Isolation of Hen Oviduct Ovalbumin and Rat Liver Albumin Polysomes by Indirect Immunoprecipitation*

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SUMMARY

The polyribosomes synthesizing ovalbumin and albumin have been isolated from total oviduct polysomes and total rat liver polysomes, respectively. The isolation was performed using an indirect immunoprecipitation technique which is based on the specific reaction which occurs between antibody against a purified native protein and the nascent peptide chains on polyribosomes synthesizing that protein. A soluble antibody-nascent chain-polyribosome complex is formed by incubating antibody with polyribosomes, and then precipitated by reaction with an anti-antibody.

The techniques developed are both efficient and highly specific. Near quantitative isolation of polysomal mRNA coding for a specific protein may be achieved. Indirect immunoprecipitation results in nonspecific precipitation of less than 0.5% of total polyribosomes. Ovalbumin mRNA isolated by indirect immunoprecipitation is 99% pure with respect to contamination by other species of mRNA and rat liver albumin mRNA is greater than 95% pure. Evidence supporting these conclusions includes: (a) incubation of oviduct polyribosomes containing labeled nascent peptide chains with anti-bovine serum albumin results in precipitation of only 0.4% of the labeled polyribosomes; (b) indirect immunoprecipitation from oviduct polyribosomes reacted with anti-ovalbumin contain essentially no nascent peptide chains larger than ovalbumin; (c) ovalbumin and albumin mRNAs extracted from immunoprecipitates are enriched for the synthesis of their respective proteins by 1.8- and 8.7-fold, exactly the degree of purification predicted from their relative rates of synthesis; (d) isolation of albumin synthesizing polyribosomes from a mixture of rat liver polyribosomes and hen oviduct polyribosomes resulted in nonspecific precipitation of only 0.4% of the ovalbumin synthesizing polyribosomes when the immunoprecipitated RNA was assayed for its ability to synthesize ovalbumin in vitro.

Indirect immunoprecipitation appears to be a general method widely applicable to the separation and isolation of polyribosomes and messenger RNAs coding for specific proteins.

A major approach to the investigation of differential gene expression and regulation of protein synthesis in animal cells is through the study of messenger RNA. Recently, intense efforts have centered on the isolation, characterization, and quantitation of the mRNAs coding for specific proteins. The mRNAs for several proteins including, hemoglobin (1, 2), myosin (3), immunoglobulin light chains (4, 5), ovalbumin (6, 7), histones (8–10), α crystallines (11), albumin (12), and silk fibroin (13) have been identified and in some cases partially characterized. Most of these mRNAs code for proteins which represent a major fraction of cell protein synthesis. They have usually been separated from other mRNAs by fractionation on the basis of size. However, most mRNAs, especially those which are not unique in size or code for proteins representing a small percentage of cell protein synthesis, cannot be isolated free of contaminating mRNAs by size fractionation.

We have developed an immunological approach based on the ability of antibody prepared against native protein to react with nascent peptide chains in polyribosomes. Our previous studies have demonstrated that labeled anti-ovalbumin and anti-albumin bind specifically to their respective polyribosomes (14, 15). Ovalbumin-synthesizing polyribosomes may be precipitated by sequential addition of ovalbumin and anti-ovalbumin (16) or by immunoadsorption to a cross-linked ovalbumin matrix (17). The three primary considerations which have emerged during development of these immunological methods of polyribosome isolation are: (a) the amount of nonspecific contamination; (b) the yield of isolated polyribosomes; (c) the amount of antigen and antibody required. Direct precipitation and immunoadsorption both require large amounts of pure protein antigen which is difficult to obtain in most cases. Direct precipitation requires a large excess of antibody and produces a large precipitate increasing the possibility of extensive contamination by adsorbed polyribosomes. Only about one-third of the available polyribosomes were isolated by immunoadsorption (17). This paper describes a polyribosome precipitation method based on indirect immunoprecipitation which overcomes these problems. Antibody against the purified native protein is reacted with polyribosomes and
that the antibody-nascent chain-polyribosome complex is precipitated with an anti-antibody. The small amount of antigen required and extremely high specificity and yield should make this method widely applicable to cell and viral mRNAs coding for the many important proteins which constitute less than 20% of cell protein synthesis.

