Isolation of Rat Liver Albumin Messenger RNA*

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Rat liver albumin messenger RNA has been purified to apparent homogeneity by means of polysome immunoprecipitation and poly(U)-Sepharose affinity chromatography. Specific polysomes synthesizing albumin were separated from total liver polysomes through a double antibody technique which allowed isolation of a specific immunoprecipitate. The albumin-polysome immunoprecipitate was dissolved in detergent and the polysomal RNA was separated from protein by sucrose gradient centrifugation. Albumin mRNA was then separated from ribosomal RNA by affinity chromatography through the binding of poly(U)-Sepharose to the polyadenylate 3' terminus of the mRNA.

Pure albumin mRNA migrated as an 18 S peak on 85% formamide-containing linear sucrose gradients and as a 22 S peak on 2.5% polyacrylamide gels in sodium dodecyl sulfate. It coded for the translation of authentic liver albumin when added to a heterologous protein-synthesizing cell-free system derived from either rabbit reticulocyte lysates or wheat germ extracts. Translation analysis in reticulocyte lysates indicated that albumin polysomes were purified approximately 9-fold from total liver polysomes, and that albumin mRNA was purified approximately 74-fold from albumin polysomal RNA. The total translation product in the mRNA-dependent wheat germ system, upon addition of the pure mRNA, was identified as authentic albumin by means of gel electrophoresis and tryptic peptide chromatography.

The study of the regulatory mechanisms in eukaryotic gene expression would be greatly facilitated by the isolation of specific messenger RNAs. A purified mRNA could be examined in structure-function studies and used for the preparation of a complementary DNA hybridization probe for quantitative analyses and gene isolation.

Three different experimental approaches to mRNA purification have been employed. The unique physical and chemical properties of certain mRNA molecules can be exploited, as in the purification of the guanine-rich silk fibroin mRNA by means of density gradient centrifugation and size fractionation (1). Size fractionation on linear sucrose gradients has also been utilized for the purification of several mRNAs such as that for hemoglobin (2-5), myosin (6), immunoglobulins (7-10), ovalbumin (11, 12), and histones (13-15). These mRNA species frequently represent a major fraction of their cellular mRNA population, which has facilitated isolation. A different approach to the purification of individual mRNAs involves antibody techniques, which achieve the immunoprecipitation of specific polysomes synthesizing a single protein. These immunological methods have been employed in the isolation of the mRNAs for chicken ovalbumin (16, 17), mouse myeloma immunoglobulin light chain (18, 19), chicken histone V (20), and ewe casein (21).

This report describes the isolation of specific albumin-synthesizing polysomes from rat liver by immunoprecipitation and the purification of albumin mRNA by affinity chromatography with poly(U)-Sepharose. The isolation of albumin-synthesizing polysomes involves the incubation of liver polysomes with an antibody prepared against native albumin, which binds to the nascent albumin peptide chains on the ribosomes. This reaction is followed by the incubation of the polyclonal antibody complex with a second antibody, which has been prepared against the first antibody (an anti-antibody). The polysome-antibody-anti-antibody complex is then sedimented through a discontinuous sucrose gradient to remove unreacted polysomes and unreacted antibody. The immunoprecipitated pellet is then dissolved in detergent, the polysomal RNA is isolated by gradient centrifugation, and the mRNA is purified with poly(U)-Sepharose.

The isolated albumin mRNA has been shown to be homogeneous by gel electrophoresis, sucrose gradient centrifugation, and translation in a mRNA-dependent cell-free protein-synthesizing system derived from wheat germ extracts. In previous work, we have described the specific binding of albumin antibody to rat liver polysomes through an immunological recognition of nascent albumin peptide chains (22). The specificity of the polysome immunoprecipitation technique has also been examined (23).

EXPERIMENTAL PROCEDURES

Animals—Male albino Sprague-Dawley rats, weighing from 150 to 200 g each, were used. They were fed water and Purina rat chow ad libitum.

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To a column (6.8 x 1 cm) of poly(U)-Sepharose (Pharmacia), equilibrated in the above final buffer, at a flow rate of 20 ml/h. The column was washed with 20 ml of the above buffer, followed by 20 ml of the same buffer minus the added NaCl. The mRNA was eluted with 70% formamide (vacuum redistilled) containing 1 mm Hesper, pH 7.2, and 2 mm EDTA. The eluate was adjusted to 0.2 mm NaCl and 2.5 volumes of ethanol, vortexed, precipitated overnight at -20°C, and collected by centrifugation (16). The mRNA eluate, as prepared here, was usually repassed over a second poly(U)-Sepharose column to remove trace contamination by RNA, which was occasionally observed after only one pass through the column.

