# ISOLATION OF EUKARYOTIC MESSENGER RNA ON CELLULOSE AND ITS TRANSLATION IN VITRO

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## SUMMARY

Chromatography on plain cellulose of RNA from rabbit reticulocyte polysomes or chicken oviduct results in the retention and enrichment of a RNA fraction with messenger properties. Addition of appropriate amounts of this RNA to a cell-free system from Krebs-II-ascites cells, supplemented with rabbit reticulocyte initiation factors, leads to 20- to 30-fold stimulation of protein synthesis. The product of the reaction directed by RNA purified from rabbit reticulocytes was shown to consist of both $\alpha$- and $\beta$-globin chains, as judged by SDS-gel electrophoresis and CM-cellulose chromatography. RNA isolated from chicken oviduct directs the synthesis of ovalbumin which has been identified by SDS-electrophoresis of the released chains after immunoprecipitation with antiovalbumin.

## INTRODUCTION

Polynucleotide sequences rich in adenylic acid at the 3'-end of messenger RNA from cellular and viral origin have been recognized as a distinctive characteristic of eukaryotic messenger RNAs, [1-10] the messengers for the histones being the only thus far reported exception. [11] This unique property of animal messenger RNA has enabled their selective isolation by absorption to Millipore filters [2,12] and by binding to poly-(U) [6,7] or oligo d-(T) [13] covalently linked to cellulose. The latter method has been successfully employed for the isolation of the mRNA for globin [14] and a myeloma light chain. [15] Recently, Kitos et al [16] have demonstrated that at high ionic strength cellulose per se is able to retain poly-(A) and poly-(A)-containing RNA. In this communication we present evidence indicating

the ability of nucleotide-free cellulose to selectively retain mRNA for globin and ovalbumin, and recommend this procedure as a very simple way for the isolation of eukaryotic messengers.

## MATERIALS AND METHODS

Sigmacell type 38, obtained from Sigma Chemical Co., was used as cellulose. ($^3$H)-Leucine (31.6 Ci/mmole) and ($^3$H)-Serine (2.23 Ci/mmole) were purchased from New England Nuclear Co.

Extraction of RNA. a) Rabbit reticulocytes: the reticulocyte ribosomes were isolated from rabbits treated with phenylhydrazine.[17] The ribosomes were suspended in 50 mM Tris-HCl (pH 8.3), 5 mM EDTA, 75 mM NaCl, 0.5% sodium dodecyl sulfate (SDS), and an equal volume of buffer saturated phenol, adjusted to pH 8.3, was added. The mixture was shaken for 5 min at 4°C and the aqueous phase thrice extracted with phenol, then made 0.3 M with respect to LiCl, and the RNA precipitated with 2 volumes of ethanol at -20° C. The precipitated RNA was washed twice in ethanol and dissolved in $H_2O$.

b) Chicken oviduct: 20 g of the magnum portion of the oviduct from laying hens were homogenized in 130 ml of cold 50 mM Tris-HCl (pH 8.3), 5 mM EDTA, 75 mM NaCl, 0.5% SDS, and 150 ml of cold buffer saturated phenol, pH 8.3, in a Waring Blender for 1 min. The RNA was extracted as described above, the only difference being that the phenolization was repeated 5 times.

Cellulose Chromatography. 100-300 $A_{260}$ units of total oviduct RNA and reticulocyte polysomal RNA in 1 ml of $H_2O$ were diluted to 10 ml with Tris-HCl (pH 7.6), 500 mM KCl, 0.2 mM $MgCl_2$ and applied to a 2-4 ml cellulose column (dry weight about 0.5-1 g) which had been equilibrated with 10 mM Tris-HCl (pH 7.6), 500 mM KCl, 0.2 mM $MgCl_2$. The column was washed with application buffer until the $A_{260}$ had dropped below 0.01 and fractions of 1 ml were collected. The absorbed RNA was eluted with neutralized water. The non-absorbed and absorbed material was brought to 0.3 M in $LiCl_2$, precipitated with ethanol, washed twice with ethanol, dissolved in $H_2O$, and used as such for the *in vitro* protein synthesis. The chromatography was performed at room temperature.