MATERIALS AND METHODS

Materials—Chemicals were obtained from the sources indicated: ovalbumin, five times recrystallized, Nutritional Biochemicals; bovine serum albumin, Miles Laboratories; heparin, Triton X-100, and rat liver serum albumin, Sigma; sodium deoxycholate, sucrose, ribonuclease-free, Schwarz-Mann; [3H]leucine, New England Nuclear; cyanogen bromide Sepharose, Pharmacia; sodium dodecyl sulfate, BDH Chemicals Ltd.; Freund’s adjuvant, Difeo. Ovalbumin and albumin were further purified as described (12, 18).

Animals—White Leghorn laying hens were obtained from Kimberly Farms (Fremont, Calif.). Female New Zealand rabbits, 2 to 3 kg, were used for preparation of antibodies and reticulocyte lysate. Male neutered goats were used for preparation of goat anti-rabbit antibody. Male, 150 to 200 g, Sprague-Dawley rats were used.

Polyribosome Preparation—Oviduct and rat liver polyribosomes were prepared as described previously (12, 18). Polyribosomes were used immediately or stored in liquid nitrogen.

Preparation of Antibodies—Antibodies against ovalbumin, rat liver albumin, and bovine serum albumin were prepared as described previously (12, 14, 18). Goat anti-rabbit antibody was prepared by intramuscular injection of 5 mg of a partially purified ammonium sulfate fraction (18) of rabbit y-globulin in complete Freund’s adjuvant. Three injections at 2-week intervals were administered and y-globulin fraction prepared as described (14).

Purification of Anti-Ov and Anti-RSA Antibodies by Affinity Chromatography—Ovalbumin-Sepharose was prepared by addition of 20 mg of ovalbumin (5 mg per ml) in 0.1 M sodium carbonate, pH 9.0, to 1 g of washed cyanogen bromide Sepharose. The mixture was stirred gently for 1 hour and left overnight at 4°C. Sepharose was sedimented by centrifugation and 50 ml of 1 M ethanamine in pH 8.0 sodium carbonate were added per g of Sepharose. The mixture was left 4 to 6 hours at 4°C to allow complete reaction with remaining CNBr-Sepharose. After sedimentation and washing twice with 0.01 M sodium phosphate, pH 7.2, containing 0.01 M sodium chloride, the ovalbumin-Sepharose was poured into a small column (1.5 X 4 X 6 cm). To remove absorbed antibody, but not ovalbumin, the column was washed successively with 50 ml of 0.1 M sodium acetate, pH 4.8, containing 1.0 M NaCl; then with 50 ml of 0.1 M sodium carbonate, pH 7.6, containing 1.0 M NaCl. After three washing cycles, the column was equilibrated with 50 ml of the above phosphate buffer and the antibody applied. The antibody remained in contact with the ovulumin-Sepharose for at least 30 min. Bound antibody was visible as an opaque white band. The column was washed with 50 ml of the above phosphate buffer followed by three washes with the above acetate and carbonate buffers. Antibody was eluted either with 4.5 M MgCl2 or with 0.1 M glycine, pH 2.8. Anti-RSA was purified by the same procedure. Purified antibodies were made ribonuclease-free by one or two passages through sterile columns of 3 ml of CM-cellulose over 3 ml of DEAE-cellulose (14).

Determination of Relative Proportion of Albumin Synthesis—Male 150-g Sprague-Dawley rats were injected intraperitoneally with 500 μCi of [3H]leucine (specific radioactivity 43 Ci per mmole) and killed after 10 min. Livers were excised and homogenized in 3 volumes of 0.25 M sucrose, and Triton X-100 and sodium deoxycholate were added to a final concentration of 0.1%. The relative proportion of albumin synthesis was determined by precipitation of labeled albumin with anti-RSA (12, 19). Iso-protein incorporation into total protein was measured in a trichloroacetic acid precipitate (19). Both immunoprecipitates and trichloroacetic acid precipitates were treated as described for reticulocyte lysate.

