Preparation and Preliminary Characterization of Purified Ovalbumin Messenger RNA from the Hen Oviduct†

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ABSTRACT: Preparation of milligram amounts of purified ovalbumin mRNA was accomplished by a sequential combination of precise sizing techniques with the selective purification of the poly(A) containing RNA by etiher affinity chromatography or adsorption to nitrocellulose filters. Several new techniques were applied to the purification of ovalbumin mRNA including Sepharose 4B chromatography and agarose gel electrophoresis in the presence of 6 M urea at pH 3.5. All the procedures used were adapted on a preparative scale to the fractionation of large quantities of RNA. The purity of the ovalbumin mRNA was assessed by several independent criteria. (1) Purified ovalbumin mRNA migrated as a single band during both agarose–urea and formamide–polyacrylamide gel electrophoresis at pH 3.5 and 7.4, respectively. A single absorbance peak containing all of the ovalbumin mRNA activity was also found using linear formamide–sucrose gradients. (2) Determination of both total mRNA activity and ovalbumin mRNA activity in the wheat germ cell-free translation assay revealed that 92% of the total peptides synthesized were specifically immunoprecipitable with an ovalbumin antiserum. (3) Analysis of the total peptides synthesized in the wheat germ assay by sodium dodecyl sulfate polyacrylamide gel electrophoresis demonstrated the presence of a single radioactive peak that corresponded exactly to a specifically immunoprecipitable ovalbumin standard. Thus, based on these observations ovalbumin mRNA appears to be greater than 95% pure. A preliminary estimation of the molecular weight of purified ovalbumin mRNA by formamide-containing sucrose gradients yielded a value of 520,000 or approximately 1600 nucleotides. This value was considerably less than the value of 900,000 obtained by gel electrophoresis under denaturing conditions. Analysis of the poly(A) content by a hybridization assay with [^{3}H]poly(U) revealed the presence of a poly(A) region containing approximately 70 adenosine residues. Thus, the size of the ovalbumin mRNA is considerably greater than that required to code for a protein of 387 amino acids. The availability of large quantities of purified ovalbumin mRNA should now permit a more thorough analysis of its physical and chemical properties.

Female sex steroids have been known to exert regulatory effects on the synthesis, activity, and possibly even degradation of tissue enzymes and structural proteins (O'Malley and Means, 1974). We have previously described and utilized the chick oviduct as a model system for investigating the mechanism of estrogen and progesterone action (O'Malley and Means, 1974; O'Malley *et al.*, 1967, 1969). Administration of estrogenic compounds to the new-born chick stimulates oviduct growth and differentiation and results in the appearance of a number of new specific intracellular proteins (Kohler *et al.*, 1969; Oka and Schimke, 1969; O'Malley *et al.*, 1967). In this context, estrogen has been shown to induce the synthesis of the egg-white protein, ovalbumin, to a level where it represents approximately 60% of the total synthesized protein in oviduct tubular gland cells (Palmiter *et al.*, 1970). Our previous studies have shown that a number of specific alterations in oviduct gene transcription precede this induction (O'Malley and Means, 1974; O'Malley *et al.*, 1969; O'Malley and McGuire, 1968) and that the rate-limiting event for induction of ovalbumin synthesis is the intracellular accumulation of biologically active ovalbumin mRNA (Means *et al.*, 1972; O'Malley and Means, 1974; Rhoads *et al.*, 1973).

Studies from several laboratories including our own (Rosenfeld *et al.*, 1972; Rosen *et al.*, 1974; Palacios *et al.*, 1973; Haines *et al.*, 1974) have indicated that a substantial purification of ovalbumin mRNA can be accomplished by a combination of precise sizing techniques with the selective purification of poly(A) containing ovalbumin mRNA by either affinity chromatography (Palmiter, 1973, Haines *et al.*, 1974) or adsorption to nitrocelluose filters (Rosenfeld *et al.*, 1972; Brawerman *et al.*, 1972). RNA extracted from either whole cells (Rosen *et al.*, 1974), total polysomes (Haines *et al.*, 1974), or specifically immunoadsorbed polysomes (Palacios *et al.*, 1973) has been used as the starting material for subsequent purification procedures. In all cases several problems inherent in most mRNA isolation procedures were encountered. First, a small amount of contamination of the ovalbumin mRNA with similarly sized 18S rRNA was found even using the best affinity chromatography methods (Palmiter, 1973; Haines *et al.*, 1974); secondly, following phenol extraction of RNA, ovalbumin mRNA had the tendency to form extremely stable higher molecular weight aggregates (Rosen and O'Malley, 1975; Haines *et al.*, 1974); third, product analysis in cell-free protein synthesizing systems was limited to specific immunoprecipitation methods and no effort was made to analyze for the presence of additional biologically active mRNAs. Finally, each of the procedures yielded only limited (μg) quantities of the purified ovalbumin mRNA. Isolation of milligram quantities of a purified ovalbumin mRNA is desirable for gene isolation experiments in which a specific mRNA is co-

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valently linked to an inert matrix (Shih and Martin, 1973) or for extensive structural studies and sequence analysis.

In order to overcome some of these difficulties we have developed several methods which are applicable on a preparative scale to the isolation and purification of ovalbumin mRNA. We have used both partially denaturing and fully denaturing conditions during the fractionation of ovalbumin mRNA by sucrose gradient centrifugation and by agarose gel electrophoresis. The purity of the ovalbumin mRNA was estimated by a careful product analysis of the total protein synthesized in a wheat germ cell-free protein synthesizing assay. In addition, a comparison was made between the specifically immunoprecipitable ovalbumin released from polysomes and the total released peptides synthesized *in vitro*. Finally, two different analytical gel electrophoretic procedures using denaturing conditions at both neutral and acidic pH's were employed to assess the purity of the ovalbumin mRNA. Using all of these criteria we estimate that the ovalbumin mRNA is of greater than 95% purity. Thus, it is a suitable template for the generation of a specific complementary [^{3}H]cDNA probe for ovalbumin mRNA sequences (S. E. Harris *et al.*, in preparation) and for preliminary structural and sequence analysis.

Experimental Procedures

Preparation of Total RNA. Extraction of RNA from the magnum portion of frozen hen oviducts was performed essentially as previously described (Rosenfeld *et al.*, 1972; Rosen *et al.*, 1974). Frozen tissue was stored in liquid N_2, broken into small pieces, and homogenized at room temperature in a small Waring Blendor in 5 volumes (v/w) of 0.5% sodium dodecyl sulfate (SDS),[1] 0.025 M Na_2EDTA, and 0.075 M NaCl (pH 8.0) containing 5 volumes of buffer-saturated phenol, pH 8.0. The resulting emulsion was chilled for 30 min in ice and centrifuged in 150-ml glass bottles at 6500*g* for 30 min. The aqueous upper phase and protein interphase were removed and reextracted with an equal volume of buffer-saturated phenol (pH 8.0) for 5 min at room temperature. Following centrifugation the aqueous phase was carefully removed, made 0.2 M in NaCl, and overlayered with an equal volume of cold 95% ethanol. The DNA was then carefully removed by spooling onto a glass rod and the remaining RNA precipitated by the addition of a second volume of cold ethanol and stored at −20° overnight. This total RNA extract was reprecipitated several times, rinsed with 100% ethanol, and dissolved in cold distilled water. The total extract still contained between 5 and 10% DNA as measured by a diphenylamine assay (Burton, 1968). Between 80 and 100 g of tissue can easily be processed in 1 day by a single person and routinely yielded between 400 and 500 mg of total RNA extract. Homogenization of the frozen tissue directly in phenol and SDS helped minimize RNase activity and the consequent loss of mRNA activity. This was a critical problem during oviduct polysome isolation procedures even using vast amounts of heparin as an RNase inhibitor (Palacios *et al.*, 1972, Rhoads *et al.*, 1973). In many instances what appeared to be intact polysomes by analytical sucrose gradient centrifugation contained ovalbumin mRNA which was biologically inactive.

