SYNTHESIS OF NASCENT PROTHROMBIN AND ALBUMIN IN A HETEROLOGOUS SYSTEM USING RAT LIVER MESSENGER RNA PURIFIED ON OLIGO (dT)-CELLULOSE

Nicholas J. Nardacci, John P. Jones, Anne L. Hall, and Robert E. Olson

Department of Biochemistry
St. Louis University School of Medicine
St. Louis, MO 63104

Received March 3,1975

SUMMARY: Highly active m-RNA was prepared by phenol extraction of rat liver polysomes followed by oligo (dT)-cellulose chromatography. This m-RNA preparation stimulated total protein synthesis in rabbit reticulocyte lysates and in wheat germ extracts. Nascent prothrombin and albumin synthesized in the reticulocyte system programmed with this m-RNA were precipitated with specific antibodies and identified by their electrophoretic mobilities on SDS-acrylamide gels.

INTRODUCTION: Success in the translation of various mammalian m-RNAs in a number of cell-free systems has been reported (1). One of our objectives has been the translation of nascent prothrombin from rat liver m-RNA in a heterologous system in order to study the properties of the translation product. We have estimated from rates of synthesis of prothrombin in microsomes and polyribosome preparations, that prothrombin m-RNA represents less than 1% of the total rat liver m-RNA (2). The need for an effective method of purifying active hepatic m-RNA in good yield was thus of ultimate importance. Oligo (dT)-cellulose has been found to be a better matrix than either cellulose or millipore filters for specificity of binding and recovery of ovalbumin m-RNA (3). We have observed that when liver RNA is prepared by SDS[1]-phenol extraction of whole livers or Mg^{+} precipitated polysomes, it binds poorly to oligo (dT)-cellulose when compared to RNA extracted from polysomes sedimented through 2 M sucrose. In this communication we wish to report that nascent rat prothrombin has been translated from ol-

[1]The abbreviation used is: SDS, sodium dodecyl sulfate.

igo (dT)-cellulose purified m-RNA in the rabbit reticulocyte system and partially characterized.

MATERIALS AND METHODS: Isolation of Polysomes and Extraction of RNA. Whole rat livers were homogenized at low speed with a Brinkman Polytron homogenizer for 5 min. at 4° C in 5 volumes of 0.25 M sucrose containing 50 mM Tris-HCl, pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$ and 0.05% Na heparin (750,000 units/l), followed by 6-7 strokes in a tight fitting Teflon glass homogenizer. The homogenate was centrifuged at 14,600 x g in a Sorval SS-34 rotor for 10 min. at 4° C. To nine volumes of the supernate was added one volume of a 10% Na deoxycholate - 10% triton x-100 solution and the mixture was stirred for 30 min. at 4° C. The supernate was then layered over a discontinuous gradient consisting of 10 mls of 2 M sucrose overlaid with 5 mls of 0.5 M sucrose both containing 50 mM Tris-HCl, pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$ and 0.05% Na heparin. The polyribosomes were sedimented through the gradient at 105,000 x g for 16 hrs. at 4° C and suspended by gentle homogenization in 50 mM Na acetate, pH 5.0, 10 mM EDTA, and 0.5% SDS. This suspension was adjusted to a concentration of 1-2 mg/ml and extracted with an equal volume of clear, reagent grade 88% phenol:chloroform (1:1 v/v) by shaking vigorously in a separatory funnel for 5 min. per extraction at room temperature. The aqueous phase was made 0.1 M with NaCl and the RNA precipitated with 2 volumes of absolute ethanol. After 12 hours at -23° C the RNA precipitate was washed twice with cold 95% ethanol and the RNA dissolved in a small volume of sterile demineralized water. A solution of 10 mM Tris-HCl, pH 7.5, containing 0.5 M KCl was added to give a final RNA concentration of 6-8 mg/ml. Two alternate procedures were used for the extraction of hepatic RNA. Polysomes were precipitated from detergent treated crude homogenates with $MgCl_2$ as described by Palmiter (4) and extracted by the SDS-phenol method described above. Also whole rat livers were directly homogenized in 50 mM Na acetate, pH 5.0, 10 mM EDTA, and 0.5% SDS in a Waring blender by the method of Schutz et al (5), and extracted with phenol as described above.

