In Vitro Translation of Globin: Effect of Proteins Purified by Affinity Chromatography on Polyadenylate–Sepharose†

Hiroichi Fukami‡ and Harvey A. Itano*

ABSTRACT: By means of affinity chromatography on poly(adenylic acid) (poly(A))-fixed Sepharose, protein fractions having strong affinity to poly(A) were prepared from postribosomal supernatants of rabbit reticulocyte and rat liver. These fractions contained several proteins similar by electrophoretic analysis to rabbit globin messenger ribonucleoprotein. Protein fractions from both sources were shown to form ribonucleoprotein complexes with rabbit globin mRNA, and these complexes sedimented at the same rate as native globin messenger ribonucleoprotein. Binding of the proteins to RNA was not highly specific, since not only poly(A) but also other polynucleotides as poly(C) or poly(U) were bound to these proteins. Ribosomal RNAs, tRNA, or DNAs did not bind the proteins. In order to ascertain the function of the poly(A)–Sepharose purified proteins, their effects on translation of globin mRNA was studied in vitro. Addition of rabbit reticulocyte protein to globin mRNA resulted in no more than a slight stimulation of both α- and β-chain synthesis. Poly(A)–Sepharose purified protein from rat liver, however, caused a marked preferential reduction of α-chain synthesis. These results showed that at least some proteins in the poly(A)–Sepharose purified proteins affect the translation of globin. This inference suggested a possibility that protein moiety in globin mRNP might be involved in control of globin synthesis.

One of the characteristics of the mRNA from eukaryotic cells is the presence of a poly(A) sequence on its 3′ terminus. This sequence is also found in heterogeneous nuclear RNAs and in certain animal virus RNAs. The function of the poly(A) sequence remains, however, obscure (Lim and Canellakis, 1970; Mendecki et al., 1972; Sheiness and Darnell, 1973; Williamson et al., 1974; Huez et al., 1974). Another specific characteristic of eukaryotic mRNA is that it occurs as a complex with several proteins when dissociated from polysomes by treatment with EDTA[1] (Lebleu et al., 1971; Kumar and Lindberg, 1972; Olsnes, 1971) or with puromycin (Blobel, 1972).

The species of proteins in these mRNP complexes appear to be markedly similar among different varieties of eukaryotes (Blobel, 1973; Bryan and Hayashi, 1973; Morel et al., 1973). The biological function of these proteins is obscure. At least one of these proteins was suggested to be required for the binding of globin mRNA to smaller ribosomal subunits (Lebleu et al., 1971); however, there is disagreement as to whether or not this protein is identical to one of the initiation factors for translation (Ilan and Ilan, 1973; Nudel et al., 1973). Since the presence of both a poly(A) sequence and the binding proteins is a characteristic common to eukaryotic mRNAs, interrelationship of the two phenomena has been suggested, namely that the proteins bind to mRNA on its poly(A) part (Kwan and Brawerman, 1972; Blobel, 1973).

The low cellular concentration of these complexes poses a major difficulty in the study of their function. Investigating the possibility that the same proteins might also be present within a supernatant fraction free from polysomes, we subjected a postribosomal supernatant to affinity chromatography on poly(A)-fixed Sepharose. A protein fraction with strong affinity to the immobilized poly(A) was shown by gel electrophoresis to contain a major component of 78 000 molecular weight as well as several minor components. Similar protein fractions were also obtained in this study from the supernatant of homogenized rat liver cells. There are several reports on purification of cytoplasmic proteins with high affinity to poly(A) by means of poly(A)-Sepharose. Schweiger and Mazur (1974, 1975) have prepared several protein fractions from cytoplasm or nucleoplasm of rat liver. They reported that these were also comprised of several proteins involving a 78 000-dalton major protein band, as indicated by sodium dodecyl sulfate-gel electrophoresis. Blanchard et al. (1974) isolated a protein from the postribosomal supernatant of HeLa cells by affinity chromatography on poly(A)-fixed Sepharose. The chromatographic properties, as well as molecular weight of this protein, differed markedly from those of the rabbit reticulocyte or rat liver proteins obtained in the present work. When the latter two protein fractions were combined with 9S globin mRNA, the resulting mRNP complex sedimented at the same rate as native globin mRNP. Translation of the synthetic complex was compared to that of 9S globin mRNA in the Krebs II cell-free system. The combination of rabbit globin mRNA with poly(A)-binding protein from rat liver was shown to bring about a marked change in the rate of synthesis of globin chains.

Materials and Methods

Cellular Fractionation of Rabbit Reticulocyte and Rat Liver. Female rabbits (ca. 3-kg body weight) were injected subcutaneously with 2.5% neutralized phenylhydrazine (0.3 ml kg weight^{-1} day^{-1}) for 5 consecutive days. Blood was taken out by cardiac puncture with the use of 0.3 M Na-EDTA (pH 7.0) as anticoagulant. The following operations were carried

† From the Department of Pathology, School of Medicine, University of California, San Diego, La Jolla, California 92093. *Received October 24, 1975.* This work was supported by National Institutes of Health Grants GM 17702 and AM 14982.

‡ Present address: Department of Agricultural Chemistry, The University of Tokyo, Yayoi, Bunkyo, Tokyo, Japan 113.

[1] Abbreviations used are: mRNP, messenger ribonucleoprotein; PASP, poly(A)–Sepharose purified protein; TKE, 10 mM Tris-HCl (pH 7.6), 15 mM KCl, 1 mM EDTA; EDTA, ethylenediaminetetraacetic acid; poly(A), poly(adenylic acid); ATP, GTP, adenosine and guanosine triphosphates; DEAE, diethylaminoethyl.

out at 0–4 °C unless otherwise stated. The blood was centrifuged at 4000*g* for 10 min, and pelletted cells were washed three times with 20 mM Tris-HCl (pH 7.6), 5 mM KCl, 0.14 NaCl, 4 mM $MgCl_2$. After the buffy coat was removed, the packed cells were agitated for 5 min with 1.5 volumes of cold distilled water. The resulting lysate was centrifuged at 15 000*g* for 20 min to remove debris. Polysomes were spun down from the initial supernatant by centrifugation at 78 000*g* for 3 h. The upper four-fifths of the supernatant from the second centrifugation were dialyzed against 10 mM Tris-HCl (pH 7.6), 0.1 M NaCl, and stored at −70 °C in aliquots for later use in the preparation of poly(A)-binding proteins.