1 The abbreviations used are: anti-Ov, antibody against ovalbumin; BSA, bovine serum albumin; anti-BSA, antibody against bovine serum albumin; DOC, deoxycholate; Ov, ovalbumin, RSA, rat liver serum albumin; anti-RSA, antibody against rat liver serum albumin.

The observed rate of albumin synthesis was the same whether crude homogenate or supernatant fluid (15,000 X gmax for 7 min) was used. Previous studies have demonstrated that anti-RSA precipitates only albumin from labeled liver homogenates under those conditions (12). Duplicate antibody and trichloroacetic acid precipitations were performed on the crude homogenate and 15,000 X gmax supernatant fluid from two rats and the results of the experiments averaged.

Preparation of Labeled Nascent Peptide Chains—Small explants of hen oviduct were incubated with [3H]leucine for 5 min (18). The [3H]leucine is incorporated exclusively into nascent peptide chains not completed molecules or ribosomal proteins (16).

Isolation of Ovalbumin and Albumin Synthesizing Polyribosomes—Incubation mixtures contained 10 to 20 A260 units of polyribosomes per ml of Tris, pH 7.1, 5 mM MgCl2, 150 mM NaCl, 500 μg per ml of hemin, and 100 to 125 μg of anti-Ov or anti-RSA per ml. Polyribosomes were incubated 45 min at 0°C with anti-Ov or anti-RSA. Anti-antibody (1 mg/15 μg of anti-Ov, RSA, or BSA) was then added and the incubation continued for an additional 1 hour at 0°C. The entire incubation mixture (0.4 to 5 ml) was carefully layered over a discontinuous sucrose gradient consisting of 3 ml of 0.5 M sucrose layered over 50 ml of 1.0 M sucrose, both in the above incubation buffer which contained 1% Triton X-100 and 1% sodium deoxycholate. The antibody-polyribosome complex was sedimented by centrifugation for 20 min at 16,000 X gmax in a Sorvall HB-4 rotor. The pellet was resuspended in 1 ml of the incubation buffer and sedimented through a second sucrose gradient as above. In one experiment (Table II), polyribosomes were freed of unreacted anti-Ov or anti-RSA by resolation of polyribosomes using the cushion method described previously (14). The minimum concentration of anti-Ov for precipitation of preformed antibody-polyribosome complex is 20 μg per ml. Anti-Ov (20 μg per ml) was therefore added to permit precipitation of the preformed antibody-polyribosome complex, and indirect immunoprecipitation was then carried out as described above.

Deproteinization, Ethanol Precipitation, and Washing of RNA—RNA was deproteinized with sodium dodecyl sulfate sucrose gradients, collected, and ethanol-precipitated as described previously (17). To remove impurities which frequently prevent achievement of a linear relation between the amount of RNA added and in vitro protein synthesis, some ethanol precipitates (e.g. Fig. 7 and Table II) were washed successively with 2.0 M LiCl containing 5 mM EDTA, 3 mM sodium acetate, pH 5.5, containing 5 mM EDTA and ethanol-water, 2.5:1, containing 0.15 M NaCl. This treatment solubilized heparin, transfer RNA, tRNA, and other impurities which frequently interfere with the assay of high concentrations of RNA in an in vitro protein synthesizing system.

Reticulocyte Lysate Assays—The mRNA activity of RNA preparations was measured by in vitro synthesis of albumin (12) and ovalbumin (6) in a rabbit reticulocyte lysate protein-synthesizing system. Incubations and immunological precipitations were carried out as described (19) except that no carrier leucine was used in incubations, and the antibody precipitates were pelleted through 30 μl of 0.5 M sucrose, containing 0.05 μl leucine, 1% Triton X-100, 1% sodium deoxycholate, layered over 100 μl of 1 M sucrose containing 0.05 μl leucine, 1% Triton X-100, and 1% sodium deoxycholate.

RESULTS

Purification of Anti-Ov and Anti-RSA—To minimize the size of immunoprecipitates and decrease the extent of nonspecific adsorption, the antibodies were purified by affinity chromatography on antigen-Sepharose. Anti-ovalbumin was purified 2- to 3-fold by chromatography on ovalbumin-Sepharose (Fig. 1), and anti-albumin was purified 5- to 6-fold after chromatography on albumin-Sepharose.