Cell-Free Protein Synthesis. A cell-free protein synthesizing system was prepared from Krebs-II-ascites cells as described[18] with the modification introduced by Metafora *et al.*[19]

Routine assays contained in 50 µl: 30 mM Tris-HCl (pH 7.5), 95 mM KCl, 3 mM magnesium acetate, 1 mM dithiothreitol, 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 5 mM creatine phosphate, 0.16 mg/ml creatine kinase, 0.04 mM of 19 unlabeled amino acids, 2.5 or 5 µC of ($^3$H)-serine or ($^3$H)-leucine, respectively, 0.15 $A_{260}$ of tRNA isolated from chicken liver, 0.3-0.4 $A_{260}$ of Krebs lysate, and 0.3 $A_{280}$ of a 0.5 M KCl wash fraction from rabbit reticulocyte ribosomes, prepared as described in (19). Incubation was at 37° C for the

times indicated. For determination of the radioactivity incorporated into proteins, the method described by Bollum (20) was used.

Characterization of Cell-Free Products. Following the incorporation period, the ribosomes were removed by centrifugation of the assay mixture at 150,000 g for 60 min and the supernatant used for characterization of the products. The specific immunoprecipitation for ovalbumin was done in a total volume of 0.335 ml, which contained: 10 mM sodium phosphate (pH 7.0), 0.14 M NaCl, 2% Triton X-100, 100-150 $\mu$l of the supernatant of the in vitro reaction, 10 $\mu$g of carrier ovalbumin and antiovalbumin rabbit $\gamma$-globulin in an amount to give optimal precipitation. The mixture was kept at room temperature for 30 min and then overnight at 4° C. The immunoprecipitates were separated by centrifugation and then washed 3 times with a volume of 0.35 ml of 10 mM sodium phosphate (pH 7.0) and 0.14 M NaCl. The precipitates were dissolved in 10 mM sodium phosphate (pH 7.0), 1% SDS and 1% $\beta$-mercaptoethanol, and electrophoresed as described below.

For analysis of the reticulocyte RNA-directed products, 0.2 ml of 0.1 M KOH was added to the released chains. After incubation for 20 min at 37° C, 1 ml of cold 10% $Cl_3CCOOH$ was added. The precipitate was washed twice with cold 5% $Cl_3CCOOH$ and twice with acetone. The precipitate was finally dissolved in 10 mM sodium phosphate (pH 7.0), 1% SDS and 1% $\beta$-mercaptoethanol, and electrophoresed following the procedure of Weber and Osborn.(21) Gels were cut into 1.6 mm slices and placed in scintillation counting vials. One ml of $H_2O_2$ was added and incubated overnight to dissolve the gel slices. The samples were then counted in Aquasol.

Hemoglobin uniformly labeled with ($^{14}$C)-valine was prepared using reticulocytes from phenylhydrazine-treated rabbits and the globin chains prepared by acid-acetone extraction. The isolated globin had a specific activity of 20,000 cpm/mg. The proteins synthesized in vitro in response to reticulocyte mRNA were mixed with ($^{14}$C)-labeled globin and the globin chains prepared as above. They were then separated on CM-Cellulose following the procedure of Rabinowitz and Fischer.(22)

## RESULTS

Chromatography of RNA on Cellulose. As can be seen in Figure 1, 1.1% of the RNA from rabbit reticulocyte polysomes is retained by cellulose under conditions of high salt. The retained material can be eluted with water as a sharp peak. A pattern similar to the one described was obtained when total RNA from chicken oviduct was fractionated on the column except that, in this case, about 2% of the RNA was retained by the cellulose. The unfractionated