Livers were obtained from female rats (about 5 weeks old) starved for 40 h. Each animal was anesthetized with chloroform, and its liver was perfused with 20 mM Tris-HCl (pH 7.6), 5 mM KCl, 0.14 M NaCl, 4 mM Mg Cl_2, before removal. The livers were rinsed with 0.25 M sucrose, 10 mM KCl, 10 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$. Chopped liver (10 g) was homogenized with 25 ml of the same buffer in a 50-ml glass homogenizer by one or two strokes with a motor-driven, loosely-fitted, Teflon pestle. It is unlikely that cell nuclei were disrupted by this procedure, since the number of nuclei observed by microscopy in the homogenate was virtually unchanged by an increase of up to ten in the number of strokes of the pestle. Leakage of nuclear RNP particles into cytoplasm, however, may be more or less unavoidable. The resulting lysate was centrifuged at 15 000*g* for 3 h, and the top one-fifth of the supernatant was pipetted off. The next three-fifths were dialyzed against 10 mM Tris-HCl (pH 7.6), 0.1 NaCl overnight, and stored at −70 °C in aliquots.

Rabbit Globin Messenger Ribonucleoprotein and Globin mRNA. The method of Huez et al. (1967) with slight modifications was followed for the preparation of globin mRNP. The polysomal pellet was rinsed once with 10 mM Tris-HCl (pH 7.6), 15 mM KCl, 1 mM EDTA (hereafter, TKE), and suspended in the same buffer to give A_{260} of around 200. A half-volume of 0.1 M Na-EDTA (pH 7.0) was added, and the suspension was allowed to stand for 30 min at 0 °C. About 2000 A_{260} units of the polysomes in 10–20 ml was loaded on a linear gradient of 10–30% sucrose in TKE, which had been set in a Spinco Ti-14 zonal rotor. After centrifugation at 47 000 rpm for 12 hr at 2 °C, fractions containing 15S mRNP complex were pooled and concentrated by centrifugation at 260 000*g* for 7 h. The resulting pellet was suspended in TKE and purified by centrifugation in another sucrose gradient (10–30% sucrose, TKE) in a Spinco SW 27 rotor. The mRNP complex was concentrated from the fractions by centrifugation as above and was dialyzed against TKE to remove sucrose.

Rabbit globin mRNA was prepared from mRNP by the procedure of Lane et al. (1971) and Huez et al. (1967). A solution of rabbit globin mRNP was dialyzed against 10 mM Tris-HCl (pH 7.6), 15 mM NaCl, 1 mM EDTA; then sodium dodecyl sulfate was added at ambient temperature to a concentration of 0.5%. This solution was layered on a 10–30% sucrose gradient in the same buffer without sodium dodecyl sulfate and centrifuged in a Spinco SW 27 rotor at 27 000 rpm for 24 h at 16 °C. Fractions containing 9S RNA were pooled, and 2 volumes of ethanol and 0.01 volume of sodium acetate (pH 5.0) were added to precipitate the RNA. The precipitation was repeated, and the final pellet was dissolved and dialyzed with distilled water. The mRNA obtained here was essentially free from protein; however, small amounts of protein in mRNP may remain even after extraction with sodium dodecyl sulfate and phenol (Evans and Rosenfeld, 1975; Brawerman et al., 1972).

Ribosomal RNAs, R17 Bacteriophage RNA. Ribosomal subunits were collected from the pooled fractions of the sucrose gradient centrifugation of the EDTA-treated polysomes. The subunits, which sedimented as 36S and 26S particles (Huez et al., 1967) in the gradient in the Ti-14 zonal rotor, were concentrated separately by centrifugation at 265 000*g* for 4 h. The pellets were suspended in 10 mM Tris-HCl (pH 7.6), 0.1 M KCl, 5 mM $MgCl_2$, and then extracted twice with phenol in the presence of 0.5% sodium dodecyl sulfate. The RNAs were precipitated from the combined aqueous phases by the addition of 2 volumes of ethanol and 0.01 volume of 3 M potassium acetate (pH 5.0). The precipitation was repeated once, and the RNAs were dialyzed against distilled water. Isolation of bacteriophage R17 and extraction of its RNA followed the procedure of Gesteland and Boedtker (1964).

Affinity Chromatography on Poly(A)-Fixed Sepharose. Poly(A) was fixed on Sepharose-4B according to published methods (Poonian et al., 1971; Cuatrecacas and Anfinsen 1971) with slight modifications. Sepharose-4B gel (10 ml) was activated with 2 g of cyanogen bromide at pH 11 at 15 °C; then the gel was chilled to 0 °C, filtered and washed quickly, and mixed with 10 mg of poly(A) (Miles Laboratories, Inc.) in 0.05 M potassium phosphate (pH 8.0). This mixture was stirred slowly in a rotary shaker overnight at 4 °C, filtered once, resuspended in 1 M ethanolamine–HCl (pH 8.0), and stirred for another 2 h at 22 °C. Finally, the gel was washed thoroughly with 0.05 M potassium phosphate (pH 8.0), distilled water, and 10 mM Tris-HCl (pH 7.6)-0.1 M NaCl. Absorbance at 260 nm of the wash indicated that more than 98% of the added poly(A) was fixed on the gel matrix by this procedure.

Chromatography on the poly(A)-fixed gel was carried out at a temperature of 22 °C and a flow rate of 30 ml/h. About 20 ml of the dialyzed reticulocyte supernatant fraction was thawed and applied onto a column of the gel (1.8 × 9 cm) that had been equilibrated with 10 mM Tris-HCl (pH 7.6), 0.1 M NaCl. The column was extensively washed first with the same buffer, then with 0.5 M KCl, 25 mM Tris-HCl (pH 7.6), until the A_{280} of each effluent buffer was negligible. The required volumes of the buffers were approximately 500 and 100 ml, respectively. The tightly bound protein fraction was then eluted out with 20 ml of 50 mM Tris-HCl (pH 7.6), 2.0 M LiCl, 1.0 M urea. After dialysis against TKE overnight, the protein fraction was concentrated with Amicon PM30 membrane under liquid-propane pressure.

