of proteins of salt washed (slot 3) and crude ribosomes (slot 2) and of proteins removed from crude ribosomes by the salt wash (slot 1). Bovine albumin (slot 4) and beef pancreas chymotrypsinogen A (slot 5) are included as markers. The arrows point to the two mRNA proteins visible as faint bands in slots 2 and 3 (see also Fig. 3). Proteins in slots 2 and 3 correspond to 4.0 A_{260} of ribosomes and those in slot 1 were washed off from ~12 A_{260} of ribosomes.

used in the puromycin-induced dissociation. Detection of an mRNA-protein complex by buoyant density analysis is dependent on the formation of covalent bonds between RNA and protein by aldehyde treatment in order to prevent their subsequent dissociation in CsCl gradients. The results of more recent experiments have shown that this condition is difficult to achieve and have suggested that it may not have been met previously. An alternative way to solve this discrepancy (protein-complexed vs. protein-free state of mRNA) is to analyze the puromycin-salt released mRNA for associated proteins, using acrylamide gel electrophoresis. The results of the experiments reported in this paper show that mRNA released by the puromycin-salt method is also bound to two proteins.

METHODS

The preparation of ribosomes from reticulocytes of anaemic rabbits was as previously described (2).

Salt wash of ribosomes: All operations were performed in the cold (2-4°C). A ribosome pellet (∿150 A_{260}) was resuspended in 2 ml of water and 2 ml of a compensating buffer were added to give a final concentration of 500 mM KCl - 50 mM triethanolamine·HCl (pH 7.5 at 20°C) - 5 mM $MgCl_2$ - 2 mM DTT. This suspension was layered over a cushion of 2 ml 30% sucrose in 500 mM KCl - 50 mM triethanolamine·HCl - 5 mM $MgCl_2$ - 2 mM DTT. Centrifugation for 1 hr at 4°C in the A321 rotor of the IEC centrifuge at 50,000 rpm (225,000 g_{max}) yielded a pellet of salt-washed ribosomes. The top 4 ml of the gradient corresponding to the load zone were saved and mixed with 8 ml ethanol. After 24 hr at -20°C a precipitate was collected by low speed centrifugation.

Dissociation of ribosomes by salt-puromycin: The previously published procedure (2) was modified as follows. To a pellet of salt washed ribosomes (∿150 A_{260}) 2 ml of a solution of 500 mM KCl - 50 mM triethanolamine·HCl - 2 mM $MgCl_2$ - 1 mM DTT - 1 mM puromycin (pH 7.0) was added. Incubation for 10 min at 37°C with occasional gentle swirling resulted in a rapid suspension

adsorbed to ribosomes after standard isolation techniques in low ionic strength and which after ribosome dissociation may adsorb or cosediment with the released mRNA, ribosomes were first subjected to a salt wash. The composition of the salt-solution was chosen on the basis of earlier studies (7), which showed that under these conditions polysomes were not dissociated, nor was their ability for endogenous amino acid incorporation significantly reduced. From Fig. 1 it can be seen that the salt wash indeed removed a great number of proteins, mostly of large molecular weight.