Oligo (dT)-cellulose Chromatography. Poly A containing m-RNA was separated from the bulk of polysomal RNA using an oligo (dT)-cellulose (type 2 Collaborative Research Inc., Waltham, Mass.) column by the method of Scolnick et al (6) except that the m-RNA fraction was eluted with sterile demineralized water. The water eluted fraction was adjusted to 0.3 M with solid LiCl and the RNA precipitated with 2 volumes of absolute ethanol at -23° C for 12 hours. The RNA pellet was collected at 2,000 x g max and dissolved in sterile water at a concentration of approximately 1 mg/ml and stored for weeks at -23° C or for months at -70° C.

Cell-Free Protein Synthesis. Assays for biological activity of the (dT)-cellulose m-RNA preparations were carried out in protein synthesizing systems derived from wheat germ by the method of Roberts and Patterson (7) and rabbit reticulocytes. Reticulocyte lysates were prepared by the method of Adamson et al (8). The reticulocyte protein synthesizing system contained the following components: 2 µM [4,5-^{3}H] leucine (55 Ci/mm), 2 µM [4,5-^{3}H] isoleucine (65 Ci/mm), 6 µM [2,3-^{3}H] valine (17 Ci/mm), 40 µM unlabeled complete amino acid mixture minus leucine, isoleucine, and valine, 5 mM Tris-HCl, pH 8.1, 50 mM NH_4 acetate, 1 mM Mg acetate, 80 mM KCl, 2.5 mM 2-mercaptoethanol, 1 mM ATP, 20 µM GTP, 80 mM creatine phosphate, 40 µg/ml creatine phosphokinase, (dT)-cellulose prepared m-RNA, 0-0.2 µg/µl reaction mixture and reticulocyte lysate 50% of total reaction volume.

Antibodies. Antibodies were raised in rabbits to rat serum albumin and rat plasma prothrombin (9).

Characterization of the In Vitro Products. After incubation for 60 min. at 25° C the reaction was stopped by the addition of 1/10 volume of 20% Triton containing 100 mM unlabeled leucine, isoleucine, and valine. Purified carrier prothrombin (5 µg) or rat albumin (10 µg) was added and the reaction mixture was cen-

trifuged at 105,000 x g for 90 min. to remove polyribosomes. Specific anti-plasma was added at equivalence point to the supernate and allowed to react for 45 min. at 30° C, then for 18 hrs. at 4° C. The immunoprecipitates were centrifuged through a discontinuous sucrose gradient containing 1% Na deoxycholate and 1% triton as described by Taylor and Schimke (10). The precipitate was dissolved in 3% SDS, 0.1 M Na phosphate buffer 7.8, and 2% 2-mercaptoethanol and heated to 90-95° C in a hot water bath for 5 min. After cooling mercaptoethanol was added back to samples and 8% SDS gel electrophoresis was performed by the method of Kobylka et al (11). Gel slices were incubated in 1 ml of Protosol:H_2O (1:1 v/v) for 2 hrs. at 50° C, cooled, and counted in Omnifluor-toluene in a liquid scintillation spectrometer. Total incorporation of label into protein was determined by pipetting 50 µl of carrier BSA (500 µg) in 1 ml of 5% TCA containing 0.1% unlabeled leucine, isoleucine, and valine. Five microliters of reaction mixture was added and the precipitate was washed 4 times at 2,000 x g for 3 min. with TCA solution using a 20' heat step at 90-95° C after the first wash to deacylate the t-RNA. The precipitate was dissolved in 0.5 ml of Protosol and 0.2 ml of benzoyl peroxide (20 mg) in toluene was added and the red color was bleached by incubating for 30 min. at 50° C. The digest was counted in 10 ml of Omnifluor-toluene with an efficiency of 48%. A calibration curve of molecular weight versus mobility was determined for each electrophoresis experiment by the method of Weber and Osborn (12) using proteins of known molecular size. ^{125}I-prothrombin and ^{125}I-albumin standards were prepared by the chloramine T procedure (13).