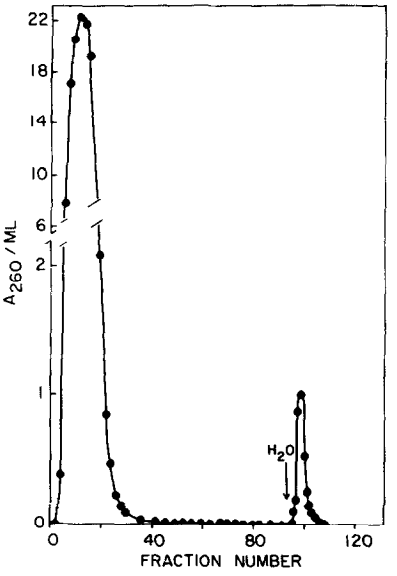


Figure 1

Chromatography on cellulose of rabbit reticulocyte polysomal RNA. 260 $A_{260}$ were applied in 20 ml of 10 mM Tris-HCl (pH 7.6), 500 mM KCl, and 0.2 mM $MgCl_2$, and extensively washed with the same buffer. Elution with water is indicated by arrow.

RNA and the RNA retained by the column were each checked for its ability to direct protein synthesis in a Krebs-II-ascites cell-free system. Whereas the material retained by the column stimulates total protein synthesis 20- to 30-fold, the unfractioned RNAs are much less active in increasing the rate of protein synthesis (Fig. 2), demonstrating that enrichment of RNA with messenger properties has occurred during cellulose chromatography. The efficiency of translation of these two mRNA fractions is dependent upon the 0.5 M KCl salt wash fraction from rabbit reticulocyte ribosomes, as has been described for the translation of globin mRNA (19) (Table 1). As indicated in Figure 3 protein synthesis is dramatically stimu-

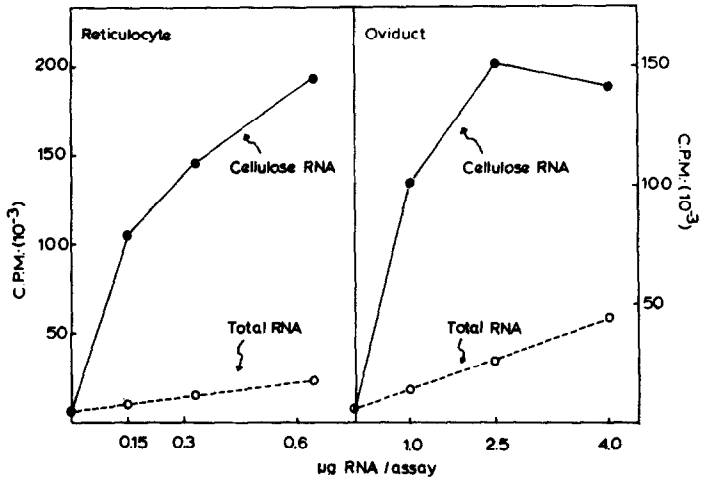


Figure 2

Effect of unfractionated and cellulose-purified RNA on protein synthesis. Incorporation of ($^3$H)-leucine into protein was measured in 50 µl reaction mixtures after 60 min incubation in the presence of the indicated amounts of unfractionated (o---o) or cellulose-purified RNA (●——●).

TABLE 1

EFFECT OF RABBIT RETICULOCYTE RIBOSOMAL SALT WASH (RSW) ON CELLULOSE RNA-DIRECTED PROTEIN SYNTHESIS IN THE KREBS-II-ASCITES CELL SYSTEM

| Cellulose-RNA Added (µg/50 µl) | RSW | KCl* (mM) | ($^3$H)-Leucine Incorporated (cpm/50 µl) |
|---|---|---|---|
| None | - | 65 | 760 |
| Oviduct (2.5) | - | 65 | 1,770 |
| Reticulocyte (0.3) | - | 65 | 3,055 |
| None | + | 95 | 10,740 |
| Oviduct (2.5) | + | 95 | 258,100 |
| Reticulocyte (0.3) | + | 95 | 234,200 |

* Previous experiments had shown that in the absence of RSW the optimal KCl concentration for ($^3$H)-leucine incorporation was 65 mM. When RSW was added the optimal concentration of KCl shifted to 95 mM.