Determination of Optimum Conditions for Indirect Immunoprecipitation—The method developed for isolation of polyribosomes synthesizing a single protein entails: (a) incubation of polyesomes with antibody prepared against a purified protein, resulting in binding to nascent peptide chains on polyribosomes synthesizing that protein; (b) incubation of the soluble antibody-nascent
Fig. 1. Purification of rabbit anti-ovalbumin by affinity chromatography on ovalbumin-Sepharose. Rabbit γ-globulin fraction, 100 mg, was applied to a column (1.6 × 4 cm) of ovalbumin-Sepharose prepared and run as described under "Materials and Methods." Precipitation curves were obtained using constant amounts of antibody, 1900 cpm of [3H]ovalbumin (specific radioactivity 1980 cpm per μg) prepared as described previously (17), and varying amounts of antigen. Data are for the starting γ-globulin fraction (O––O), the unbound antibody combined with a phosphate buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2) wash (■––■), and the purified antibody eluted with 4.5 M MgCl₂ (○––○).

Fig. 2. Effect of antibody concentration on the indirect immunoprecipitation of [3H]leucine-labeled oviduct polysomes. A₂₆₀ units, 5.2, of oviduct polysomes with [3H]leucine-labeled nascent chains (65,000 cpm per A₂₆₀ unit) were incubated in 0.75 ml with the indicated amounts of rabbit anti-Ov (○––○) or rabbit anti-BSA (O––O) as described under "Materials and Methods," and sedimented once through the discontinuous sucrose gradient. Indirect immunoprecipitates were dissolved in 0.7 ml of NCS solubilizer (Amersham) and counted in 10 ml of a toluene-based scintillation fluid (17).

The specificity of indirect immunoprecipitation—The specificity of indirect immunoprecipitation was demonstrated by comparison of the nascent polypeptide chains associated with total polysomes and specific and nonspecific immunoprecipitates (Fig. 3). The specific immunoprecipitate contains essentially no radioactivity in polypeptides larger than ovalbumin. The nascent chains nonspecifically adsorbed to the anti-BSA-anti-antibody precipitate exhibit a pattern on sodium dodecyl sulfate acrylamide gel electrophoresis which parallels that of total nascent chains (Fig. 3), indicating that nonspecific precipitation represents random low level adsorption of labeled polysomes.

The specificity of indirect immunoprecipitates was also investigated by examination of precipitated RNA. RNA was separated from protein and heparin in precipitates by sodium dodecyl sulfate extraction and sodium dodecyl sulfate-sucrose gradient ultracentrifugation. No RNA was visible when anti-BSA was used instead of anti-Ov (Fig. 4). In vitro synthesis demonstrated that RNA extracted from the nonspecific BSA precipitate contained less than 1% of the ovalbumin synthesizing activity of RNA from anti-Ov precipitates. The precise ovalbumin synthesizing activity of nonspecific immunoprecipitates was so low it could not be determined accurately.

A direct demonstration that indirect immunoprecipitation results in separation of ovalbumin polysomes and mRNA from total polysomes was achieved by measurement of the capacity of RNA isolated by indirect immunoprecipitation to direct ovalbumin synthesis in a heterologous cell-free protein synthesizing system. The specific activity (counts of ovalbumin synthesized per μg of RNA) of RNA extracted from purified ovalbumin antibody was then added and immunoprecipitation carried out. Although resolation and subsequent dialysis resulted in a significant loss of polysomes, substantial improvement in the ratio of specific to nonspecific precipitation was achieved (Table I). Due to the complexity of this procedure, it was not employed in any of the studies described.

Resuspension of the precipitate and resedimentation through the discontinuous sucrose-detergent gradient produced little loss of specific precipitate and decreased the amount of nonspecific precipitate by almost 50% (Table I). This method was therefore employed in all subsequent experiments.