Cell-free Protein Synthesis in Reticulocyte Lysates — The albumin mRNA activity of various RNA preparations was assayed in vitro in a rabbit reticulocyte lysate protein-synthesizing system as described (24). The albumin translation product, labeled with [3H]leucine, was measured immunologically with the previously characterized goat anti-albumin (24).

Cell-free Protein Synthesis in Wheat Germ Extracts — Wheat germ extracts were prepared and the translation assay was used as described by Roberts and Paterson (27). The 27,000 x g supernatant fluid was preincubated for 15 min at 30°C and stored as frozen beads in liquid nitrogen. The assay system (27) was supplemented with 40 mm spermine, and [3H]leucine was used to label the translation product. Purified mRNA at 1 to 5 µg/ml was incubated for 2 h at 25°C. Following the incubation, ribosomes were removed by centrifugation at 134,000 x g for 60 min at 4°C. The postribosomal supernatant fluid was then treated with RNase A and EDTA, dialysed overnight to remove free [3H]leucine, and examined by polyacrylamide gel electrophoresis as described (11). The capacity of the wheat germ protein-synthesizing system to translate albumin mRNA has been examined in detail (7). The assay, as used here, was found to be dependent on the addition of exogenous mRNA, resulting in a 25- to 50-fold stimulation of total radioactivity incorporated into acid-precipitable protein when compared to a control reaction without added mRNA.

Polyacrylamide Gel Electrophoresis — RNA electrophoresis was performed with 5% polyacrylamide gels in 50 mm Tris, 29 mm NaOAc, 2 mm EDTA, and 0.1% sodium dodecyl sulfate at pH 7.4. Gels (0.6 x 10 cm) underwent pre-electrophoresis in this buffer for 1 h before use. RNA samples were dissolved in 1% sodium dodecyl sulfate containing 5 mm EDTA, 25 mm Tris at pH 7.4, and 10% glycerol. Solutions containing 1 to 15 µg of RNA in 25 to 100 µl were layered on the gels. Electrophoresis was performed for 4 h at 100 V. Gels were scanned with a Gilford recording spectrophotometer at 260 nm with a 0.1-mm slit width.

Protein or polysome immunoprecipitates were dissolved in Buffer A (1% sodium dodecyl sulfate, 1% dithiothreitol, and 50 mm sodium phosphate at pH 7.2). The samples were heated at 30°C for 3 min and adjusted to 10% in glycerol and approximately 0.01% bromphenol blue. The dialyzed translation products from the wheat germ protein synthesis assays were also adjusted to Buffer A conditions and treated in a similar manner. Gels (0.8 x 9 cm) underwent pre-electrophoresis for 1 h at 7.5 V/cm before use in 50 mm sodium phosphate at pH 7.2 containing 0.1% sodium dodecyl sulfate. Electrophoresis, in the same buffer, was performed for 3 to 4 h at 6 mA/gel. Gels were sliced and radioactivity was determined as previously described (24, 25).

Formamide-Sucrose Gradients — These denaturing gradient conditions were modified from the procedure of Macnaughton et al. (28). RNA samples were dissolved in 85% formamide (freshly redistilled under vacuum) containing 3 mm Hesper and 3 mm EDTA at pH 7.2 and incubated at 40°C for 20 min. Solutions of 5 to 50 µg of RNA in 360 µl of 0.3 m sucrose were layered on 15,000 to 250,000 linear gradients in 2 ml of sucrose containing 5% formamide, and 150 mm NaCl. Sodium phosphate buffer at pH 7.2 containing 0.1% sodium dodecyl sulfate. Electrophoresis, in the same buffer, was performed for 3 to 4 h at 6 mA/gel. Gels were sliced and radioactivity was determined as previously described (24, 25).

Determination of Optimum Conditions for Polysome Immunoprecipitation — The previous investigation of the binding of [3H]-anti-albumin to liver polysomes (21) indicated that a concentration of 50 µg of goat anti-albumin per ml of polysomes (containing approximately 10 A260 units) approached saturating conditions. For the second antibody, it was determined that 1 mg of rabbit anti-goat γ-
goat anti-albumin were used while the amount of second antibody per volume of 1 ml with the indicated amounts of antibodies. The immunoprecipitation volumes. In addition, polysomes were prepared from unfractionated liver homogenates (25) and from the postmitochondrial supernatant material to investigate the quantitation of albumin polysomes. Previously reported work (25) showed that in the preparation of detergent-treated postmitochondrial polysomes, substituting goat anti-chicken ovalbumin for the first antibody at 70 μg/ml resulted in less than 0.3% of the total radioactivity found in the immunoprecipitate.