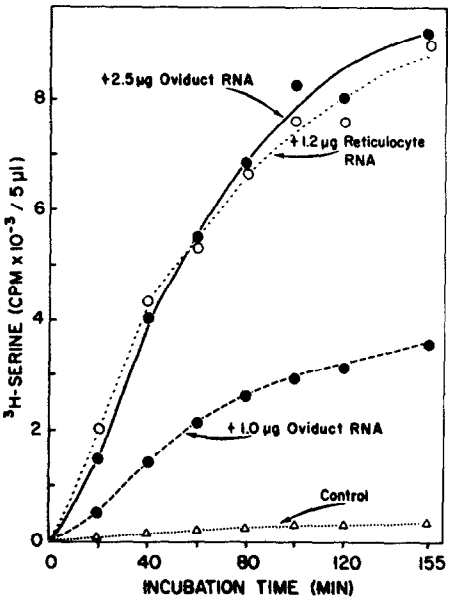


Figure 3

Time course of reticulocyte and oviduct mRNA stimulated ($^3$H)-serine incorporation into protein. Incorporation of ($^3$H)-serine was measured in 50 µl reaction mixtures in the absence of added RNA ( △---△ ), with 1.2 µg reticulocyte RNA purified on cellulose (o---o), and 1 (●---●) and 2.5 µg (●—●) oviduct RNA purified on cellulose. At the indicated times 5 µl were removed from the reaction mixture and the radioactivity incorporated into protein determined as described in Methods.

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lated by µg levels of oviductal and reticulocyte mRNA and continues well up to 150 min.

We believe that the selective retainment of a small portion of the RNA is due to the poly-(A) content of this RNA for two reasons: a) Sigmacell cellulose type 38 retains poly-(A) under these salt conditions (ref. 16 and our own unpublished results), and b) the globin-m-RNA and the ovalbumin-m-RNA both contain poly-(A).[1,5,12]

Characterization of the in vitro Products. In order to get an insight into the fidelity of translation, the in vitro products have been analyzed. The protein syn-

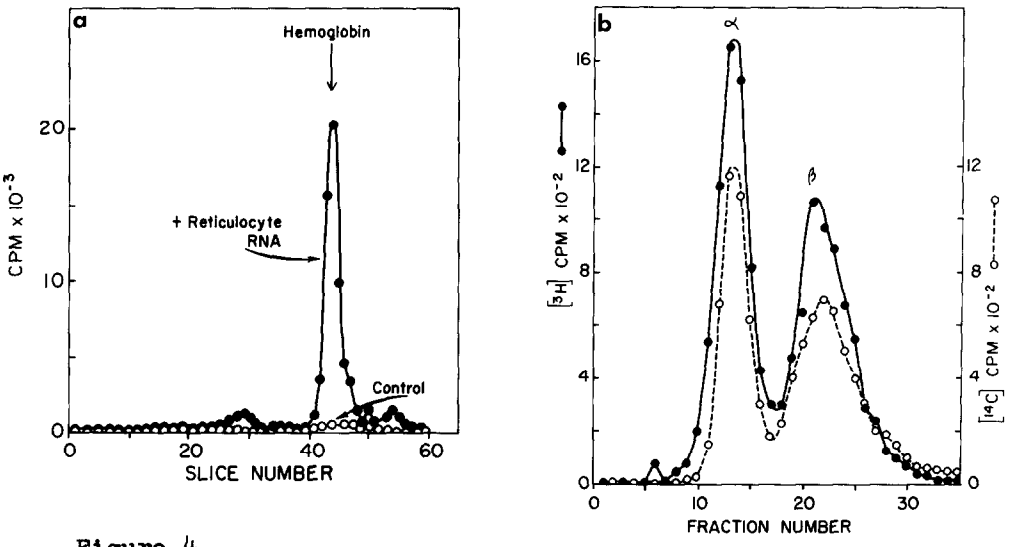


Figure 4

a) SDS-polyacrylamide electrophoresis of proteins synthesized and released in vitro in response to reticulocyte mRNA. The chains released after 100 min incubation from a 30 $\mu$l reaction mixture containing 0.72 $\mu$g of cellulose-purified reticulocyte RNA were analyzed on an SDS-polyacrylamide gel. The arrow indicates the position of hemoglobin used as internal marker. The direction of anodal migration is from left to right.
b) Separation of in vitro synthesized $\alpha$- and $\beta$-globin chains on CM-Cellulose. The globin chains released after 60 min incubation from a 100 $\mu$l reaction mixture containing 2.5 $\mu$g of cellulose-purified reticulocyte RNA were prepared after addition of 2 mg of ($^{14}$C)-labeled globin as described in Methods. The $\alpha$- and $\beta$-chains were separated on CM-Cellulose by the method described.(22)