The optimum ratio of specific to nonspecific precipitation was achieved using 1 mg of anti-antibody per 15 μg of rabbit antibody and this ratio was used in all subsequent studies.

To define the reaction conditions, oviduct polysomes containing labeled nascent peptide chains were employed. The optimum concentration of anti-ovalbumin for indirect immunoprecipitation of ovalbumin polysomes is governed by two factors; the fraction of ovalbumin polysomes precipitated and the amount of nonspecific precipitation. To determine the amount of nonspecific precipitation, antibody against a non-oviduct protein (anti-BSA) was added to oviduct polysomes with labeled nascent chains and the radioactivity in indirect immunoprecipitates determined. In the experiment shown in Fig. 2, the concentration of anti-Ov or anti-BSA was varied and the radioactivity precipitated with each antibody determined. The optimum ratio of specific (anti-Ov) to nonspecific (anti-BSA) precipitation was at about 125 μg of anti-Ov per ml.

Two methods were developed to further reduce the degree of nonspecific binding. A decrease in the amount of primary antibody used and hence the size of the precipitate produces a commensurate decrease in the amount of nonspecifically adsorbed material. We therefore separated the unreacted antibody from the antibody-polysome complex by reisolation of the polysomes prior to addition of goat anti-rabbit antibody. After dialysis to remove sucrose, the minimum amount of anti-Ov (20 μg per ml) necessary for precipitation of preformed antibody-polysome complex by anti-antibody was added.

D. J. Shapiro, unpublished observations.
To examine the effect of sedimentation, [3H]leucine-labeled hen oviduct polysomes (5 A260 units; 65,000 cpm per A260 unit) were immunoprecipitated as described under “Materials and Methods.” Rabbit anti-Ov, 105 μg, was used in specific indirect immunoprecipitation and 108 μg of rabbit anti-BSA to measure nonspecific immunoprecipitation. The precipitates were either sedimented once through the detergent-containing discontinuous sucrose gradient and dissolved and counted (Fig. 2), or resuspended and sedimented again through the gradient, dissolved, and counted. In the reisolation experiment oviduct polysomes were precipitated (9.9 A260 units; 17,000 cpm per A260 unit) or first freed of unreacted rabbit antibody by reisolation (16.0 A260 units; 17,000 cpm per A260 unit) using the cushion method described previously (14). After dialysis to remove sucrose, 20 μg per ml of rabbit anti-Ov was added to permit precipitate formation and indirect immunoprecipitation carried out as described under “Materials and Methods.”

| Sample treatment                | Total polysomes precipitated | Per cent of total polysomes | Per cent of specific precipitate | Per cent of first precipitate |
|--------------------------------|-----------------------------|----------------------------|---------------------------------|-------------------------------|
| Effect of sedimentation         |                             |                            |                                 |                               |
| Rabbit anti-Ov                  |                             |                            |                                 |                               |
| Sedimented once                 | 125,000                     | 39                         |                                 | 94                            |
| Sedimented twice                | 117,000                     | 36                         |                                 |                               |
| Rabbit anti-BSA                 |                             |                            |                                 |                               |
| Sedimented once                 | 2,680                       | 0.53                       | 2.1                             |                               |
| Sedimented twice                | 1,430                       | 0.44                       | 1.1                             | 54                            |
| Effect of polysome reisolation  |                             |                            |                                 |                               |
| Rabbit anti-Ov                  |                             |                            |                                 |                               |
| Not reisolated                  | 59,100                      | 35                         |                                 |                               |
| Reisolated                      | 63,700                      | 25                         |                                 | 71                            |
| Rabbit anti-BSA                 |                             |                            |                                 |                               |
| Not reisolated                  | 573                         | 0.84                       | 0.97                            |                               |
| Reisolated                      | 481                         | 0.19                       | 0.75                            | 55                            |

* A260 units, 15, of polysomes (17,000 cpm per A260 unit) were used for reisolation while only 9.9 A260 units (also 17,000 cpm per A260 unit) were precipitated and not reisolated. The percentages in the specific and first precipitates are normalized to the total amount of polysomes (i.e. 25/35 = 71, 19/34 = 55).