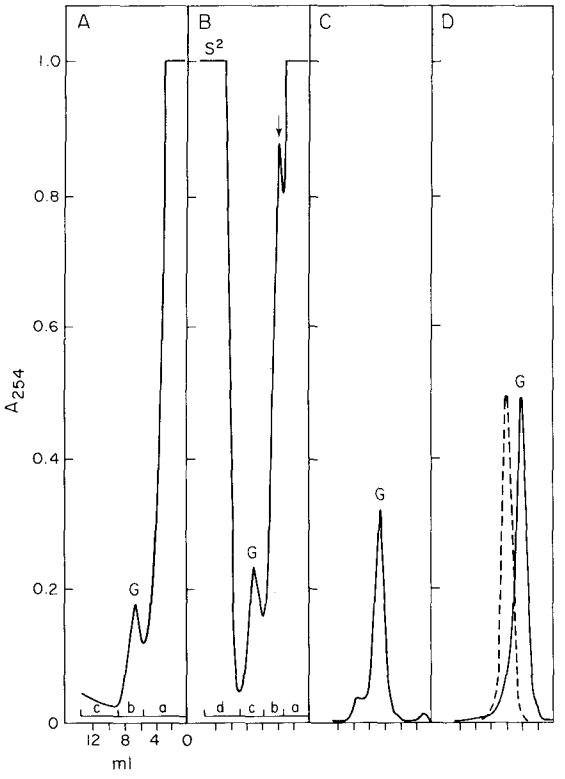

Fig. 2. Sedimentation profile of salt-puromycin dissociated ribosomes and of isolated G fractions. 0.5 ml aliquots of a solution containing ~40 A_{260} of dissociated ribosomes (see Methods) were layered on 5-20% sucrose gradients in 500 mM KCl-50 mM triethanolamine HCl (pH 7.5) and either 3 mM $MgCl_2$ (A) or no $MgCl_2$ (B). Centrifugation was for 7 hr at 20°C and at 39,000 rpm (180,000 g_{aver}). The G peak was collected from three gradients as shown in B and centrifuged for 16 hr at 4°C and 40,000 rpm in a Spinco #40 rotor. The resulting pellet was resuspended at room temp in 0.2 ml of 500 mM KCl-50 mM triethanolamine HCl and the solution was layered on a 5-20% sucrose gradient in 500 mM KCl-50 mM triethanolamine HCl (C) or on a 7-20% sucrose gradient in 10 mM KCl-10 mM triethanolamine HCl (D). Centrifugation was at 39,000 rpm for either 7 hr at 20°C (C) or 7 hr at 4°C (D). S^2 designates the derivative of the small ribosomal subunit (8). The dashed line shows the sedimentation profile in an identical gradient of the small ribosomal subunit after EDTA dissociation of ribosomes.

Both the puromycin-induced disassembly of polysomes and the subsequent separation of functional ribosomal subunits was previously found to require the presence of critical concentrations of magnesium; below these, ribosomal subunits were converted into slower sedimenting, inactive derivatives (8). In the case of the large subunit this derivatization was shown to involve the loss of 5S RNA, a 5S RNA-associated protein and several more ribosomal proteins (8). Some of these changes are evident in the sedimentation profiles shown in Figs. 2A and B. In the magnesium containing gradient (A) the small subunit (S^0) has sedimented to the bottom, whereas in a gradient containing no magnesium, its slower sedimenting derivative (S^2) occupied most of the lower third of the gradient (B). Furthermore, as a result of the derivatization of the large subunit, the 5S RNA-protein complex is clearly visible as a peak sedimenting faster than the large peak near the top of the gradient, which is due to puromycin and tRNA. It should be noted that the absence of magnesium had no effect on the sedimentation rate of the globin mRNA-containing peak (designated G).

Analysis of the proteins sedimenting in various fractions from the top to the bottom of the gradient (indicated in Figs. 2A and B), showed two distinct proteins in the G peak (Figs. 3A, slot b, 3B, slot c). The molecular weight of these two proteins was determined to be 78,000 $\pm$ 1,000 and 52,000 $\pm$ 1,000 in five separate experiments, using bovine albumin, ovalbumin and beef pancreas chymotrypsinogen A as markers. These two proteins are also present, although in much lower amounts, in the fractions sedimenting slower or faster than the G peak. This could be due to fraction overlap or to other mRNA's present in reticulocyte ribosomes, smaller or larger than globin mRNA, containing the same two proteins, and sedimenting slower or faster than G. The intensity of the stain strongly suggests that the two proteins are present in stochiometric amounts. Other proteins are present in much lower concentrations, most conspicuously one with a molecular weight of $\sim$115,000 (vertical arrows in Fig. 3A and B). Since these proteins are largely