Purification of Poly(A) Containing mRNA. Adsorption of poly(A) containing mRNA to nitrocellulose filters was performed as previously described (Rosenfeld *et al.*, 1972). Approximately 2 mg of the total RNA extract or 100–200 μg of the partially purified ovalbumin mRNA was applied per 25 mm, 0.45-μ nitrocellulose filter (Millipore Corporation, Type HAWP). Using this procedure 600 mg of a total extract can be processed by a single technician in 1 day.

Oligo(dT)-cellulose (6–18mer average) was obtained from Collaborative Research, Inc. Affinity chromatography using dT-cellulose was performed at room temperature by the method of Aviv and Leder (1972), with the omission of the 0.1 M KCl intermediate salt wash; 100 mg of the total RNA extract dissolved in 100 ml of buffer (0.5 M KCl–0.01 M Tris-HCl (pH 7.6) was applied at a flow rate of 0.5 ml/min to 10 g of the oligo(dT)-cellulose packed in a 1.6 × 10 cm column. The column was stored in 0.02% NaN_3 and stripped with 0.1 N NaOH between each run.

Analysis of mRNA poly(A) content by a specific hybridization assay with [^{3}H]poly(U) was performed essentially as described by Gillespie *et al.* (1972).

Preparative Sizing Techniques. (1) Zonal ultracentrifugation was performed using a Beckman Ti-14 rotor for 12 hr at 48,000 rpm at 21°. Approximately 30 mg of total RNA was dissolved in 0.004 M $NH_4C_2H_3O_2$ buffer (pH 7.0) containing 0.002 M Na_2EDTA and 1% SDS. The RNA was rapidly heated in this buffer to 70° for 5 min and immediately layered on a linear 10–40% (w/w) sucrose gradient in 0.1% SDS, 0.002 M Na_2EDTA, and 0.004 M $NH_4C_2H_3O_2$ buffer (pH 7.0). The sample was assayed at a rate of 20 ml/min and the absorbance monitored with an Isco UA-2 monitor at 254 nm. (2) Fractionation of total RNA was also accomplished by chromatography on Sepharose 4B in 0.1 M $NaC_2H_3O_2$–0.001 M Na_2EDTA (pH 5.0) at 4°. Approximately 100 mg of the total extract was applied to a 5 × 100 cm column and eluted at a flow rate of 30 ml/hr. When 15 mg of the filtered or dT-bound RNA was fractionated, a smaller 2.6 × 100 cm column was used and the flow rate reduced to 10 ml/hr. (3) Preparative gel electrophoresis was performed using a 2% agarose (Bio-Rad, electrophoresis grade) gel containing 6 M urea (Schwarz/Mann, ultrapure)–0.025 M citric acid (pH 3.5). Between 2 and 3 mg of the purified ovalbumin mRNA was applied to the gel. A detailed description of this technique and the apparatus used will be published elsewhere (S. L. C. Woo *et al.*, in preparation).

Analytical Sizing Techniques. (1) Analysis of RNA on 5–25% (w/w) linear sucrose gradients containing 70% formamide (Fisher F-95, $A_{270} < 1.0$), and 0.003 M Na_2EDTA, and 0.003 M Tris-HCl (pH 7.4) was performed as described by Suzuki *et al.* (1972). RNA samples were incubated at 37° for 15 min, layered, and centrifuged for 20 hr at 35,000 rpm in a Beckman SW40 rotor at 25°. (2) Characterization of RNA samples throughout the purification scheme was accomplished by electrophoresis on 1.5% agarose gels containing 6 M urea and 0.025 M citric acid (pH 3.5). RNA samples were dissolved in a urea–citrate layering buffering containing 20% sucrose (RNase free, Schwarz/Mann) and 0.005% Bromophenol Blue marker dye. The electrophoresis buffer was composed of only 0.025 M citric acid (pH 3.5). Electrophoresis was carried out for 7 hr at 2 mA/gel at 2° in a Buchler polyanalyst using 6-mm diameter gel tubes. Gels were stained for 30 min in a 1% Methylene Blue–15% acetic acid solution, destained overnight in water, and scanned at 600 nm using a Gilford 2400S spectrophotometer equipped with a Model 2410S linear transport. A slightly uneven background in the agar-

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; DTT, dithiothreitol; mRNA, messenger ribonucleic acid.

ose gel rather than discrete RNA bands accounted for the base line variability.

When ovalbumin mRNA activity was to be measured in the wheat germ assay, the RNA was extracted from unstained gels. Gels were initially scanned at 270 mm and then sliced manually with a razor blade. The slices were individually homogenized in cold 0.1 M $NaC_2H_3O_2$ buffer (pH 5.0) using a small Teflon pestle homogenizer. Following centrifugation for 15 min at 20,000*g* the aqueous upper layer was carefully removed and the gel rehomogenized in additional cold acetate buffer. The combined aqueous layers were made 0.5 M NaCl and the RNA was precipitated by the addition of 2 volumes of 100% ethanol and stored at −20° overnight. The resulting RNA was reprecipitated from acetate buffer, rinsed with 95% ethanol, and dissolved in distilled water. While recovery of both mass and mRNA activity from gels using this procedure was low (approximately 5% of starting RNA), there was adequate activity to identify ovalbumin mRNA in the wheat germ assay. Efficient extraction of microgram quantities of functional mRNA from either polyacrylamide or agarose gels especially when denaturing conditions were employed has proved to be especially difficult (J. M. Rosen, unpublished observations). These difficulties could be overcome by the use of preparative gel electrophoresis.

Purified ovalbumin mRNA was also analyzed by formamide polyacrylamide gel electrophoresis essentially as described by Boedtker *et al.* (1973). Electrophoresis was performed on 4% polyacrylamide gels containing 99% formamide and 0.02 M sodium phosphate (pH 7.4) at 5 mA/gel. Gels were again stained with a 1% Methylene Blue and 15% acetic acid solution, destained, and scanned at 60 nm. Molecular weight estimates of ovalbumin mRNA were determined by both acid-urea agarose gel electrophoresis and neutral formamide polyacrylamide gel electrophoresis using chicken 18S rRNA (MW 0.66×10^6) and 28S rRNA (MW 1.6×10^6) standards.