If the absorbance ratio, A_{260}/A_{280}, of this fraction was higher than 0.8, the solution was applied to a small column of DEAE-cellulose (Whatman DE52) to remove impurities, which according to absorption spectrophotometry were mostly fragments of poly(A). The protein was eluted from the ion-exchange column with 0.6 M KCl, 20 mM Tris-HCl (pH 7.6), and was concentrated as described above after dialysis against TKE. This fraction is called poly(A)-Sepharose purified protein (abbreviated as PASP) in this study. The concentration of each protein preparation was determined by the method of Lowry et al. (1951).

Gel Electrophoresis. Sodium dodecyl sulfate-containing polyacrylamide gel, prepared by a method modified from Davis (1964), consisted of 8.5% separation gel and 1.25% stacking gel in a 2.8-mm-thick slab. The gels and the tank buffer contained 0.5% sodium dodecyl sulfate. Aliquots of the concentrated mRNP or PASP fractions were heated for 1 min in a boiling water bath in the presence of 1% 2-mercaptoethanol and 10–20% glycerol. Samples of up to 100 μl were placed in slots on the stacking gel along with 1 μl of 0.05% bromphenol blue. Electrophoresis was performed overnight until the dye

was 0.5 cm from the end of the gel (usually at 8 mA constant current, 20 V). The gel was soaked in 5% trichloroacetic acid to allow sodium dodecyl sulfate to diffuse out of the gel, stained with 0.1% Coomassie blue in 5% trichloroacetic acid, and destained in 5% trichloroacetic acid. The molecular weights of polypeptide chains were estimated according to Weber and Osborn (1969) with *Escherichia coli* β-galactosidase, bovine serum albumin, and lactate dehydrogenase as standards.

In Vitro Amino Acid Incorporation System. A cell-free system was prepared from Krebs II ascites tumor by the procedure of Mathews and Korner (1970). The supernatant of the centrifugation at 30 000*g* for 30 min (S-30 fraction) was preincubated at 37 °C for 45 min, passed through a Sephadex G-25 column, and stored at −70 °C in small aliquots. For the assay of poly(A)-directed [^{3}H]lysine binding to ribosomes, ionic concentrations in the reaction mixture were adjusted to 80 mM KCl, 7.1 mM $MgCl_2$, to obtain maximum stimulation of the binding of Lys-tRNA by added poly(A). Other components in the reaction mixture were 10 mM Tris-HCl (pH 7.6), 7 mM 2-mercaptoethanol, 1 mM ATP, 0.2 mM GTP, 4 mM creatine phosphate, 0.1 mM in each of 19 unlabeled amino acids, 5 μg of creatine kinase, 10 μg of poly(A), 30 μl of preincubated S-30 fraction, and 2 μCi of L-[^{3}H]lysine in a total volume of 0.15 ml. The mixture was incubated at 35 °C for 30 min, and 15-μl aliquots were diluted with 2 ml of the chilled buffer (10 mM Tris-HCl (pH 7.6), 80 mM KCl, 7.1 mM 2-mercaptoethanol) to stop the reaction. The ^{3}H radioactivity trapped on a Millipore HAWP 045 filter pad was counted in a scintillation counter. For globin synthesis, the final concentrations of KCl and $MgCl_2$ were adjusted to 80 and 3.1 mM, respectively, at which concentrations the stimulation of synthesis upon the addition of globin mRNA was maximal. L-[^{3}H]Lysine (2 μCi/0.15 ml of reaction mixture) was used for the labeling of globin because the numbers of lysine residues in the α and β chains of rabbit globin are equal. Other components in the reaction mixture were the same as described above, except that 2.0 μg of rabbit globin mRNA was used instead of poly(A). The incorporation of [^{3}H]lysine into the acid-insoluble fraction was assayed in the manner described by Nathans (1968). The background incorporation (without added mRNA) of this system was 3–5% of the full incorporation in the presence of globin mRNA. The incorporation continued linearly for 50 min or longer.

Quantification of α- and β-Globin Chains. The cell-free reaction mixture (50 μl) was mixed with 1.0 ml of 0.5% rabbit globin solution and extracted by the acid–acetone procedure of Rossi-Fanelli et al. (1958). Rabbit globin separation was carried out by the procedure of Dintzis (1961) on a column (0.6 × 18 cm) of carboxymethylcellulose (Bio-Rad Laboratories, Inc.). The eluate from the column was mixed continuously with an equal volume of Aquasol scintillation fluid (New England Nuclear Corp.) and pumped through a modified flow cell (Bakay, 1975) set in a β-Mate II (Beckman Instrument Co.) scintillation counter. Radioactivity was recorded at 5-min intervals. With a window setting for maximal ^{3}H-counting efficiency, this counting system had approximately 180 cpm of background caused largely by stray light from outside the system. This background was subtracted in the figures.

Results

Poly(A)–Sepharose Chromatography. The postribosomal supernatant fraction from rabbit reticulocyte was passed through the poly(A)–Sepharose column and chromatographed as described under Materials and Methods. The result is shown in Figure 1. The corresponding cellular fraction from rat liver