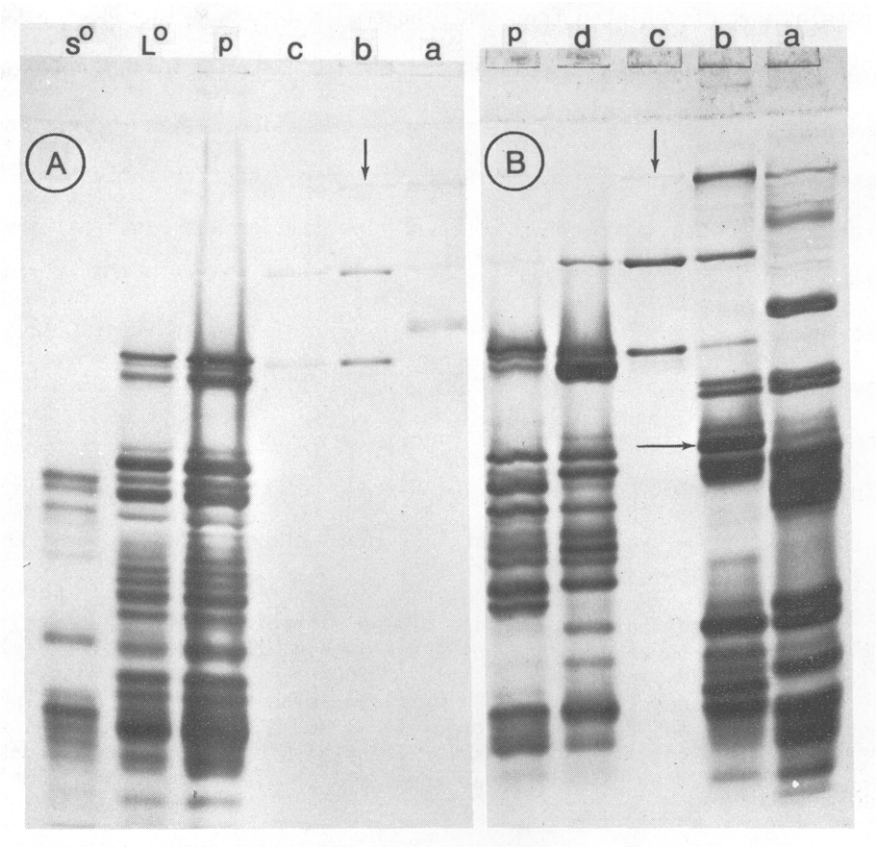

Fig. 3. Electropherogram in an 11% acrylamide gel of the proteins of sucrose gradient fractions shown in Fig. 2A and B. 3A corresponds to 2A, and 3B to 2B. In each case, fractions were pooled from 3 gradients. The fractions sedimenting slower than G were precipitated with alcohol (see Methods). The G peak and the faster sedimenting fractions were recovered as pellets (see Fig. 2). All alcohol precipitates and the G pellet were resuspended in approximately equal volumes, the other pellets, including the three pellets at the bottom of the sucrose gradients, in twice that volume. Equal volumes of all samples were loaded into the various slots of the gel. The letters a, b, c, d on top of each column correspond to the fractions indicated in the sedimentation profile of Fig. 2 and the letter p to the pellets in these gradients. S^0 and L^0 are non-derivatized, active small and large ribosomal subunits, respectively, prepared as previously described (7).

---

eliminated when G was analyzed after recentrifugation (Fig. 2C), they are for the moment considered to be contaminants. It should be noted that the two mRNA proteins remained associated with mRNA in the G peak, even in the absence of magnesium (Fig. 3B, slot c), when a distinct number of ribosomal

proteins was dissociated from the ribosomal subunits (Fig. 3B, slots a, b), among them the 5S RNA-associated protein (marked with a horizontal arrow in Fig. 3B).