The question of quantitation of specific albumin-synthesizing polysomes was investigated further by the use of a completely different antibody combination. Rabbit anti-albumin was used as the first antibody and goat anti-rabbit y-globulin was the second antibody in polysome immunoprecipitation study. The results of Fig. 2 also show that a maximum of approximately 11 to 12% of the postmitochondrial polysomes are precipitated. The agreement among these immunoprecipitation experiments supports the specificity of the reaction and suggests that this technique can be used to quantitate albumin polysomes. It can be seen that much more antibody was required in this second experiment. A comparison of standard precipitin curves (data not shown) indicated that the goat anti-rabbit antibody (Fig. 2) was less efficient than rabbit anti-goat y-globulin in per μg of first antibody.

Substituting goat anti-chicken ovalbumin for the first antibody at 70 μg/ml resulted in less than 0.3% of the total radioactivity found in the immunoprecipitate. The results of Fig. 2 also show that a maximum of approximately 11 to 12% of the postmitochondrial polysomes are precipitated. The agreement among these immunoprecipitation experiments supports the specificity of the reaction and suggests that this technique can be used to quantify albumin polysomes. It can be seen that much more antibody was required in this second experiment. A comparison of standard precipitin curves (data not shown) indicated that the goat anti-rabbit antibody (Fig. 2) was less efficient than rabbit anti-goat y-globulin (Fig. 1). In the polysome immunoprecipitation of Fig. 2, the difference in efficiency required that more of both first and second antibody be used, resulting in approximately a 4-fold greater amount of protein in the final precipitate as compared to Fig. 1. Thus, nonspecific adsorption, which was not a significant problem with the antibody combination of Fig. 1, became a problem for the antibody combination of Fig. 2. Therefore, the antibody conditions of Fig. 1 were used for the subsequent preparative isolation of albumin-synthesizing polysomes for mRNA purification.

Specificity of Polysome Immunoprecipitation — The specificity of polysome immunoprecipitation was investigated by examining the purification of albumin mRNA translational activity using a protein-synthesis assay in rabbit reticulocyte lysates (24). Albumin mRNA was found to be enriched 9-fold...
in the immunoprecipitate compared to the initial liver polysome preparation (Table I). This purification corresponds closely to the value that would be predicted based on the data presented in Figs. 1 and 2 and on the finding that albumin represents approximately 11% of the total liver protein synthesized (20). Furthermore, the observed yield of 83% of the albumin mRNA was also in the predicted range for the concentration of second antibody used in the preparative procedure as discussed above.

Purification of Albumin mRNA - The albumin-synthesizing polysomes were dissolved in 2% sodium dodecyl sulfate and the protein was separated from the polysomal RNA by sedimentation through linear sucrose gradients. The isolated polysomal RNA was then denatured by heating to destroy any possible aggregates between rRNA and mRNA (29). Albumin polysomal RNA was next passed slowly over a column of poly(U)-Sepharose in a high salt-concentration buffer. Under these conditions, the mRNA should adsorb to the gel through the poly(A) segment at its 3' terminus (24). Nonspecifically adsorbed RNA was removed by washing the gel with a low ionic strength buffer. Finally, the albumin mRNA was eluted with 70% formamide. The formamide eluate contained approximately 70% of the initial albumin mRNA and was enriched 74-fold over the starting material (Table II). None of the mRNA activity in the unbound RNA fraction would adsorb to the column when repassed twice more, suggesting that this mRNA contained either very short poly(A) segments or none at all. The low salt wash material, when examined on linear sucrose gradients, was found to be primarily rRNA. Assuming that the formamide eluate is pure mRNA and that the unbound mRNA is translated with equal efficiency, it can be estimated that the initial albumin polysomal RNA contains approximately 1.8% albumin mRNA.

Purity of Isolated Albumin mRNA - The mRNA in the formamide eluate from the poly(U)-Sepharose column was collected by NaCl/ethanol precipitation and examined directly by electrophoresis on polyacrylamide gels. The absorbance profile in Fig. 3 shows a single, symmetrical peak of mRNA migration at about a 22 S position when compared to rRNA species. This observation is especially significant in view of the fact that no size-fractionation techniques of any kind have been used in the albumin mRNA purification. Therefore, gel electrophoresis becomes a meaningful criterion of homogeneity in this case. The mRNA gel also shows no evidence of contamination by 18 S or 28 S rRNA, indicating the effectiveness of the poly(U)-Sepharose column for mRNA isolation.