polysomes should be higher than that of RNA from total polysomes. The specific ovalbumin-synthesizing activity of RNA extracted from oviduct polysomes purified by indirect immunoprecipitation was about 1.7-fold greater than that of total polysomal RNA (Fig. 5A). This is exactly the enrichment predicted since ovalbumin represents approximately 60% of hen oviduct protein synthesis (18). This experiment also demonstrates that the antibodies used are free of ribonuclease activity, since treatment of polysomes with antibody does not change the specific activity of the RNA. In addition, the sum of the ovalbumin-synthesizing activity of RNA from precipitated and nonprecipitated polysomes is about equal to that of the starting polysomes. The polysomes which did not precipitate at the concentration of anti-Ov used contained considerable ovalbumin synthesizing activity (Fig. 5A). Raising the ovalbumin antibody concentration permits the nearly quantitative isolation of ovalbumin synthesizing polysomes (Fig. 5B). About 90% of polysomal ovalbumin mRNA was recovered by indirect immunoprecipitation at the higher anti-Ov concentration (Fig. 5B).
min polysomes. This work had two aims. (a) To demonstrate the general applicability of indirect immunoprecipitation as a method for polysome isolation. Since albumin accounts for only 13% of liver protein synthesis (20), the utility of this method for a much wider range of proteins is demonstrated. Also, comparison of the increase in specific albumin synthesizing activity and that expected from its rate of synthesis provides a clear demonstration of the polysome purification achieved.

(b) To isolate albumin-synthesizing polysomes from a mixture of hen oviduct and rat liver polysomes and measure the extent of nonspecific precipitation of ovalbumin polysomes by assaying the immunoprecipitated RNA for its ability to synthesize albumin and ovalbumin in vitro.

By immunochemical precipitation of labeled albumin from rat liver homogenates, we have determined that albumin synthesis accounts for 11.3% of liver protein synthesis (see "Materials and Methods"). This is in excellent agreement with the figure of 10.9% reported by Peters and Peters (20). Separation of the polysome for a protein representing 11.3% of cell protein synthesis in the rabbit reticulocyte lysate system as described under "Materials and Methods." RNA from immunoprecipitates exhibited a 1.7-fold increase in specific ovalbumin synthesizing activity (counts per min per µg of RNA).

Fig. 5. In vitro ovalbumin synthesis by RNA extracted from indirectly immunoprecipitated oviduct polysomes. Indirect immunoprecipitates from 20 A 260 units of hen oviduct polysomes were prepared as described under "Materials and Methods." Rabbit anti-Ov, 115 µg per ml, was used in the indirect immunoprecipitation in Panel A and 290 µg per ml of rabbit anti-Ov used in the indirect immunoprecipitation in Panel B. RNA was extracted from the immunoprecipitates, applied to a 5 to 20% sucrose gradient, sedimented, collected, and ethanol-precipitated (see "Materials and Methods"). Precipitated RNA was dissolved in a small volume of water, and RNA from polysomes not treated with antibody (○—○), precipitated polysomes (●—●), polysomes that did not precipitate (▼—▼), and polysomes incubated with rabbit anti-Ov (□—□) (A) or goat anti-rabbit antibody (□—□) (B) were assayed for ovalbumin synthesis in the rabbit reticulocyte lysate system as described under "Materials and Methods." RNA from indirect immunoprecipitates exhibited a 1.7-fold increase in specific ovalbumin synthesizing activity (counts per min per µg of RNA).

The observed 8.7-fold increase in specific albumin-synthesizing activity of the precipitated RNA is in excellent agreement with the 8.8-fold increase predicted from the independently measured rate of albumin synthesis.