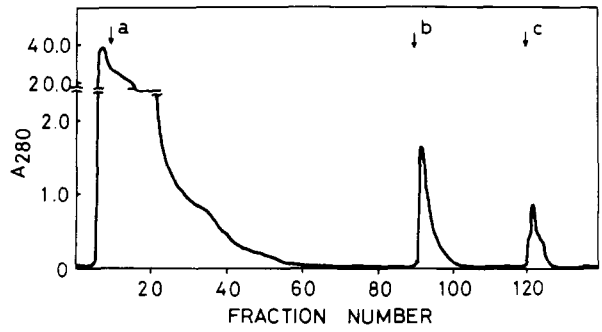

FIGURE 1: Chromatography of rabbit reticulocyte postribosomal supernatant on poly(A)–Sepharose. The reticulocyte postribosomal supernatant (20 ml) was applied on a poly(A)–Sepharose column (1.8 × 9 cm) as described under Materials and Methods. The eluate from the column was monitored by its transmittance at 281 nm by LKB Uvicord II with a 3-mm optical cell. Fractions of 8 ml were collected and those with high absorbance were measured after dilution. Arrows indicate where the elution buffers were changed: (a) 0.01 M Tris-HCl (pH 7.6), 0.1 M NaCl; (b) 0.025 M Tris-HCl (pH 7.6), 0.5 M KCl; and (c) 0.05 M Tris-HCl (pH 7.6), 2 M LiCl, 1 M urea.

was also analyzed under identical conditions (figure not shown). By extensive washing with 0.1 M NaCl, Tris-HCl (pH 7.8), the bulk of proteins including most of the hemoglobin was eluted out from the column. Further removal of proteins remaining nonspecifically bound on the gel was accomplished by washing with 0.5 M KCl. A sharp peak of absorbance at 280 nm was then eluted by 2.0 M LiCl, 1.0 M urea. The amount of PASP in the postribosomal supernatant was about twice the capacity of the gel for these proteins. At this loading ratio approximately 4 mg of PASP was obtained from 25 ml of gel.

The PASP fraction was compared to rabbit globin mRNP by electrophoresis on sodium dodecyl sulfate-containing 8.5% polyacrylamide gel. The fraction was found to contain polypeptide chains of 8 to 10 different sizes ranging in molecular weight from 21 000 to 160 000, as shown in Figure 2, slot A2. The electrophoretic mobility of a major component in the PASP agreed well with that of the protein of molecular weight 78 000 in globin mRNP (slots A1 and B1); a protein of this size has also been observed by others in eukaryotic cells (Blobel, 1973; Bryan and Hayashi, 1973). Some of the other protein bands in the PASP also corresponded well to those present in the globin mRNP.

A protein fraction prepared by the identical procedure from rat liver (slots B2 and B3) contained almost the same polypeptide components as the rabbit reticulocyte PASP fraction, except that it lacked the band of molecular weight 74 000, which is the main protein component in the reticulocyte postribosomal supernatant (slot B6). Instead, a protein band at molecular weight around 115 000 was somewhat stronger than in the reticulocyte protein. The protein fraction that was eluted from the column by 0.5 M KCl (slots B4 and 5) showed a more heterogeneous protein composition and was less like the globin mRNP standard. Thus, the elution of proteins electrophoretically similar to that of globin mRNP required a higher salt concentration than 0.5 M KCl.

Formation of mRNP Complex. The binding ability of PASP to various nucleic acids was studied by sedimentation experiments in sucrose density gradients. Figure 3 demonstrates the strong affinity of PASP to poly(A) in 10 mM Tris-HCl (pH 7.6), 80 mM KCl, 3.1 mM $MgCl_2$, the optimal ionic condition for in vitro hemoglobin synthesis. Brief incubation at 30 °C with PASP markedly changed the sedimentation pattern of poly(A); 6.3S poly(A) sedimented faster and

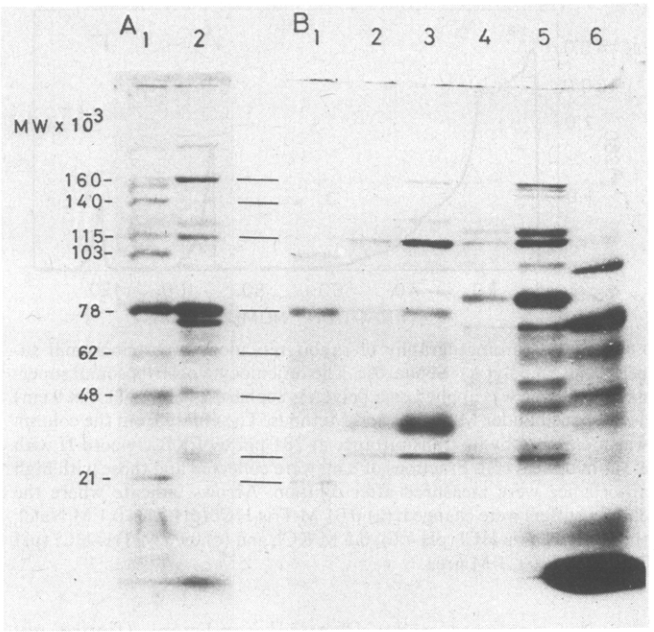

FIGURE 2: Polyacrylamide gel electrophoresis of poly(A)–Sepharose purified protein in the presence of sodium dodecyl sulfate. (A1), (B1): rabbit globin mRNP; (A2): 20 μg of rabbit reticulocyte PASP; (B2) 7 μg and (B3) 20 μg of rat liver PASP; (B4) 10 μg and (B5) 30 μg of the rabbit reticulocyte protein fraction eluted out with 0.5 M KCl, 25 mM Tris-HCl (pH 7.6); (B6) 5 μl of the rabbit reticulocyte postribosomal supernatant.

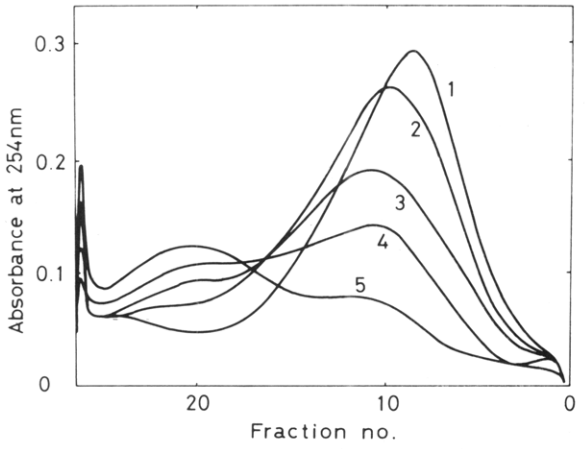

FIGURE 3: The effect of rabbit reticulocyte PASP on the sedimentation pattern of poly(A) in a sucrose density gradient. Each reaction mixture of 200 μl contained 10 μg of poly(A) and (2) 10 μg, (3) 20 μg, (4) 30 μg, (5) 50 μg each of the PASP from rabbit reticulocyte in 10 mM Tris-HCl (pH 7.6), 80 mM KCl, 3.1 mM $MgCl_2$. Line 1 represents 10 μg of poly(A) alone in the same buffer. The mixture was incubated at 30 °C for 4 min, then layered on top of the 10–30% linear sucrose gradient containing the same buffer. Centrifugation was performed in a Spinco SW 50.1 rotor at 50 000 rpm for 7.0 h at 2 °C. The gradients were fractionated with absorbance at 254 nm continuously monitored with the ISCO Density Gradient Fractionator Model 640 with a 10-mm optical path length cell. Sedimentation was from left to right.