RESULTS: Rat liver RNA prepared by SDS-phenol extraction of polysomes sedimented through 2 M sucrose was soluble and amenable to oligo (dT)-cellulose chromatography. When whole livers or Mg^{++} precipitates of polysomes from crude homogenates were extracted with SDS-phenol the RNA was not completely soluble in water nor in neutral Tris-HCl buffers and could not be effectively processed on oligo (dT)-cellulose. Table I shows variation in yield of RNA obtained by oligo (dT)-cellulose chromatography in the three different extraction procedures. Purified m-RNA obtained from procedure 1 inhibited total protein synthesis and was approximately one eighth as active per microgram in directing the incorporation of ^{3}H-amino acids into albumin immunoprecipitates in the reticulocyte system when compared to the m-RNA derived from procedure 3. The m-RNA product from procedure 2 represented only 0.06% of the total RNA applied to the column and was not tested for biological activity. The m-RNA fraction from procedure 3 stimulated total protein synthesis 30-45 fold in wheat germ extracts and 20-30 percent in crude reticulocyte lysates (Table 2). As shown in Figure 1 it directed ^{3}H-amino acid incorporation into rat serum albumin, in the reticulocyte system and was used for all of the subsequent experiments. Figure 1A shows the relationship of added m-RNA to albumin synthesized. Figure 1B shows the 8% SDS-acrylamide mi-

TABLE 1

Efficiency of Rat Liver m-RNA Isolation by Various Procedures

	Fractions Extracted with SDS-phenol	Liver Processed gm.	RNA Applied to Oligo (dT)-column mg.	RNA Recovered in m-RNA fraction µg.	Percent RNA Recovered in m-RNA fraction
1.	Whole livers	165	457	306	0.06
2.	Mg^{++} precipitated polysomes	119	520	102	0.02
3.	Polysomes sedimented through 2 M sucrose	137	67	1,715	2.60

TABLE 2

Bioassay of Rat Liver m-RNA Prepared via Oligo (dT)-cellulose

Wheat Germ System:

µg RNA Added per 100 µl Reaction Mixture	CPM x 10^{-3} in Total Protein per 100 µl Reaction Mixture
0	65
1.8	940
3.6	1,490
5.6	1,900
9.2	2,000

Reticulocyte System:

µg RNA Added per 400 µl of Reaction Mixture	CPM x 10^{-3} in Total Protein per 400 µl Reaction Mixture
0	7,870
30	8,400
50	8,600
80	9,000
110	9,510

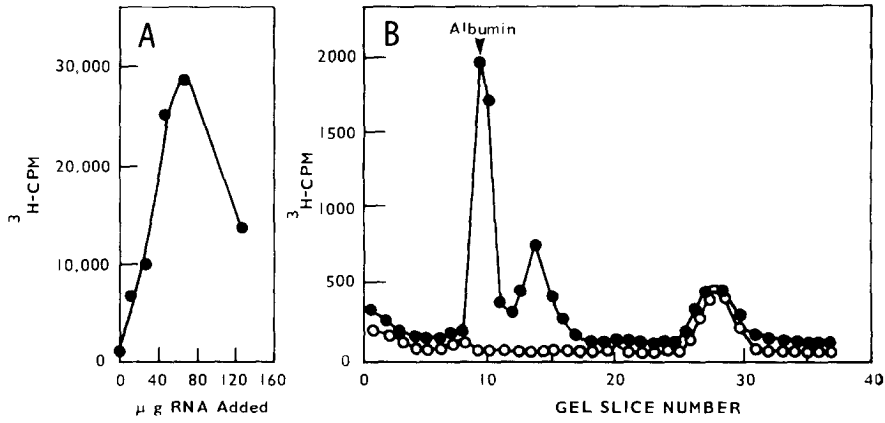

Figure 1: Messenger RNA Directed Albumin Synthesis in Reticulocyte Lysate

A. ^{3}H-amino acid incorporation into albumin immunoprecipitates as a function of m-RNA added to 400 μl total reticulocyte reaction mixture.
B. SDS-gel electrophoresis of albumin immunoprecipitates with 40 μg RNA in 400 μl total reticulocyte reaction mixture (●--●) or without added RNA (O--O).

gration patterns for proteins precipitated with antisera to rat serum albumin. The arrow in Figure 1B denotes the mobility of authentic rat serum albumin which corresponded to the major ^{3}H-protein peak. The specificity of our antibodies were individually tested for reactivity against ^{125}I-albumin and ^{125}I-prothrombin (Figure 2). Furthermore, each antibody preparation showed a single band against rat plasma by immunoelectrophoresis.