Translation Assay. Previous studies from our laboratory have utilized a heterologous cell-free protein synthesizing system derived from rabbit reticulocytes (Means *et al.*, 1972) to quantify ovalbumin mRNA activity. While this assay permitted the detection of specifically immunoprecipitable ovalbumin, high endogenous levels of globin synthesis made the analysis of total mRNA activity difficult. Therefore, in order to assess total mRNA activity as well as ovalbumin mRNA activity we have used the wheat germ cell-free system described by Roberts and Patterson, 1973. This cell-free system is characterized by low levels of endogenous protein synthesis and is able to translate exogenous mRNA with fidelity (Marcus *et al.*, 1970). Wheat germ was obtained from General Mills, Inc., Minneapolis, Minn., and stored at 4° in a vacuum desiccator until preparation of the 30,000*g* supernatant fraction (S-30). The S-30 was prepared essentially as described (Roberts and Paterson, 1973) except that the wheat germ was ground to a fine powder prior to the addition of buffer. After the homogenization buffer was added grinding was continued for an additional 2 min. The S-30 fraction was passed through the G-25 column without preincubation and stored as 50-μl aliquots in liquid N_2.

The components of the cell-free assay system were as follows: 24 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.6), 2 mM DTT (dithiothreitol), 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 40 μg/ml of creatine phosphokinase, 84 mM KCl, 2.5 mM $Mg(C_2H_3O_2)_2$, 20 μM each of unlabeled amino acids, and 4 μM [^{14}C]valine (260 Ci/mol). A 200-μl assay containing 40 μl of wheat germ S-30 was routinely employed, and the incubations were performed for 2 hr at 25°. At the end of the incubation a 50-μl aliquot was removed, diluted into 2.0 ml of 5% Cl_3CCOOH, and heated at 90° for 10 min. The samples were then chilled at 4° and the precipitates collected on glass fiber filters. The dried filters were placed in 5 ml of toluene–Spectrofluor and counted in a Beckman LS-250 liquid scintillation counter. The measurement of radioactivity incorporated into Cl_3CCOOH-insoluble material was used as an indication of total mRNA activity. An additional 100-μl aliquot of the original reaction mixture was used for the determination of ovalbumin mRNA activity by a specific immunoprecipitation procedure previously described (Means *et al.*, 1972). The specific activity of ovalbumin mRNA preparations were determined from the linear portions of the assay. Differing RNA inputs were used depending on the purity of the preparations tested. Thus, while 10–30 μg of the total RNA extract was assayed only 0.4–2.0 μg of the final purified ovalbumin mRNA was tested.

Product analysis of the total peptides synthesized in the wheat germ assay in response to exogenous mRNA was performed by SDS gel electrophoresis of the released polypeptide chains. Following a 2-hr incubation the ribosomes were removed by centrifugation at 105,000*g* for 1 hr at 4°. The postribosomal supernatant containing the released radioactive peptides was made 20 mM in Na_2EDTA and incubated with pancreatic RNase A (20 μg/ml) for 15 min at 37°. Samples were then adjusted to 1% SDS and 0.01 M DTT, heated at 90° for 5 min, and dialyzed against 500 ml of 0.1% SDS, 1 mM DTT, and 10 mM sodium phosphate (pH 7.0) overnight. Analysis on 10% polyacrylamide gels containing 0.1% SDS was performed as described by Weber and Osborn (1969). The gels were cut into 2-mm slices using a Gilson gel fractionator, digested with 0.5 ml of 30% H_2O_2 at 60° overnight, and counted in a Spectrofluor–Triton (2:1) scintillation cocktail.

Results

The application of preparative techniques to the isolation of specific mRNAs presented several problems not usually encountered during the isolation of ribosomal and transfer RNAs. As previously reported (Means *et al.*, 1972, Rosen *et al.*, 1974, Haines *et al.*, 1974) when either oviduct polysomal RNA or a total cell RNA extract was analyzed on linear sucrose gradients, the majority of ovalbumin mRNA activity was found in the 16S to 18S region of the gradients. However, in some experiments as much as 40–50% of the ovalbumin mRNA activity was found as higher molecular weight aggregates (Rosen *et al.*, 1974, Rosen and O'Malley, 1975, Haines *et al.*, 1974). Although zonal ultracentrifugation has proven to be an especially useful technique for the isolation of large amounts of hemoglobin mRNA (Williamson *et al.*, 1971), the problem of mRNA aggregation resulted in poor recoveries of ovalbumin mRNA activity during zonal ultracentrifutation using nondenaturing conditions. Attempts to disaggregate this higher molecular weight ovalbumin mRNA activity by treatment with EDTA, SDS, use of low salt gradients, or pretreatment with Me_2SO followed by reprecipitation proved unsuccessful (Rosen and O'Malley, 1975). However, this problem could be avoided by rapid heating of the total RNA extract to 70° followed by room temperature, zonal ultracentrifugation on