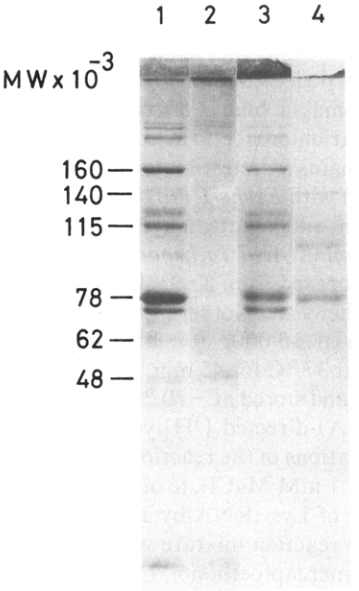

FIGURE 4: Sodium dodecyl sulfate-containing gel electrophoresis of poly(A)–PASP complex. Rabbit reticulocyte PASP (20 μg) and poly(A) (10 μg) were mixed, incubated, and centrifuged in the same condition as in line 3 of Figure 3. The centrifuged gradient was fractionated, and pooled fractions from number 1–6 and those from 10–20, respectively, were combined and lyophilized after dialysis against water. Slab gel electrophoresis was carried out as described under Materials and Methods. (1) 20 μg of reticulocyte PASP. (2) Pooled fractions 1–6. (3) Pooled fractions 10–20. (4) 20 μg of rabbit globin mRNP.

at the same time became more heterogeneous with respect to its size distribution after incubation with PASP. The extent of the changes depended on the mixing ratio of the protein to poly(A). When ten times as much protein by weight as poly(A) was added, virtually all the polynucleotide sedimented to the bottom of the tube upon centrifugation. The binding of the PASP is not, however, fully specific for poly(A). Similar binding was also observed under the same conditions with poly(U) and with poly(C). Binding of poly(G) was unascertained, since it does not dissolve reproducibly in the buffer solution (data not shown). The PASP fraction may contain impurities with affinity for the polysaccharide matrix rather than for poly(A) itself. In order to examine whether all of the components in the PASP preparation are active in binding poly(A), the poly(A)–PASP complex was recovered and its protein composition was analyzed. In an experiment identical to curve 3 of Figure 3, pools of fractions 1–6 and of fractions 10–20 were lyophilized following dialysis against distilled water. The protein compositions of the pools were compared in Figure 4. The heavier fractions 10–20 (slot 3) containing the poly(A)–PASP complex showed almost the same composition as uncentrifuged PASP (slot 1), except that the 48 000-dalton band was missing. The 48 000-dalton protein was found in the lighter fractions 1–6 (slot 2) with some weaker bands of around 200 000 daltons. This indicates that virtually all components were actively bound to poly(A) except the 48 000-dalton protein, which might have been denatured after the elution from the column.

For the standard binding assay, an equal amount by weight of PASP was mixed with polynucleotides. Ribosomal RNAs from rabbit reticulocyte were incapable of binding PASP according to the sedimentation assay, as shown in Figure 5. The sedimentation rate of the RNA from bacteriophage R17 increased slightly following incubation with PASP (Figure 5C). The sharpness of the peak remained essentially unchanged. This small increase, estimated to be about 3S by comparison with standards of 28S and 18S ribosomal RNAs, did not change with addition of PASP in up to five times excess. Bacteriophage R17 RNA may therefore have a limited number of available sites for PASP, saturation of which results in a fixed maximum increase in its sedimentation rate. Reticulocyte tRNA, native or denatured calf thymus DNA, and poly(dA)·poly(dT) did not bind PASP.

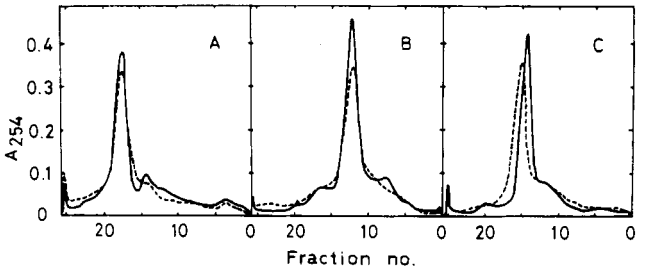

FIGURE 5: The effect of rabbit reticulocyte PASP on the sedimentation patterns of 18S and 28S rabbit reticulocyte ribosomal RNAs and bacteriophage R17 RNA. The reaction mixture (final volume 100 μl) contained: (A) 10 μg of 28S rabbit reticulocyte ribosomal RNA, (b) 10 μg of 18S rabbit reticulocyte RNA, (C) 10 μg of bacteriophage R17 RNA, each in 10 mM Tris-HCl (pH 7.6), 80 mM KCl, 3.1 mM $MgCl_2$. The RNAs were first placed in the buffer, then 10 μg of rabbit reticulocyte PASP were added (broken line). The mixture was incubated at 20 °C for 5 min and centrifuged as described in the legend to Figure 3, except that centrifugation time was 2.7 h. The solid lines represent the control mixture in which the added PASP solution was replaced by buffer.