When the products of m-RNA translation in the reticulocyte system were precipitated with prothrombin antibodies a ^{3}H-protein peak appeared on SDS gels which had a mobility less than albumin but slightly greater than that of a stable 73,000 dalton species derived from native rat prothrombin (M.W. 85,000) (9,18) as shown in Figures 3A and 3B. The molecular weight of this nascent prothrombin was estimated to be 70,000 daltons, and the amount detected was proportional to the concentration of m-RNA added in the range of 10-80 μg per 400 μl of reaction mixture. Echis carinatus venom degraded this peak without affecting the corresponding albumin peak. The specificity of the prothrombin antisera for nascent prothrombin is shown in Figure 3C by the SDS gel electrophoresis of ^{3}H-proteins im-

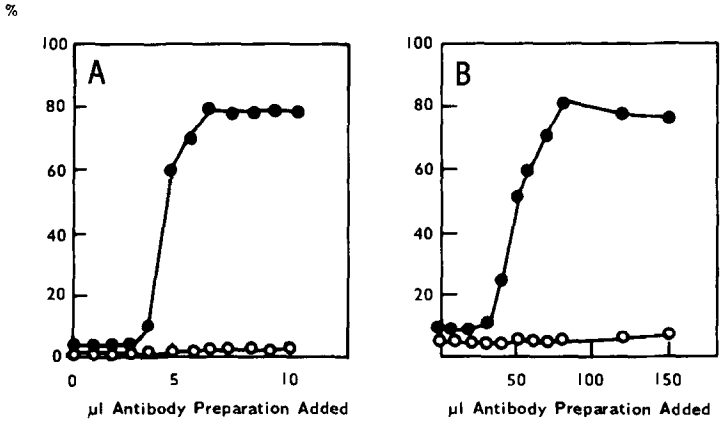

Figure 2: Precipitin Reactions for Albumin and Prothrombin

A. Percent of ^{125}I rat albumin (5 µg) precipitated with albumin antibodies (●--●). Percent of ^{125}I-prothrombin (5 µg) precipitated with albumin antibodies (0--0).

B. Percent of ^{125}I-prothrombin (5 µg) precipitated with prothrombin antibodies (●--●). Percent of ^{125}I-albumin (5 µg) precipitated with prothrombin antibodies (0--0).