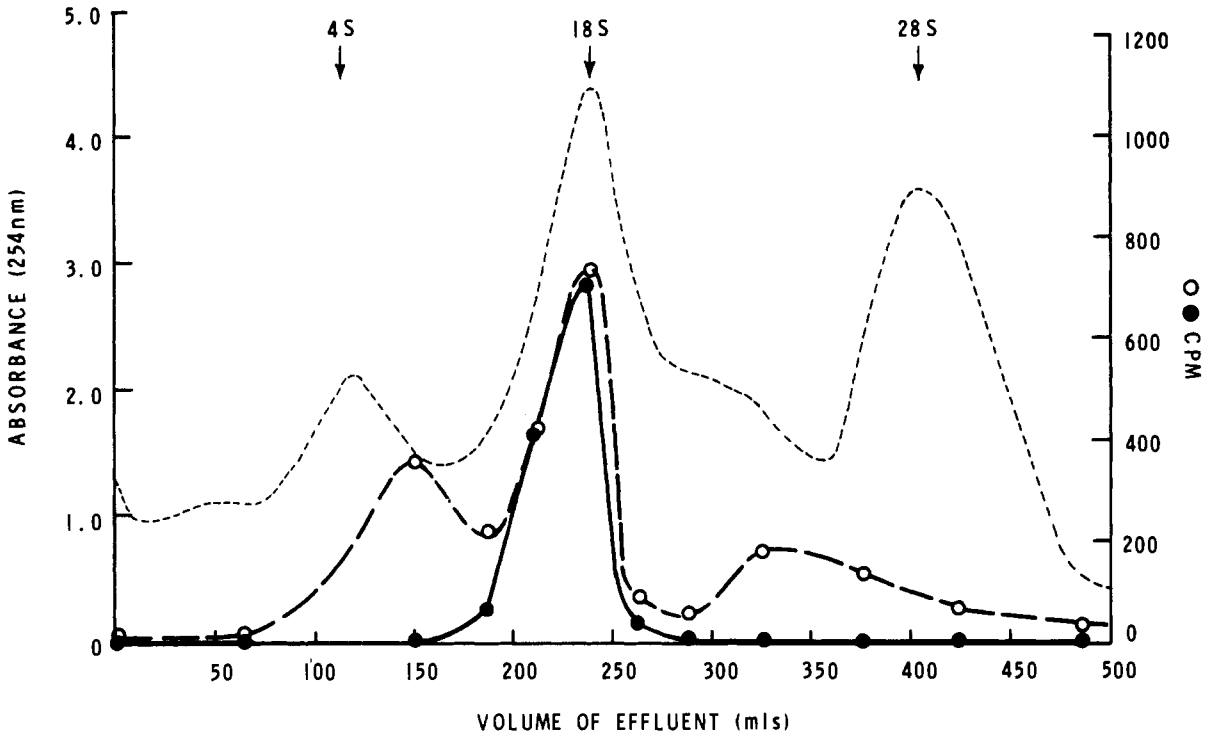

FIGURE 1: Zonal centrifugation analysis of oviduct total nucleic acid extract; 29.1 mg of hen oviduct RNA was treated with 1% SDS in 2 mM EDTA–4 mM $NH_4C_2H_3O_2$ buffer adjusted to pH 7.0 with NH_4OH. The sample was quick heated in this buffer to 70° for 5 min. Without cooling the sample was immediately layered on a linear 10–40% (w/w) sucrose gradient containing 0.1% SDS, 2 mM EDTA, and 4 mM $NH_4C_2H_3O_2$ (pH 7.0). The sample was centrifuged in a Ti-14 rotor for 12 hr at 21° at 48,000 rpm and was assayed at a rate of 20 ml/min. Absorbance was monitored with an ISCO UA-2 ultraviolet analyzer. Appropriate fractions were collected and assayed for mRNA activity as indicated under Experimental Procedures. (- - -). Absorbance at 254 mm; (●—●) ovalbumin mRNA activity × 10^{-3}; (○ - - -○) total mRNA activity × 10^{-4}.

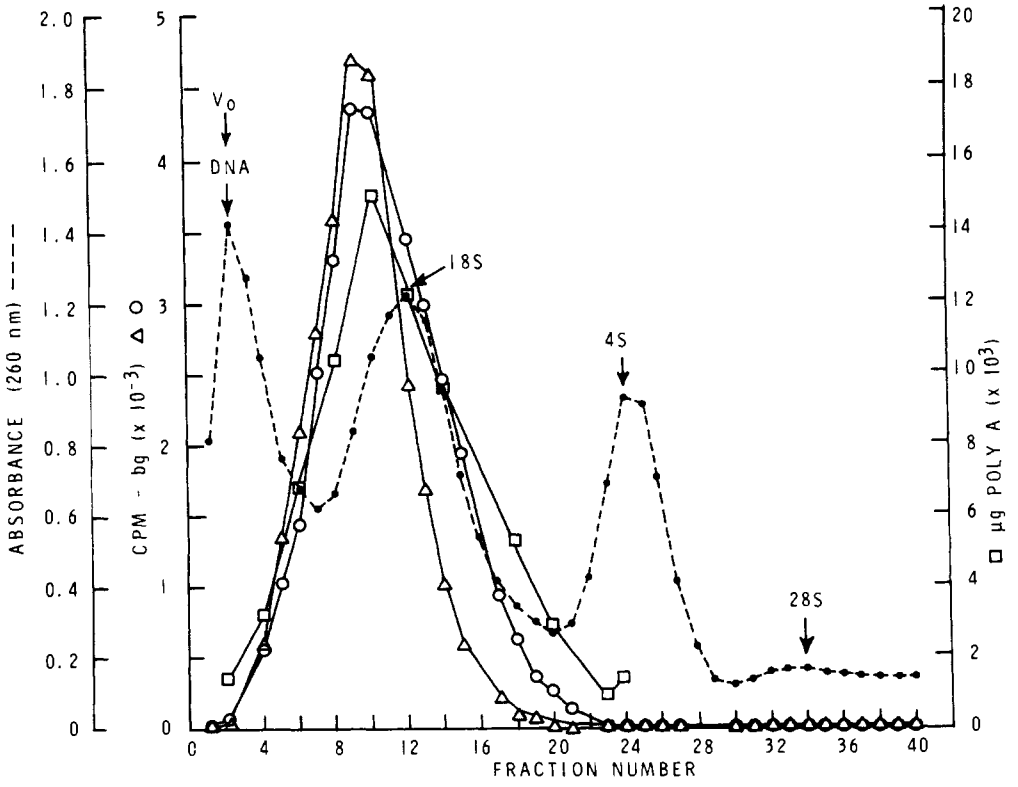

FIGURE 2: Sepharose 4B chromatography of oviduct total nucleic acid extract. Approximately 100 mg of the total RNA extract was fractionated on a 5 × 100 cm Sepharose 4B column as described under Experimental Procedures. Both total mRNA activity (○) and ovalbumin mRNA activity (△) were determined in the wheat germ assay as described. Poly(A) containing RNA (□) was detected by a specific hybridization assay with [^{3}H]poly(U) (Rosen *et al.*, 1974).

low salt containing (<10 mM) sucrose gradients (Figure 1). The ovalbumin nRNA activity sedimented as a broad band between 16S and 18S and was clearly separable from 4S to 28S RNA. Other functional mRNAs present in the 9S to 15S and 18S to 28S areas of the gradient were also separated from ovalbumin mRNA as shown by the determination of total mRNA activity in the wheat germ assay. No ovalbumin mRNA activity >18S was found indicating the lack of mRNA aggregates.

Although the preparative fractionation of oviduct RNA on linear sucrose gradients resulted in a substantial enrichment in ovalbumin mRNA activity, this method was unable to totally remove the sheared DNA present in the total nucleic acid extract. This DNA was observed as a broad band sedimenting between 14 S and 28 S. In addition, some of the DNA was adsorbed to Millipore filters under the conditions used to bind poly(A) containing mRNA. Removal of DNA was accomplished by chromatography of the total RNA extract on Sepharose 4B in 0.1 M sodium acetate buffer (pH 5.0) containing 0.001 M Na_2EDTA (Figure 2). Under these conditions, the DNA was excluded from the column and all the detectable ovalbumin mRNA activity appeared in a peak slightly preceding the 18S rRNA peak. As expected a peak of 4S RNA was eluted after the 18S rRNA peak. However, quite unexpectedly the elution of 28S rRNA was considerably retarded when the chromatography was performed in the 100 mM sodium acetate buffer. This allowed for almost the complete removal of contaminating 28S RNA from ovalbumin mRNA (see Figures 4 and 6C). This was somewhat fortuitous since 28S RNA was a major contaminant present after the adsorption of ovalbumin mRNA to nitrocellulose filters (Rosen *et al.*, 1974) or chromatography on dT-cellulose (Haines *et al.*, 1974). Furthermore, the ovalbumin mRNA was also partially separated from other smaller molecular weight mRNAs that were eluted with the trailing edge of the 18S rRNA peak. There was a reasonable coincidence between total mRNA activity determined in the wheat germ assay and poly(A) containing RNA as shown by the specific hybridization to [^{3}H]poly(U).