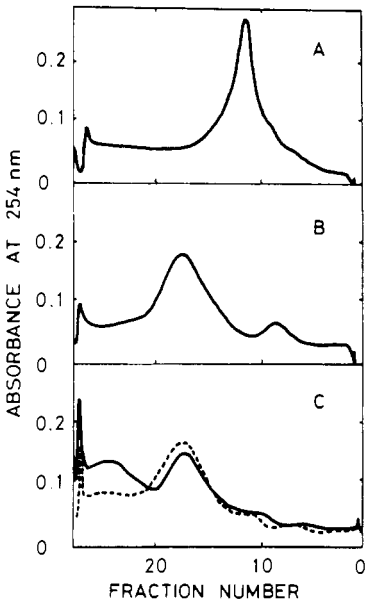

FIGURE 6: The effect of PASP on the sedimentation pattern of rabbit globin mRNA. 100 μl of solution layered on the gradients contained: (A) 10 μg of rabbit globin mRNA, (B) 20 μg of native rabbit globin mRNP, (C) 10 μg of rabbit globin mRNA, and 10 μg of either rabbit reticulocyte PASP (solid line) or rat liver (broken line). The mRNAs were first placed in 10 mM Tris-HCl (pH 7.6), 80 mM KCl, 3.1 mM $MgCl_2$, then the PASP preparations in the same buffer were added. The mixture was incubated at 20 °C for 5 min and centrifuged as in Figure 4.

When rabbit reticulocyte PASP was combined with globin mRNA in the same medium as that used in the Krebs II cell-free system (see Materials and Methods), a complex that sedimented in exactly the same position (16S) as natural globin mRNP (Figure 6B) was produced. A smaller peak was observed at around 22S but was not identified. This minor component was almost unobservable when PASP from rat liver was substituted for that from rabbit reticulocyte. The "hybrid" complex of rat liver PASP on rabbit globin mRNA was found also to have exactly the same sedimentation value as natural rabbit globin mRNP (Figure 6C). In Figure 5, the sedimentation patterns of the RNAs were essentially unchanged even after incubation with PASP. No small RNA fragments were produced by mixing PASP in Figure 6C. Contamination of PASP by ribonuclease, if any, was therefore negligible.

The Effect of PASP on the Translation of mRNA. A cell-free system prepared from Krebs II ascites tumor was used to study the effect of PASP on translation. Poly(A)-directed binding of lysyl-tRNA onto ribosomes was inhibited by the addition of PASP as shown in Figure 7A. The observed radioactivity represents the binding of both lysyl- and polylysyl-tRNA on ribosomes. PASPs from rabbit reticulocyte and rat liver showed the same inhibitory effect on the translation of poly(A).

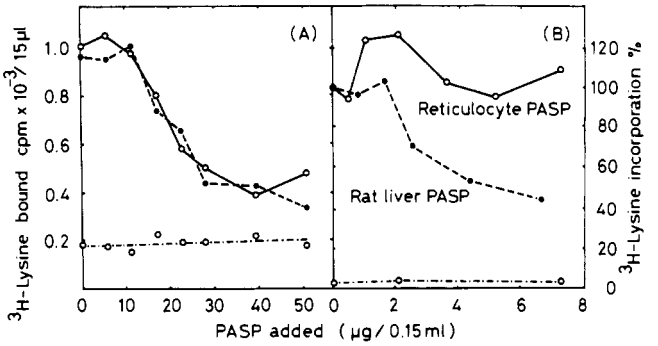

FIGURE 7: The effect of PASP on translation in the Krebs II ascites tumor cell-free system. (A) Poly(A)-directed [^{3}H]lysine incorporation on ribosomes. (B) Rabbit globin mRNA-directed [^{3}H]lysine incorporation into hot trichloroacetic acid-insoluble fraction. (A) In a volume of 100 μl, poly(A) (10 μg) was first incubated with varying amounts of rabbit reticulocyte PASP (O—O) or rat liver PASP (●- - -●) as in Figure 3. The reaction mixture for the assay of [^{3}H]lysine binding was prepared by adding components in concentrations as specified under Materials and Methods (final volume 150 μl). The reaction was started by the addition of preincubated S-30 followed immediately by incubation at 35 °C for 10 min. The dotted line (O - - - O) represents the background level without the addition of poly(A). (B) Rabbit globin mRNA was incubated with rabbit reticulocyte PASP (O—O) or rat liver PASP (● - - - ●) as in Figure 6. mRNA (2.0 μg) in 100 μl of the chilled mixture was then supplemented with the components for assay of globin synthesis. From the final volume of 150 μl, 15-μl aliquots were taken up after incubation for 30 min at 35 °C and submitted to the assay for hot trichloroacetic acid-soluble radioactivity. Radioactivity of about 3000 cpm, which was the amount incorporated without the addition of PASP, was taken at 100% on the figure. The dotted line (O - - - O) represents the background level without the addition of globin mRNA.

In Figure 7B, rabbit globin mRNA was incubated briefly with PASP as in Figure 6, and other premixed components were then added in order to obtain globin synthesis. The radioactivity incorporated for 30 min at 35 °C is accounted for almost solely by globin chain translation, since background (measured without the addition of mRNA) was just a few percent of total counts. A significant difference in the effect of adding PASP was revealed between proteins from rabbit reticulocyte and rat liver. The former brought about no remarkable change in translation of the message except 10–20% stimulation at a weight ratio of PASP to mRNA of about unity, an effect reproduced in four experiments. Further addition of reticulocyte PASP did not change the amount of globin synthesis significantly. In contrast, incorporation of amino acids was reduced by about one-half by the presence of rat liver PASP at a weight concentration three to four times that of mRNA. Specific inhibition of α-chain synthesis by rat liver PASP accounted for this decrease. Figure 8 represents the chromatographic separation of α and β rabbit globin chains synthesized in vitro. Synthesis of both chains was increased slightly by the addition of rabbit reticulocyte PASP, as in the experiment of Figure 7B. On the other hand, the addition of rat liver PASP resulted in a marked reduction of α-chain production to as low as 40% of normal. At the same time the amount of β chain translated was normal or slightly elevated. This unequal sensitivity to rat liver protein converted the ratio