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thesized in response to the reticulocyte RNA retained by the cellulose column has been analyzed by SDS-polyacrylamide gel electrophoresis. More than 90% of the labeled polypeptides released into the supernatant after spinning out the ribosomes co-migrated with rabbit globin (Fig. 4a). The synthesis of $\alpha$- and $\beta$-globin chains in approximately equal amounts was confirmed by chromatography of the ($^{3}$H)-leucine-labeled products on CM-Cellulose with uniformly labeled ($^{14}$C)-rabbit globin as a marker (Fig. 4b).

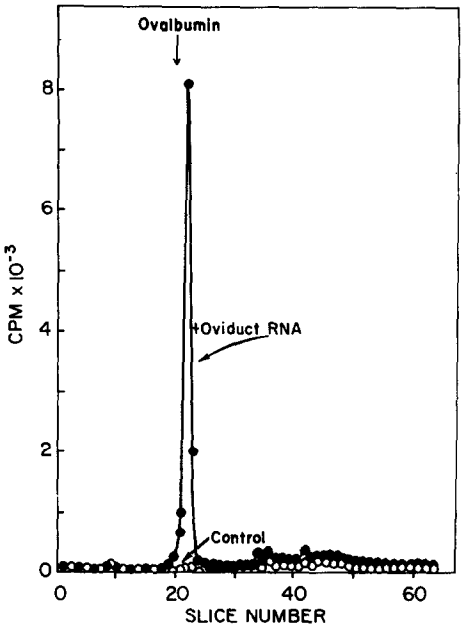


Figure 5

SDS-polyacrylamide electrophoresis of the proteins synthesized in vitro in the presence of oviduct mRNA and immunologically isolated. The released polypeptide chains after 60 min incubation period of a 90 $\mu$l reaction system containing 4.5 $\mu$g oviductal mRNA were isolated immunochemically with rabbit antiovalbumin $\gamma$-globulin. The immunoprecipitate was subjected to SDS-polyacrylamide electrophoresis (see Methods). The arrow indicates the position of authentic ovalbumin used as an internal marker. The direction of migration is from left to right.

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The products directed by the fraction of chicken oviduct RNA retained by cellulose was analyzed for ovalbumin content by specific immunoprecipitation with rabbit anti-ovalbumin $\gamma$-globulin and subsequent SDS-polyacrylamide electrophoresis of the immunoprecipitate. As shown in Figure 5, this RNA directs the synthesis of a product which forms a specific immunoprecipitate with antiovalbumin and which, in SDS gels, migrates as a single peak slightly ahead of the ovalbumin marker. This may be due to the absence of the carbohydrate moiety of 1500 molecular

weight of ovalbumin which may not be synthesized or incorporated into the nascent ovalbumin polypeptide under these *in vitro* conditions. (23, 24, 25)

Three conclusions can be drawn from these studies: 1) Cellulose can be used for the isolation of the messenger RNA for globin and ovalbumin. The same procedure was successfully employed for the isolation of RNA with messenger properties from rat and chicken livers. A complementary sequence of oligo-(dT) of poly-(U) is not required for the selective binding of these mRNAs. Therefore, we suggest chromatography of RNA on cellulose as an effective procedure for the purification of eukaryotic messenger RNAs. 2) The Krebs-II-ascites cell-free protein synthesis system allows the faithful translation of the messenger RNA for ovalbumin as well as globin. 3) Supplementation with 0.5 M KCl extract of rabbit reticulocyte ribosomes not only stimulates total protein synthesis, but also selectively enhances the translation of heterologous messenger RNA in the Krebs-II-ascites system. These findings provide additional evidence against the need of messenger-specific initiation factors.

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