Further evidence that mRNAs as well as transfer and ribosomal RNAs could be fractionated by chromatography on Sepharose 4B was obtained by analysis of the peptides synthesized in the wheat germ system (Figure 3). RNA fractions obtained from the front side of the mRNA activity peak synthesized predominantly ovalbumin as demonstrated by the comigration of the radioactive peptides on SDS gels with a specifically immunoprecipitated ovalbumin standard. Moreover, a progressive increase in the proportion of smaller molecular weight peptides and a corresponding decrease in radioactivity in the region of ovalbumin was observed when RNA fractions from the peak and trailing side of the mRNA activity peak were assayed. Thus, different size classes of mRNA could be separated by chromatography on Sepharose 4B and a selective enrichment of ovalbumin mRNA (approximately six- to eightfold compared to the total RNA extract) could be obtained by pooling the appropriate fractions between the DNA and 18S peaks. Sepharose 4B chromatography did not require the sophisticated instrumentation necessary for zonal ultracentrifugation and also permitted the removal of the majority of contaminating DNA from ovalbumin mRNA; 100 mg of a total RNA extract was easily and reproducibly fractionated by this procedure. This technique was also utilized for the separation of mRNAs that had been previously enriched by

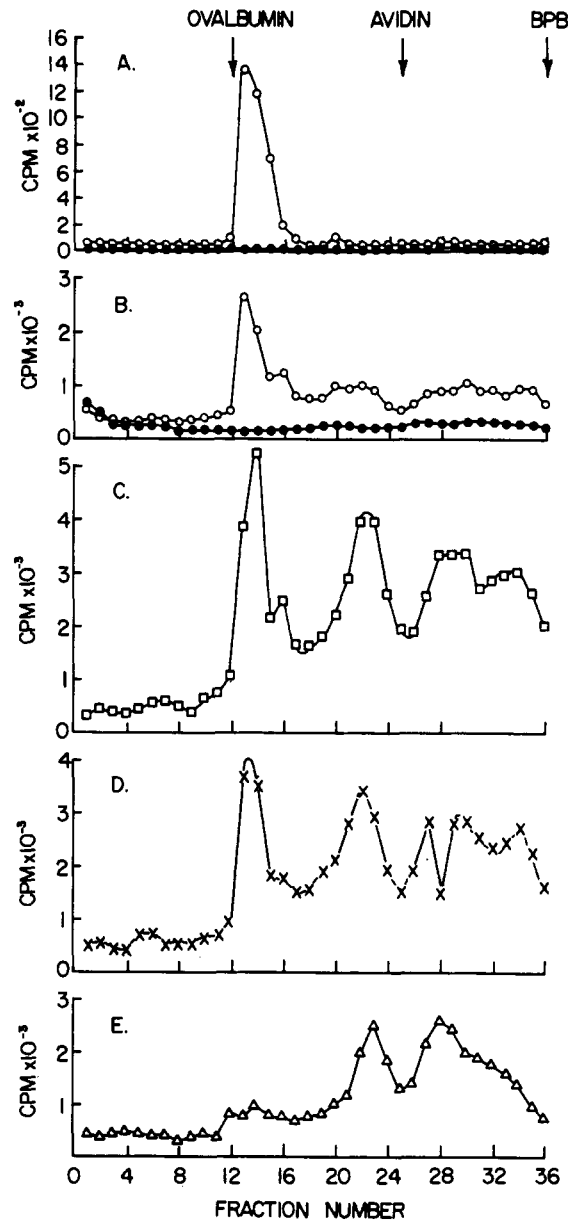

FIGURE 3: Product analysis of Sepharose 4B mRNA fractions. The radioactive peptides released into the postribosomal supernatant after a 2-hr incubation were analyzed on 10% polyacrylamide gels containing 0.1% SDS as described under Experimental Procedures. (A) Ovalbumin standard obtained by the specific immunoprecipitation of the cell-free products with monospecific ovalbumin antiserum (○); (B) Sepharose fraction 6 (○); (C) fraction 9; (D) fraction 12: (E) fraction 15. Panels A and B also show control gels (●) with no mRNA added to the wheat germ assay.

adsorption to nitrocellulose filters or dT-cellulose chromatography.

The application of Millipore filtration and dT-cellulose chromatography on a preparative scale to the isolation of poly(A) containing mRNA is summarized in Table I. Both techniques resulted in a substantial enhancement of ovalbumin mRNA specific activity compared to the total RNA extract. However, dT-cellulose chromatography resulted in an increased recovery of total ovalbumin mRNA activity (60–80% *vs.* 30% for Millipore filtration) and a slightly greater increase in specific activity (25-fold *vs.* 16-fold). Analysis of nitrocellulose- and dT-bound RNA by agarose gel electrophoresis under denaturing conditions revealed that both fractions were still contaminated with 28S and

TABLE I: A Comparison of Millipore Filtration and dT-Cellulose Chromatography.

	RNA Applied[a] (mg)	RNA Bound (mg)	Sp Act. (cpm/μg)	Purification (Fold)	Total Activity Recovered (%)
Millipore filtration	100	1.95	4000	16	31
dT-Cellulose chromatography	100	2.8	6250	25	70

[a] The specific activity of the total RNA extract applied was 250 cpm/μg.

18S rRNA (Figure 6B). Some DNA was still present in the Millipore-bound RNA (Figures 4 and 6B), but was absent after dT-cellulose chromatography (data not shown). A new absorbance peak in the 20S to 21S region of the gel was found with both preparations well separated from the 18S rRNA peak (Figure 6B).

In order to ascertain if the 21S RNA band contained ovalbumin mRNA activity, RNA was extracted from the gel and assayed in the wheat germ cell-free system (Figure 4). Ovalbumin mRNA that had been purified approximately 30-fold by a combination of Sepharose 4B chromatography and adsorption to nitrocellulose filters was applied to the gel. A single peak of both ovalbumin mRNA activity and total mRNA activity was found to coincide exactly with the 21S RNA peak. Although this technique permitted the detection of ovalbumin mRNA activity for analytical purposes, poor recoveries of mRNA activity from the gel by homogenization did not allow its application on a preparative scale.

Since ovalbumin mRNA had previously been reported to migrate as a 16S to 18S moiety on sucrose gradients (Means *et al.*, 1972; Rosen *et al.*, 1974) its migration as a 21S species during agarose gel electrophoresis was quite unexpected. In an attempt to resolve this discrepancy, the Sepharose-filtered ovalbumin mRNA preparation was also analyzed by sucrose gradient centrifugation under denaturing conditions (Figure 5). A sharp peak of ovalbumin mRNA activity was found sedimenting at 16 S in the formamide-containing sucrose gradients. This again coincided with the majority of total mRNA activity, although some additional mRNA activity was observed in the 6 S to 15S region of the gradients. The specific activity of the ovalbumin mRNA recovered from the formamide sucrose gradients was approximately 1.6 times greater than the Sepharose-filtered starting RNA. However, the resolving power of the sucrose gradient was not adequate to completely remove contaminating 18S rRNA. This ability of ovalbumin mRNA to migrate as a 21S species during gel electrophoresis and a 16S species during sucrose gradient centrifugation provided an additional analytical tool with which to test for contaminating 21S rRNA in purified ovalbumin mRNA preparations.