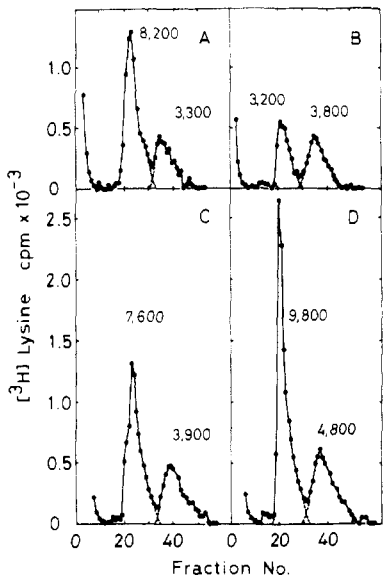

FIGURE 8: In vitro synthesis of α- and β-globin chains in the presence of globin mRNA–PASP complexes. Rabbit globin mRNA (2.0 μg) was mixed with 2.0 μg of (B) rat liver PASP or (D) rabbit reticulocyte PASP, as in Figure 6. Globin synthesis was assayed as in Figure 7. Figures A and C represent control experiments (without addition of PASP) of B and D, respectively. The products of translation were analyzed as described under Materials and Methods. The numbers in the Figures are total radioactivities of the fractions containing α- and β-globin chains.

of α- to β-chain production from 2.5 to 0.86, whereas reticulocyte PASP caused no significant change (1.9 to 2.1) in this ratio.

Lodish and Nathans (1972) reported that globin chain synthesis was selectively inhibited by homopolynucleotides or by changes in Mg^{2+} concentration; however, in the present experiments, the ratio A_{260}/A_{280} of rat liver PASP was no higher than that of the reticulocyte protein, i.e., lower than 0.8. From the absorptions of poly(A) ($E_{260}^{1\%} = 227$, $E_{280}^{1\%} = 63$, at pH 7.6) and protein in general ($E_{260}^{1\%} = 5 \pm 2.5$, $E_{280}^{1\%} = 10 \pm 5$), possible contamination of the rat liver PASP by poly(A) from the affinity column was estimated to be less than 2% by weight. Poly(A) in PASP may, therefore, contaminate the in vitro translation system by a maximum of 1–1.5 μg/ml. This concentration is less than one-tenth of the reported level for inhibition by polynucleotides (one-half inhibition at 20–50 nmol of phosphorus/ml). The dialyzing buffer solution from the last step of rat liver PASP preparation was substituted for rat liver PASP in the control experiment.

Discussion

The experiments presented in this study provide evidence that proteins having a strong affinity for poly(A) are present in postribosomal supernatants of rabbit reticulocyte and rat liver. These proteins were released from the poly(A)–Sepharose column with 2 M LiCl, 1 M urea, 50 mM Tris-HCl (pH 7.6) after the column was washed with a buffer containing 0.5 M KCl. Schweiger and Mazur (1974, 1975) prepared proteins with high affinity for poly(A) from rat liver cytoplasm under different chromatographic conditions and studied the composition of these proteins. The rat liver proteins consisted of several polypeptides that included a 78 000-dalton band as indicated by sodium dodecyl sulfate-containing gel electrophoresis. PASP obtained in this study was also heterogeneous, showing about ten protein bands by electrophoresis. In contrast, poly(A)-affinity protein from HeLa cell ribosomal wash (Blanchard et al., 1974) is released from poly(A)–Sepharose with a buffer containing 0.2 M NaCl, and shows a single band of molecular weight 38 000 by sodium dodecyl sulfate gel electrophoresis. Morel et al. (1973) reported that a mRNP complex from duck reticulocyte remains undissociated even in 0.5 M LiCl. Rabbit globin mRNP was reported also to be stable in 0.5 M KCl (Blobel, 1972). The binding of our proteins to poly(A)–Sepharose is similarly stable. The ten-banded electrophoretic pattern of our rabbit globin mRNP (Figure 2, slots A1 and B1) resembles that of globin mRNP from duck reticulocyte (Morel et al., 1973). Five of about nine observed bands in our rabbit reticulocyte PASP preparation corresponded well to globin mRNP bands, as did four out of eight bands in the rat liver protein. One of these bands corresponds to the 78 000 molecular weight polypeptide that occurs as a major component in eukaryotic cells (Blobel, 1973; Bryan and Hayashi, 1973). Partial homology between PASPs and the protein components of globin mRNP is suggested by these results.

The starting material used in the present affinity chromatography, namely the postribosomal supernatant, is known to contain a fraction of subribosomal mRNA in a form of mRNP complex (Jacobs-Lorena and Baglioni, 1972; Gander et al., 1973; Bag and Sarkar, 1973). The poly(A)–Sepharose purified proteins may, therefore, result from the protein components of this complex, being dissociated from the mRNA during the chromatographic process. However, our PASP fractions seem to be free of mRNA because the absorbance ratio of A_{260}/A_{280} is lower than 0.8 (Materials and Methods), and because no amino acid was incorporated in a Krebs II cell-free system upon addition of the PASP (Figure 6).

When PASP was mixed with RNAs, some RNAs formed complexes with the proteins as observed in sedimentation experiments (Figures, 3, 5, 6). The specificity with which these proteins bound to RNA seemed not to be high. Globin mRNA and R17 bacteriophage RNA bound reticulocyte PASP, but neither ribosomal RNAs, tRNA, nor DNA bound the proteins. Complexes were formed also with poly(U) or poly(C), as well as poly(A). Proteins thus distinguished some RNAs and polynucleotides; however, the structural or conformational basis for binding was not evident. Dubochet et al. (1973) showed by electron microscopy that the proteins in a natural globin mRNP complex were bound along the whole mRNA strand rather than on only one end. The poly(A) part of the mRNA is therefore only one of the several sites to which the proteins attach. The low-binding specificity of PASP is thus consistent with this previously observed behavior of mRNP. The synthetic complex from globin mRNA and PASP sedimented at the same rate as a natural globin mRNP. Though the results suggested stoichiometric association, another heavier and unidentified complex was produced in the case of rabbit reticulocyte PASP (Figure 6C). Therefore, in order to produce complexes of more natural structure, more moderate binding conditions, e.g., dialysis of the mixture of PASP and mRNA from high salt–urea solution, might have been more appropriate but was not attempted in the present study. An earlier report that natural globin mRNP binds more efficiently than free mRNA to deoxycholate-washed ribosomal subunits (Lebleu et al., 1971), implied that the protein moiety influenced globin translation under certain conditions. Evidence was not presented, however, to prove direct involvement of the protein moiety in the control of globin translation. Globin mRNP stimulated amino acid incorporation with essentially the same efficiency as free mRNA in a cell-free system from mouse liver (Sampson et al., 1972). Our present study has