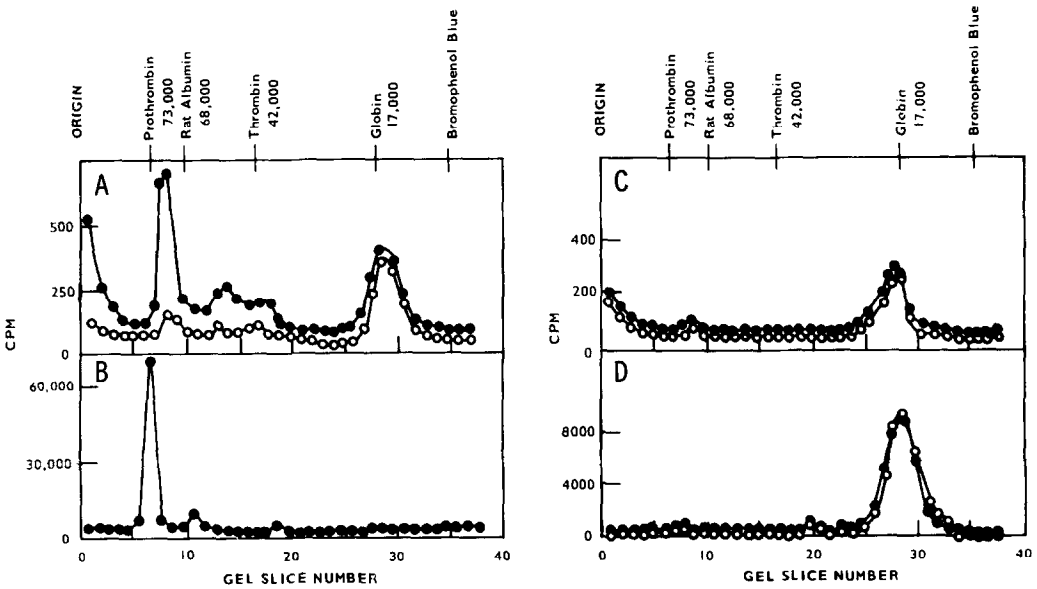

Figure 3: SDS-gel electrophoresis of prothrombin standard and translation products of rat liver m-RNA in Reticulocyte Lysate (●--●) denotes 50 µg m-RNA added in 400 µl total reaction mixture and (0--0) denotes no RNA added.

A. Prothrombin immunoprecipitates in 400 µl reaction mixture.
B. Mobility of ^{125}I-plasma rat serum prothrombin.
C. Ovalbumin immunoprecipitates in 400 µl reticulocyte reaction mixture.
D. TCA precipitates of 0.5 µl total reaction mixture.

munoprecipitated with ovalbumin antibodies. Only traces of ^{3}H-globin chains (M.W. 17,000) nonspecifically attached to the immunoprecipitates were detected representing less than 0.03% of the total globin synthesized in the reticulocyte lysate. Likewise when the reticulocyte lysate was incubated with or without rat liver m-RNA and precipitated with TCA, only globin chains (Figure 3D) were detected.

DISCUSSION: Oligo (dT)-cellulose provides an excellent matrix for the purification of hepatic m-RNA, provided the RNA has been isolated from purified polysomes. Recently Rosen et al (14) has extracted RNA from hen oviducts by direct SDS-phenol extraction and has shown that the total extract contained between 5 and 10% DNA as measured by the diphenylamine assay. The inability of our total rat liver RNA extracts to adequately bind to oligo (dT)-cellulose may be due to DNA contaminants which inhibit binding. In our hands, precipitation of polysomes with 100 mM Mg^{++} from crude liver homogenates gave visibly contaminated precipitates which may have accounted for the inability of these preparations to properly bind to (dT)-cellulose.

The (dT)-cellulose m-RNA prepared from polysomes sedimented through 2 M sucrose was active in stimulating total protein synthesis in wheat germ extracts and crude reticulocyte lysates. In addition it was active in directing incorporation of ^{3}H-amino acids into rat albumin and nascent prothrombin in the reticulocyte lysate. The molecular weight of the major ^{3}H-protein in albumin immunoprecipitates was identical to rat serum albumin which is in agreement with the observations of Taylor and Schimke (10).

As noted, the major ^{3}H-protein precipitated with prothrombin antibodies had a slightly greater mobility than a 73,000 dalton fragment derived from rat prothrombin and a corresponding greater mobility than the labile native 85,000 dalton species (not shown in Figure 3). Traces of possible degradation products (55,000 daltons) and thrombin (40,000 daltons) were also noted (Figure 2A). Since prothrombin is 10-12% carbohydrate by weight (15), the nascent peptide observed would correspond to a mature plasma protein of the order of 80,000

daltons. Ovalbumin, a glycoprotein which contains 3.2% carbohydrate by weight (16), has been synthesized in a reticulocyte system and had a slightly higher mobility than egg ovalbumin (17).

Convincing evidence has now been provided by Stenflo (18) and others (19, 20) that bovine plasma prothrombin is a carboxylated derivative of an inactive precursor protein made in the liver and secreted into the plasma in dicumarolized cows. If the rat is similar to the cow, the nascent prothrombin we have detected should be the unmodified preprothrombin which accumulates in the microsomes of vitamin K-deficient and warfarin-anticoagulated rats (21,22). Experiments are in progress to test this prediction.

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