Having examined the properties of ovalbumin mRNA we were able to combine a series of alternate sizing and filtration procedures to achieve a 140-fold increase in the specific activity of ovalbumin mRNA compared to the total RNA

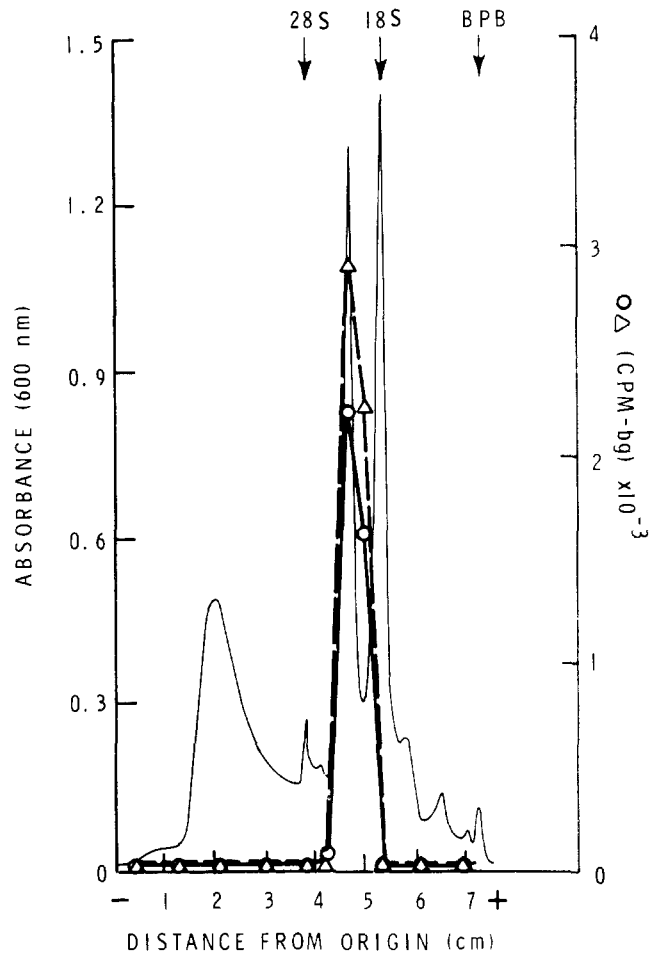

FIGURE 4: Determination of ovalbumin mRNA activity after agarose gel electrophoresis. Electrophoresis of 34 μg of ovalbumin mRNA partially purified by Sepharose 4B chromatography and adsorption to nitrocellulose filters. Extraction of the RNA and assay in the wheat germ translation assay was performed as described under Experimental Procedures. Total mRNA activity (Δ – – Δ); ovalbumin mRNA activity (O–O); absorbance at 600 mm of a parallel stained gel (·). The gel used for the determination of ovalbumin activity was scanned at 270 nm prior to extraction, but the high background due to the opaque nature of the agarose gel made the tracing less sensitive than stained gels scanned at 600 nm. The activity profile was therefore superimposed at the same relative position on the stained gel tracing. The first peak present at 2 cm is DNA as shown in Figure 6. The amount of DNA was unusually high in this particular preparation because of a slight overloading of the Sepharose column. It is completely absent in Figure 6C.

extract (Table II); 1 mg of purified ovalbumin mRNA can be generated by this procedure in 1 week starting with 2 g of total nucleic acid extract. Preparative agarose gel electrophoresis was used as the final step to remove the remaining contaminating 18S rRNA and some smaller molecular weight mRNAs (S. L. C. Woo *et al.*, in preparation). The percentage of immunoprecipitable ovalbumin in the wheat germ postribosomal supernatant fraction compared to the total peptides synthesized increased during purification from 45 to 92%. This is at best a minimal estimate since short, incomplete ovalbumin chains may not have been recognized by the ovalbumin antibody. Thus, essentially all of the detectable biologically active mRNA present in the purified preparation consisted of ovalbumin mRNA. Analysis of the poly(A) content throughout purification showed a reasonable correspondence with the determination of ovalbumin mRNA specific activity in the wheat germ assay. The final purified ovalbumin mRNA contained 4.2%

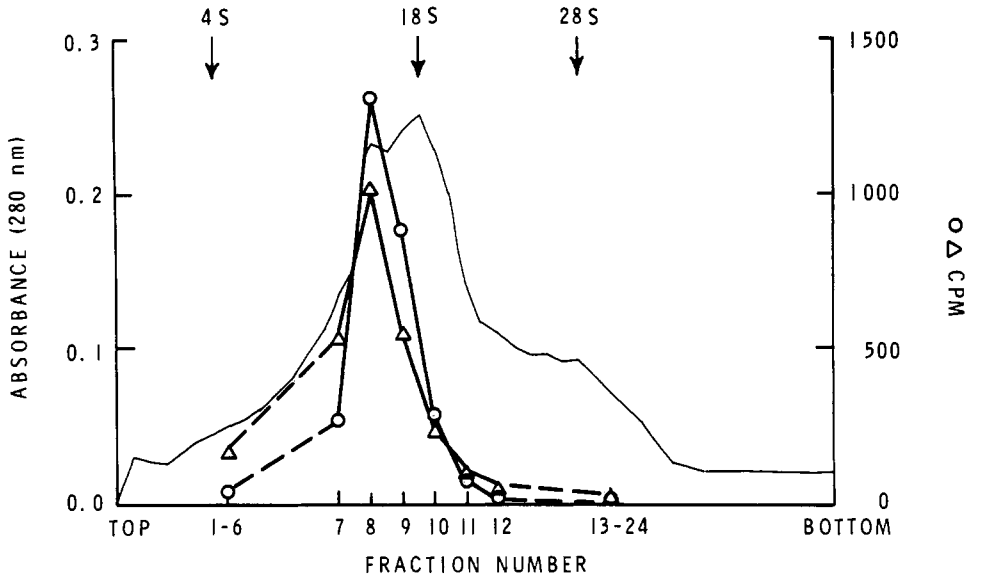

FIGURE 5: Purification of ovalbumin mRNA on formamide-containing sucrose gradients; 150 mg of Sepharose-filtered ovalbumin mRNA was fractionated on a 5–25% linear sucrose gradient containing 70% formamide. Centrifugation was performed in a SW40 rotor at 35,000 rpm for 23 hr at 25°. Gradients were fractionated into 20-drop fractions and the absorbance at 280 nm (–) was determined using an ISCO UA-4 absorbance monitor. Following alcohol precipitation of each fraction, the RNA pellets were rinsed with 95% ethanol, dried, and dissolved in cold deionized water. The determination of total mRNA activity (Δ – –Δ) and ovalbumin mRNA activity (O–O) was performed using the wheat germ translation assay as described.

TABLE II: Purification of Ovalbumin mRNA.

	Sp Act. (cpm/μg)	Yield (%)	Purification (Fold)	Ovalbumin Synthesized[a] / Total Protein Synthesized (%)	Poly(A) Content (%)
Total extract	305	100.0	1	45	0.061
Nitrocellulose-adsorbed RNA	4,810	31.0	16	65	1.29 (21×)
Sepharose peak	10,800	20.8	35	72	1.94 (32×)
Nitrocellulose-readsorbed RNA	11,910	11.3	39	73	3.09 (51×)
Preparative gel	42,452	6.7	140	92	4.21 (69×)

[a] These are the corrected values. Using a [^{14}C]ovalbumin internal standard only 85–90% of the Cl_3CCOOH-precipitable radioactivity was specifically immunoprecipitable. Therefore a correction factor of 10% was added to each value.

poly(A) as determined by the hybridization assay with [^{3}H]poly(U).

When RNA preparations at each step of the purification procedure were analyzed by agarose gel electrophoresis an increase in the amount of 21S RNA was observed that correlated with the increased activity of ovalbumin mRNA (Figure 6). The final purified RNA migrated as a single band at 21S on both agarose–urea gels at pH 3.5 (Figure 6E) and 99% formamide-containing polyacrylamide gels at neutral pH (Figure 6F). It was completely free of any contaminating 18S and 28S rRNA and DNA. The estimated molecular weight of ovalbumin mRNA using either of these gel electrophoretic systems was 900,000, corresponding to approximately 2800 nucleotides. When the purified ovalbumin mRNA was analyzed on formamide-containing sucrose gradients it sedimented as a sharp band at 16 S (data not shown). No 21S RNA was detected by absorbance at 270 nm and all of the ovalbumin mRNA activity was again present in the 16S peak. Molecular weight analysis on denaturing sucrose gradients resulted in an estimated value of 520,000 or approximately 1600 nucleotides.