provided data relevant to this problem. Addition of PASP to globin nRNA had markedly different effects on the in vitro translation of the message, depending on the origin of the PASP. The effect of homologous protein was less pronounced in that both α- and β-chain translation increased about 15% over the translation by free mRNA. On the other hand, heterologous protein from rat liver combined with rabbit globin mRNA to form a hybrid mRNP that directed α- and β-chain synthesis unequally. While β-chain translation was normal or slightly enhanced, α-chain translation was lowered to 50% or less. This dissimilar action of the respective PASP on the synthesis of the two chains suggests that PASP may be involved in one of the mechanisms that control the translation of globin. As reported on the protein constituents of mRNP from *Tenebrio* (Ilan and Ilan, 1973), some of the protein components in PASP could be identical with the initiation factors of translation. The results of centrifugation experiments (Figure 5) also suggested that both α-chain mRNA and β-chain mRNA bound PASP, and essentially no free mRNA remained in the presence of an equivalent amount of PASP. Therefore, the differential effect of the protein operates after it binds to globin mRNA. Whether all of the components in the PASP fraction bind to globin mRNA and whether or not their binding site on the mRNA is limited to the poly(A) part of the mRNA remain to be determined.

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References

Bag, J., and Sarkar, S. (1975), *Biochemistry 14,* 3800–3807.

Bakay, B. (1975), *Anal. Biochem. 63,* 87–98.

Blanchard, J. M., Brissac, C., and Jeanteur, P. H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1882–1886.

Blobel, G. (1972), *Biochem. Biophys. Res. Commun. 47,* 88–95.

Blobel, G. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 924–928.

Brawerman, G., Mendecki, J., and Lee, S. Y. (1972), *Biochemistry 11,* 637–641.

Bryan, R. N., and Hayashi, M. (1973), *Nature (London), New Biol. 244,* 271–274.

Cuatrecasas, P., and Anfinsen, C. B. (1971), *Methods Enzymol. 22,* 345–378.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci., 121,* 404–427.

Dintzis, H. M. (1961), *Proc. Natl. Acad. Sci. U.S.A. 47,* 247–261.

Dubochet, J., Morel, C., Lebleu, B., and Herzberg, M. (1973), *Eur. J. Biochem., 36,* 465–472.

Evans, G. A., and Rosenfeld, M. G. (1975), *Biochem. Biophys. Acta 390,* 342–351.

Fukami, H., and Imahori, K. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 570–573.

Gander, E. S., Stewart, A. G., Morel, C. M., and Scherrer, K. (1973), *Eur. J. Biochem. 38,* 443–452.

Gesteland, R. F., and Boedtker, H. (1964), *J. Mol. Biol. 8,* 496–507.

Huez, G., Burny, A., Marbaix, G., and Lebleu, B. (1967), *Biochim. Biophys. Acta 145,* 629–636.

Huez, G., Marbaix, G., Hubert, E., Leclercq, M., Nudel, U., Soreq, H., Salomon, R., Lebleu, B., Revel, M., and Littauer, U. Z., (1974), *Proc. Natl. Acad. U.S.A. 71,* 3143–3146.

Ilan, J., and Ilan, J. (1973), *Nature (London), New Biol. 241,* 176–180.

Jacobs-Lorena, M., and Baglioni, C. (1972), *Proc. Natl. Acad. U.S.A. 69,* 1425–1428.

Kumar, A., and Lindberg, U. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 681–685.

Kwan, S-W., and Brawerman, G. (1972), *Proc. Natl. Acad. Sci. U.S.A., 69,* 3247–3250.

Lane, C. D., Marbaix, G., and Gurdon, J. B. (1971), *J. Mol. Biol. 61,* 73–91.

Lebleu, B., Marbaix, G., Huez, G., Temmerman, J., Burny, A., and Chantrenne, H. (1971), *Eur. J. Biochem. 19,* 264–269.

Lim, L., and Canellakis, E. S. (1970), *Nature (London) 227,* 710–712.

Lodish, H. F., and Nathans, D. G. (1972), *J. Biol. Chem. 247,* 7822–7829.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.

Mathews, M. B., and Korner, A. (1970), *Eur. J. Biochem. 17,* 328–338.

Mendecki, J., Lee, S. Y., and Brawerman, G. (1972), *Biochemistry 11,* 792–798.

Morel, C., Gander, E. S., Herzberg, M., Dubochet, J., and Scherrer, K. (1973), *Eur. J. Biochem. 36,* 455–464.

Nathans, D. (1968), *Methods Enzymol. 12B,* 787–791.

Nudel, U., Lebleu, B., Zehavi-Willner, T., and Revel, M. (1973), *Eur. J. Biochem. 33,* 314–322.

Olsnes, S. (1971), *Eur. J. Biochem. 23,* 557–563.

Poonian, M. S., Schlabach, A. J., and Weissbach, A. (1971), *Biochemistry 10,* 424–427.

Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1958), *Biochim. Biophys. Acta 30,* 608–615.

Sampson, J., Mathews, M. B., Osborn, M., and Borghetti, A. F. (1972), *Biochemistry 11,* 3636–3640.

Sheiness, D., and Darnell, J. E. (1973), *Nature (London) New Biol. 241,* 265–268.

Schweiger, A., and Mazur, G. (1974), *FEBS Lett. 46,* 255–259.

Schweiger, A., and Mazur, G. (1975), *FEBS Lett. 60,* 114–118.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406–4412.

Williamson, R., Crossley, J., and Humphries, S. (1974), *Biochemistry 13,* 703–707.