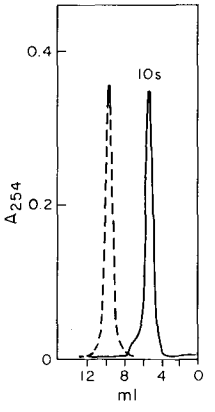

Fig. 4. Sedimentation profile of RNA extracted from the G peak. G was collected from three gradients as shown in Fig. 2B, sedimented (see legend, Fig. 2) and resuspended at room temp in 200 µl of H_2O and 20 µl of a solution of 10% sodium dodecyl sulfate-0.1 M triethanolamine HCl (pH 7.5). The suspension was layered on a 5-20% sucrose gradient in 50 mM NaCl-10 mM triethanolamine HCl (pH 7.5). Centrifugation was for 16 hr at 6°C and 39,000 rpm. Dashed line shows sedimentation profile in an identical gradient of 18S ribosomal RNA.

Since previously the G peak was considered protein-free mRNA, its sedimentation rate (11.3S) in high salt gradients was related to marker RNA rather than to ribonucleoprotein. When G was recentrifuged into a low salt gradient (Fig. 2D) it sedimented at ∿20S when compared to the sedimentation rate of the small subunit, obtained after EDTA dissociation. After deproteinization the RNA in the G peak sedimented at 10S (Fig. 4) when compared to 18S ribosomal RNA. Thus, in a low salt gradient the sedimentation rate of the mRNA protein complex was twice that of its protein-free RNA.

DISCUSSION

There are already two reports (4, 5) in the literature in which it has been shown that globin mRNA, released upon dissociation of reticulocyte ribosomes by EDTA in low salt, is bound to two distinct proteins. These findings are strongly supported by the results of the experiments reported

here, in which dissociation of reticulocyte polysomes was achieved by a different procedure, i.e. puromycin-high salt treatment. The resistance of the mRNA-protein complex to dissociation by salt concentrations so high as to cause complete dissociation of a few distinct ribosomal proteins from ribosomal subunits points to a specific interaction between mRNA and these two proteins rather than to a nonspecific adsorption of protein to RNA, which is known to occur at low ionic strength and therefore could not be ruled out in the case of mRNA-protein complexes extracted by EDTA in low concentrations of salt. The molecular weights of 78,000 and 52,000 for the two proteins reported here differ significantly from the 130,000 and 68,000 in ref. 4. On the other hand, they are close to the molecular weights of 73,000 and 49,000 in ref. 5, although the ribosomes in the latter case were isolated from duck reticulocytes, whereas those in ref. 4 and in the present work were obtained from rabbit reticulocytes. The large discrepancy in the estimates of the molecular weights cannot be the result of the different isolation procedures, since in our experiments the mRNA-protein complex isolated by the EDTA-low salt method (data not shown) also contained two major proteins of molecular weights of 78,000 and 52,000. In addition, however, the complex contained a large number of other proteins, particularly if the ribosomes were not salt-washed before their dissociation by EDTA.

REFERENCES

1. Huez, G., A. Burny, G. Marbaix and B. Lebleu. Biochim. Biophys. Acta, 629, 145 (1967).
2. Blobel, G. Proc. Nat. Acad. Sci., USA, 68, 832 (1971).
3. Petermann, M.L. and A. Pavlovec. Biochemistry, 10, 2770 (1971).
4. Lebleu, B., G. Marbaix, G. Huez, J. Temmerman, A. Burny and H. Chantrenne. European J. Biochem. 19, 264 (1971).
5. Morel, C., B. Kayibanda and K. Scherrer. FEBS Letters, 18, 84 (1971).
6. Maizel, J.V. in Fundamental Techniques in Virology, ed. K. Habel and N.P. Salzman (Academic Press, New York, 1969). Chap. 32, p. 334.
7. Blobel, G. and D. Sabatini. Proc. Nat. Acad. Sci., USA, 68, 390 (1971).
8. Blobel, G. Proc. Nat. Acad. Sci., USA, 68, 1881 (1971).