An additional proof of the purity of the final ovalbumin mRNA preparation was obtained by a careful product analysis of the total peptides synthesized in the wheat germ assay (Figure 7). A single peak was detected on SDS gels that corresponded exactly to the ovalbumin synthesized in the wheat germ translation system and then precipitated with a specific antibody to authentic ovalbumin run on a parallel gel. There were no detectable smaller molecular weight peptides such as had been observed when the peptides synthesized in response to the ovalbumin mRNA preparation partially purified by Sepharose 4B chromatography were analyzed (Figure 3). Furthermore, greater than 95% of the radioactivity present in this single band was immunoprecipitable with a monospecific ovalbumin antiserum.

Discussion

Purification of a specific mRNA, such as ovalbumin, can be approached in a manner analogous to that of the purification of an enzyme such as calf thymus RNA polymerase. While there is a slight increase in initial polymerase specific activity when isolated nuclei are used as the source of en-

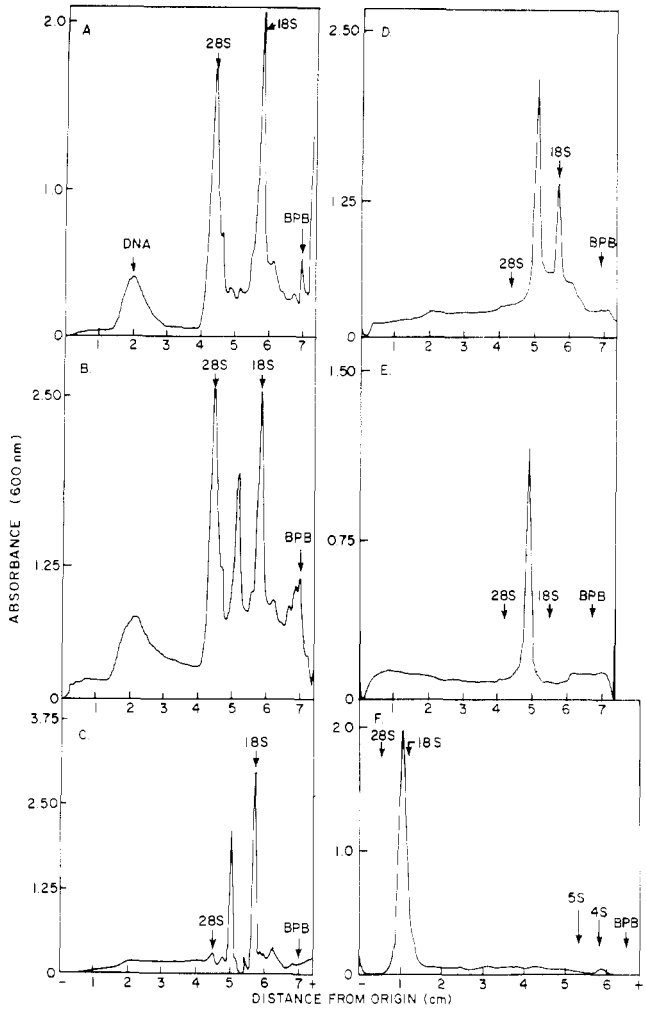

FIGURE 6: Sequential purification of ovalbumin mRNA. Panels A–E are scans of 1.5% agarose gels containing each of the RNA fractions described in Table II. (A) Total RNA extract (30 μg). (B) Millipore-bound RNA (25 μg). (C) Sepharose 4B peak activity fraction (15 μg). (D) Absorption of Sepharose 4B peak fraction (15 μg). (E) Preparative gel 21S fraction (10 μg). Panel F is a formamide-containing polyacrylamide gel profile of 10 μg of the same RNA fraction analyzed in E. The details of the gel electrophoretic procedures are described under Experimental Procedures.

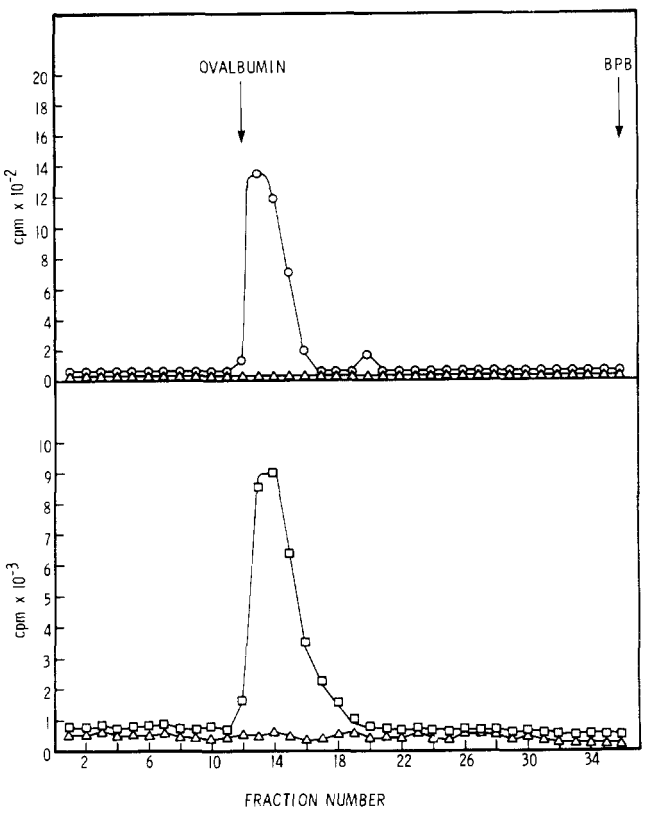

FIGURE 7: Product analysis of the peptides synthesized in response to pure ovalbumin mRNA. The top panel again contains a specifically immunoprecipitable ovalbumin standard (O). The bottom panel depicts the peptide analysis obtained when 1 μg of pure ovalbumin mRNA was assayed in a 100-μl wheat germ assay. Control gels with no mRNA added (Δ) are shown in each panel. The detailed procedures of the wheat germ assay and SDS gel electrophoresis are described under Experimental Procedures.

zyme, recovery of total polymerase activity is only one-third that when total cells are used as the starting source (Kedinger *et al.*, 1972). Thus, although the total oviduct RNA extract has a lower specific activity than oviduct polysomal RNA, the loss of mRNA activity due to RNase degradation inherent in subcellular fractionation procedures is minimized. In addition, large quantities of the total RNA extract can easily be obtained allowing for the subsequent preparation of milligram amounts of purified ovalbumin mRNA. While immunoadsorption of ovalbumin-synthesizing polysomes has been successfully used to prepare small amounts (μg) of partially purified ovalbumin mRNA (Palacios *et al.*, 1973), it is not easily adaptable to preparative scale procedures.