The albumin mRNA preparation was also examined, under denaturing conditions, on linear gradients of 5 to 20% sucrose containing 85% formamide and low ionic strength buffers (Fig. 4A). Albumin mRNA sediments as an 18 S molecule (compared to the behavior of 18 S rRNA on similar gradients) and again shows no evidence of rRNA contamination (i.e. no 28 S rRNA). The mRNA can be recovered from these gradients and is translated efficiently in both reticulocyte lysates and wheat germ extracts. The mRNA molecule, then, is apparently an intact, undamaged single chain. Under similar experimental conditions, normal liver rRNA species appear to be considerably degraded, with a significantly decreased amount of intact 28 S rRNA present. Total liver mRNA, prepared by phenolchloroform extraction and poly(U)-Sepharose chromatography (25), sediments with a much broader size distribution (Fig. 4B) than albumin mRNA, centering at a 14 to 15 S region on the gradients. On linear sucrose gradients in the absence of formamide (data not shown), albumin mRNA migrates at approxi-
mately a 17 S position, instead of 18 S, probably due to differences in the secondary structure and hydrodynamic properties of the molecule in the different gradient solutions. Based on the mobility of the albumin mRNA in sucrose gradients and polyacrylamide gels, a molecular weight range of 700,000 to 900,000 can be estimated.

**Translation of Purified Albumin mRNA in Reticulocyte Lysates.** The purified albumin mRNA from the poly(U)-Seph- arose column was examined for translational capacity in the cell-free protein-synthesizing system with rabbit reticulocyte lysates as described previously (24). The translation product was labeled with [3H]leucine and was recognized by immunoprecipitation with specific anti-albumin antibody in the presence of added unlabeled albumin carrier (24). The immunoprecipitate was dissolved in 1% sodium dodecyl sulfate and examined on polyacrylamide gels. Fig. 5 shows that greater than 90% of the radioactivity migrates as a single sharp peak. This material co-migrates with a [14C]albumin standard prepared from rat serum (24). Antibody methods are required for the detection of the translation product of an exogenous mRNA in this system due to the high endogenous background from reticulocyte mRNAs, particularly globin mRNA.

**Translation of Purified Albumin mRNA in Wheat Germ Extracts.** The unfractionated RNA from the formaldehyde eluate of the poly(U)-Sepharose column was added to a cell-free protein-synthesizing system derived from wheat germ extracts (27). Following translation, the ribosomes were removed by centrifugation, and the total supernatant fluid was digested with RNAse, adjusted to 1% sodium dodecyl sulfate, and dialyzed to remove free [3H]leucine. The total translation product, released from ribosomes, was then examined on polyacrylamide gels. Fig. 6 shows only one sharp peak of radioactivity located in the albumin position. Small amounts of other radioactive peptides are found in lower molecular weight regions. More than 90% of the total translation product reacts with the specific albumin antibody, including this small peptide material, except for the very small peptides around Fraction 40 on the gel. Premature termination of translation has been found to be a significant problem with large mRNA species such as albumin mRNA, and the low molecular weight material has been identified as incomplete albumin peptides. Essentially no radioactive material can be identified on gels in the absence of added exogenous mRNA, since the wheat germ extract preparation used here is a mRNA-dependent system. It should be emphasized that no preliminary purification procedures such as size fractionation or immunoprecipitation have been applied to the ribosome-released total albumin translation product. Therefore, the examination of this total translation material by gel electrophoresis (following protein synthesis in a mRNA-dependent system) represents an excellent criterion of homogeneity for the purified albumin mRNA, assuming that all mRNA species are translated with a similar efficiency under these conditions.

The nature of the albumin total translation product was examined further by tryptic peptide analysis. This material, which was labeled with [3H]leucine, was combined with standard [14C]albumin, and reduced, carboxamidomethylated, and digested by trypsin according to previously described techniques (24). The peptides were partially resolved by an automated ion exchange chromatography method (30), and the radioactivity in eluted column fractions was measured (Fig. 7). The close correspondence of each 14C peak with a 3H peak clearly demonstrates that the putative albumin mRNA is directing the synthesis of authentic rat albumin in the wheat germ extracts. No peptide derived from the standard [14C]albumin is without a corresponding 3H-labeled peptide from the translation product. The variation in the 3H/14C ratios is most likely due to the prematurely released incomplete albumin peptides, with peptides derived from the NH2-terminal region being enriched for 3H-labeled material. Con-
considerable variation in $^3$H/$^14$C peptide ratios has been observed in various cell-free systems by others for different mRNAs (3, 7-9, 31, 32). At Fraction 12 in Fig. 7, a $^3$H-labeled tryptic peptide derived from the translation product is identified without a corresponding $^14$C peak. A similar finding has also been made for immunologically isolated albumin translation product from reticulocyte lysates (24). This extra peptide might be derived from the NH$_2$-terminal precursor region of the albumin product (33, 34). Similar precursor regions for other proteins have been shown to contain leucine (35). If the albumin precursor were only slightly larger than native serum albumin (95,000 molecular weight), the difference in molecular size might not be detected on standard polyacrylamide gels.