The ideal measurement of nonspecific precipitation would be to determine the amount of nonspecific mRNA isolated in the indirect immunoprecipitation of a specific polysome. We have approached this question by measurement of the amount of ovalbumin mRNA precipitated during the isolation of albumin polysomes from a mixture of equal amounts of hen oviduct and rat liver polysomes. Ovalbumin and albumin mRNAs can be assayed in vitro simultaneously without detectable cross-reaction. Only 0.43% of the ovalbumin synthesizing activity was precipitated (Table II). Since the oviduct polysomes which were not precipitated retained over 90% of their ovalbumin synthesizing activity (Table II) and the precipitated RNA was enriched 8.8-fold for rat liver albumin synthesis (Table II), ovalbumin mRNA could not have nonspecifically precipitated and escaped detection because of degradation during isolation. Nonspecific precipitation apparently results from random low level adsorption of polysomes (Fig. 4). Therefore, the level of nonspecific adsorption of total polysomes should be similar to the 0.4% observed for ovalbumin polysomes. The purity of the precipitated albumin mRNA can be calculated from the amount of RNA precipitated, and the fraction of the precipitate due to nonspecific adsorption of 0.4% of total polysomes. These results indicate that albumin mRNA isolated from liver polysomes is greater than 95% pure with respect to contamination by other mRNAs.1

1 Isolation of albumin-synthesizing polysomes from 100 A 260 units of total liver polysomes: nonspecific precipitation is 100 X 0.0043 = 0.43 A 260 unit. The total precipitated RNA (Fig. 7) was 8.9 A 260 units. The per cent purity is given by the amount of

Fig. 6. In vitro albumin synthesis by RNA extracted from immunoprecipitated rat liver polysomes. Indirect immunoprecipitation was performed on 100 A 260 units of rat liver polysomes, using 115 µg per ml of rabbit anti-RSA and the RNA was extracted as described under "Materials and Methods." To remove minor contaminants which interfere with in vitro protein synthesis assays at high RNA concentrations, the ethanol precipitates were washed as described under "Materials and Methods." Precipitated RNA was dissolved in a small volume of water and RNA from polysomes that were not treated with antibody (○—○), immunoprecipitated polysomes (●—●), and polysomes that did not precipitate (▼—▼) were assayed in vitro in the rabbit reticulocyte cell-free protein synthesizing system.

The observed 8.7-fold increase in specific ovalbumin synthesizing activity (counts per min) of RNA in the immunoprecipitate was 8.7-fold greater than the specific activity of RNA extracted from total rat liver polysomes (Fig. 6).

The observed 8.7-fold increase in the specific albumin-synthesizing activity of the precipitated RNA is in excellent agreement with the 8.8-fold increase predicted from the independently measured rate of albumin synthesis.

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It seems probable that polysome losses on resolation were due primarily to the small amount of polysomes used and that losses would be minimized if resiolation was used in large scale preparative immunoprecipitations. Although it was not employed in these studies, resolation of the polysomes and addition of the minimum amount of antibody required to form a small precipitate should prove useful for isolation of polysomes synthesizing proteins which represent a very small percentage of cell protein synthesis. Polysome resolation also permits use of unpurified antibodies, since the large excess of unreacted nonspecific antibody is separated from the antibody-polysome complex on resolation.

Quantitation of the degree of nonspecific binding and demonstration of the specificity of indirect immunoprecipitation were achieved by the following experiments. (a) Reaction of oviduct polysomes containing labeled nascent chains with anti-RSA results in precipitation of only 0.4% of the labeled polysomes (Table I). (b) Although oviduct polysomes possess many labeled nascent peptide chains larger than ovalbumin, indirect immunoprecipitates show essentially no nascent chains larger than ovalbumin and exhibit the pattern expected for nascent ovalbumin chains on sodium dodecyl sulfate-acylamide gel electrophoresis (Fig. 3). (c) Direct immunoprecipitates prepared with oviduct polysomes and anti-BSA contain no detectable RNA and have less than 1% the in vitro ovalbumin synthetic capacity of polysomes precipitated with anti-Ov (Fig. 4). (d) The capacity of precipitated oviduct polysomes to synthesize ovalbumin mRNA is increased by 1.7-fold (Fig. 5), exactly as expected for a protein comprising 60% of protein synthesis (1.66-fold). (e) Since ovalbumin represents 60% of ovalbumin protein synthesis, we extended these observations to rat liver albumin which represents only 11.3% of liver protein synthesis. The 8.7-fold enrichment achieved is in excellent agreement with the value of 8.8-fold predicted for the purification of a protein synthesizing a protein comprising 11.3% of cell protein synthesis. (f) Isolation of albumin-synthesizing polysomes from a mixture of hen oviduct and rat liver polysomes, followed by in vitro assay of the capacity of the isolated albumin mRNA to synthesize albumin and ovalbumin permits a direct demonstration of the specificity of indirect immunoprecipitation. In two experiments, approximately 0.4% of the ovalbumin polysomes were nonspecifically precipitated (Table II). Since nonspecific binding appears to represent uniform low level adsorption, the level of nonspecific adsorption of total ovalbumin or rat liver polysomes should be 0.4%.