DISCUSSION

The primary consideration in establishing the conditions for the immunoprecipitation of albumin-synthesizing polysomes was to minimize the size of the final immunoprecipitate. It has previously been shown (23) that the amount of nonspecific adsorption in the precipitate is approximately proportional to the bulk size of the immunoprecipitate and not to the amount of polysomal material isolated. We investigated several antibody preparations in two different combinations (Figs. 1 and 2) and established polysome immunoprecipitation conditions that proved to be consistently reproducible through several mRNA preparations.

Although we have demonstrated the specificity of intact immunoglobulins in the antibody technique for polysome identification, here and in earlier work (22, 23), it should be emphasized that each antibody preparation can have unique reaction properties in immunological procedures. Other laboratories have found it necessary to employ the Fab fragments of immunoglobulins because of nonspecific adsorption problems with the intact molecules (36, 37); but these problems are probably a result of antibody variability rather than the polysome immunoprecipitation technique itself. Not only albumin mRNA, but several other mRNA species (16-21) have been purified by this and similar procedures.

The results of Figs. 1 and 2 with $^3$H-leucine-labeled nascent peptide chains in polysomes show that approximately 11 to 12% of the liver polysomes are engaged in albumin production. This finding correlates with the observation that approximately 11% of the total protein made by the rat liver under normal conditions is albumin (25, 38). If all liver proteins were translated at similar rates, one might expect that approximately 11% of the polysomal mRNA would be albumin mRNA and that its isolation should be technically feasible. Therefore, a 9-fold purification of albumin translational activity, as found in the polysome immunoprecipitate (Table I), should yield a reasonably homogeneous mRNA.

The separation of albumin mRNA from rRNA was efficiently performed with poly(U)-Sepharose. Passing the mRNA over the column twice effectively removed all traces of rRNA while allowing excellent recovery. Compared to oligo(dT)-cellulose, we have routinely found poly(U)-Sepharose to give higher recoveries of more highly purified mRNA. The finding that about one-fourth of the total albumin mRNA does not adsorb to the column, even upon repassing several times, suggests that some of this material have very short lengths of poly(A) segments or none at all. This has been a reproducible observation for both albumin mRNA and total liver mRNA as well. Similar findings have been reported for other mRNA species, such as ovalbumin (16, 39) and globin mRNAs (40).

Several criteria of homogeneity were applied to the purified albumin mRNA. Gel electrophoresis showed a single narrow band of mRNA (Fig. 3) migrating at $n = 22 S$ position. Since the migration of a RNA species in polyacrylamide gels and sucrose gradients will be strongly influenced by nucleotide composition and secondary structure, only a molecular weight range was estimated here. This problem is currently under study. Sedimentation in denaturing gradients containing 85% formamide (Fig. 4) demonstrated that we had isolated an intact molecule that retained its translational capacity in subsequent protein synthesis experiments. Furthermore, albumin mRNA could be divided into two equal fractions on sucrose gradients (the front and back halves of the peak), and each fraction was found to give identical translation products when examined in wheat germ extracts.

Translation of the purified albumin mRNA in wheat germ extracts was found to be useful, since this protein-synthesizing system is essentially dependent upon the addition of exogenous mRNA. Analysis of the translation product by gel electrophoresis and tryptic peptide fingerprinting clearly demonstrated that the albumin mRNA had been isolated and that no major mRNA contaminants existed in our preparations. Furthermore, the tryptic peptide chromatogram suggested that albumin may be translated as a leucine-rich precursor, a possibility suggested by the work of others (33-35). Wheat germ extracts probably do not contain the normal post-translational processing system that apparently exists in the liver endoplasmic reticulum, so that identification of the albumin precursor might be possible. However, our gel electrophoresis studies have not detected a precursor, suggesting that it may not be much larger than native serum albumin.

An important aspect of this work is that size fractionation techniques have not been employed in our mRNA isolation procedures. The experimental approach to mRNA purification used here, therefore, allows the use of a wide range of criteria for mRNA homogeneity and characterization, including gel electrophoresis and sucrose gradient sedimentation. Studies are currently in progress on the physical characterization of the albumin mRNA.

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