Using the 0.4% level of nonspecific adsorption which was independently determined by precipitation of in vivo labeled nascent peptide chains and by in vitro translation of contaminating ovalbumin mRNA (see a and f above), the purity of the isolated ovalbumin and albumin mRNAs was calculated.³ Ovalbumin mRNA isolated by indirect immunoprecipitation is 99% pure with respect to contamination by other species of mRNA and rat liver albumin is greater than 95% pure. While it seems unlikely that mRNA or nuclear RNA which sediments with polysomes but is not being translated in vivo or in vitro contaminates our immunoprecipitates, we cannot absolutely exclude this possibility. In contrast to direct precipitation (16) and immobilized immunoadsorbents (17), indirect immunoprecipitation does not require purified antigen in the precipitation reaction and is therefore suitable for proteins which cannot be purified in large amounts. The amount of specific antibody required is very small and other antibodies may be used to elicit anti-antibody.
production. (Goat anti-rabbit antibody prepared against anti-Ov was used to precipitate the albumin antibody-polysome complex.) The precipitate produced is relatively small and the purified antibodies are free of ribonuclease activity. The yield of mRNA was 60 to 70% for ovalbumin polysomes and 70 to 80% for serum albumin polysomes compared to the 30% obtained previously by immunoadsorption of ovalbumin synthesizing polysomes (17). Nearly quantitative precipitation of ovalbumin-synthesizing polysomes may be achieved by increasing the amount of anti-Ov (Fig. 5B). However, the increase in immunoprecipitate size decreases the purity of the precipitated polysomes from 99% to about 95%. The major advantage of the indirect immunoprecipitation technique described is that the level of nonspecific precipitation (0.4%) is extremely low relative to that obtained in earlier studies. Unenoyma and Ono (21) have published on the immunoprecipitation of albumin and catalase polysomes. The extent of nonspecific contamination was not measured, however, and the isolated RNA was partially degraded. Recently Schechter (22) has reported the isolation of immunoglobulin light chains by an anti-antibody method. Although the recovery of polysomes and procedure were not described in detail, the level of nonspecific precipitation was found to be 2.0 to 2.1%. This is the same as the level of nonspecific adsorption (2.0%) on immunoadsorption of eukaryotic polysomes (17), and is about five times greater than that obtained by our techniques (0.4%). Since the yield of polysomes is roughly twice that obtained following previous immunoadsorption procedures, the RNA isolated by our indirect immunoprecipitation technique contains about 10-fold less contaminating mRNA.

The isolation of the mRNA coding for a single protein requires separation from other RNAs and from rRNA. Separation of these RNAs from RNA is based on the occurrence of a polyadenylate sequence at the 3' end of almost all eukaryotic mRNAs (23-25). The polyadenylate sequence binds to cellulose (26) and nitrocellulose filters (27) and hybridizes with oligo(dT) coupled to cellulose (28, 29) or poly(U) coupled to agarose or cellulose (30). Both ovalbumin and albumin mRNAs appear to contain poly(A) sequences since they can be separated from rRNA by binding to nitrocellulose filters (12, 17) and oligo(dT) cellulose (12).

Ovalbumin mRNA purified by immunoadsorption and separated from most rRNA by binding to nitrocellulose filters has been successfully used as a template for the synthesis of a complementary DNA using Rous sarcoma virus RNA-dependent DNA polymerase (31). Rat liver albumin mRNA, here separated from other liver mRNAs for the first time, will be used as template for the synthesis of a complementary DNA sequence.

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