Purification of any mRNA is facilitated by choosing a tissue containing high levels of the mRNA in question and by the availability of a sensitive assay for mRNA activity. Since ovalbumin comprises about 60% of the protein synthesized on oviduct polysomes (Palmiter *et al.*, 1970) its mRNA would be expected to be the principal mRNA species present in a 16S fraction on sucrose gradients or a 21S fraction on gels. Evidence supporting this conclusion comes from the observation that the translation rates of each of the egg-white proteins appear to be similar (Palmiter, 1972). Furthermore, the activity of each of the extractable mRNAs for ovalbumin, conalbumin, lysozyme, and ovomucoid can be roughly correlated with the levels of these proteins in the oviduct (Palmiter and Smith, 1973). The sedimentation properties of each of these mRNAs are also different on sucrose gradients. Thus, a purification procedure based on precise sizing techniques and adsorption of poly(A) rich mRNA has the capability of generating a highly purified ovalbumin mRNA. We have successfully applied two new preparative sizing techniques to the purification of ovalbumin mRNA. Sepharose 4B chromatography was capable of fractionating messenger RNAs based on their respective sizes and allowed for the removal of both DNA as well as 28S, 5S, and 4S RNAs. In addition, agarose gel electrophoresis permitted the clear separation of ovalbumin mRNA from contaminating 18S ribosomal RNA and some other lighter molecular weight mRNAs. Preparative agarose gel electrophoresis has the capacity to permit rapid separation of large amounts of RNA (Weil and Hampel, 1973).

Agarose gel electrophoresis in 6 M urea at pH 3.5 had several advantages over polyacrylamide gel electrophoresis. Agarose gels require no polymerizing agents which may affect RNA. They are easily poured, physically firmer than low percentage acrylamide gels, and are directly adaptable to preparative gel electrophoresis (Weil and Hampel, 1973;

S. L. C. Woo et al., in preparation). In addition, agarose gel electrophoresis permitted the fractionation of high molecular weight RNA under denaturing conditions that minimized mRNA aggregation with comparable resolution to that obtained using acrylamide gels. Although 6 M urea did not appear to be an effective denaturing agent at neutral pH, no mRNA aggregates were found under the acidic conditions employed. No significant effect of the protonation of cytosine or adenosine residues at pH 3.5 was observed on the migration of high molecular weight RNA during agarose gel electrophoresis. Thus, ovalbumin mRNA migrated at 21S in both the urea–citrate buffer at pH 3.5 and the formamide–phosphate buffer at pH 7.4. Finally, small amounts of contaminating high molecular weight DNA did not clog the agarose gels as is prone to happen with polyacrylamide gels of greater than 3%.

The formation of stable aggregates of ovalbumin mRNA presented a serious problem in both molecular weight estimation and in preparative procedures designed to yield a discrete band of mRNA activity (Rosen and O'Malley, 1975; Haines *et al.*, 1974). mRNA aggregation may result from phenol extraction (Sedat and Sinsheimer, 1970) and can be prevented by the use of partially or fully denaturing conditions during fractionation procedures. A similar phenomenon has been observed for immunoglobulin light chain mRNA (Schechter, 1974) and may in that case be advantageous for the further purification of the light chain mRNA. However, as a rule it would appear that mRNA purification procedures, and especially the analytical methods used to assess mRNA purity, should incorporate denaturing conditions. Only then can the separation of mRNAs into discrete molecular species and the identification of any higher molecular weight precursors be assessed. Such conditions were found to be necessary during the purification of ovalbumin mRNA.

While ovalbumin mRNA is routinely observed as a 16S species in sucrose gradients under both nondenaturing (Means *et al.*, 1972; Palmiter, 1973) and denaturing (70% formamide) conditions (Figure 5), it migrates slower than 18 S on both agarose and acrylamide gels (Figure 5, Haines *et al.*, 1974). Although the reason for this discrepancy is not completely understood, a similar phenomenon has been observed for hemoglobin mRNA (Williamson *et al.*, 1971) and it does provide a basis for the separation of ovalbumin mRNA from 18S rRNA. It is possible that this difference between the molecular weight estimate by gel electrophoresis of 900,000 and that obtained by formamide–sucrose gradients of 520,000 reflects a slower migration of poly(A) containing mRNA than rRNA during gel electrophoresis (Morrison *et al.*, 1973). Accordingly, rRNAs may not be the appropriate molecular weight standards for mRNAs during gel electrophoresis. Even under the denaturing conditions of 99% formamide high GC regions in 28S and 18S rRNA may not be completely dissociated.

Determination of the true molecular weight of ovalbumin mRNA may be obtained by electron microscopy (Robberson *et al.*, 1971) or by chemical methods in which the length of the poly(A) segment is quantitated and compared to the percentage of the RNA that is poly(A) by hybridization. We have recently performed such experiments (S. L. C. Woo *et al.*, in preparation) and confirmed by three separate methods that ovalbumin mRNA is composed of $\sim$ 1600 nucleotides. This is greater than would be expected for a mRNA coding for a protein containing 387 amino acids (1161 nucleotides) even considering the addition of a poly(A) region containing 70 adenosine residues. It is clear that there are almost 400 additional noncoding nucleotides present in ovalbumin mRNA. Although similar discrepancies between the length of a mRNA and the size of the encoded protein have been observed for other eukaryotic mRNAs (Gaskell and Kabat, 1971; Brownlee *et al.*, 1973; Favre and Bertazzoni, 1974) the function of any additional nucleotide sequences remains to be elucidated. It is conceivable that ovalbumin mRNA may code for a much longer precursor molecule (Mach *et al.*, 1973). However, at present there is no evidence for the synthesis of precursors to ovalbumin in cell-free translation systems.

The length of the poly(A) region in ovalbumin mRNA can be estimated from the [^{3}H]poly(U) hybridization data (Table II) to be approximately 70 adenosine residues (4.2% $\times$ 1600 nucleotides). This length of poly(A) may partially reflect the use of Millipore filtration to purify the ovalbumin mRNA. This procedure only retains mRNAs with poly(A) tails longer than 50 adenosine residues (Lingrel, personal communication). When dT-cellulose chromatography was used ovalbumin mRNA containing shorter poly(A) regions of 20–50 nucleotides (Morrison *et al.*, 1973) would also be purified. This may account for the improved efficiency of dT-cellulose chromatography during ovalbumin mRNA isolation. In addition, while dT-cellulose chromatography permitted an increased recovery of ovalbumin mRNA activity, it was equally effective in the isolation of other contaminating mRNAs. Thus, Millipore filtration resulted in a larger increase in the ovalbumin mRNA activity to total protein ratio presumbly due to the partial loss of other mRNAs during the Millipore procedure. Indeed, a heterogeneity of length of poly(A) regions in mouse hemoglobin mRNA has recently been reported (Mansbridge *et al.*, 1974).

The absolute purity of a specific mRNA is difficult to assess without detailed sequence analysis (Suzuki *et al.*, 1972). Obviously, the determination of mRNA specific activity alone is not sufficient to assess mRNA purity. Inhibitors of translation activity may be removed during purification rather than increasing the actual mRNA concentration. Furthermore, determination of mRNA activity in heterologous cell-free translation systems with high levels of endogenous protein synthesis, *e.g.*, the rabbit reticulocyte lysate, may reflect the ability of an exogenous mRNA preparation to complete with endogenous mRNA for available ribosome binding sites rather than the true mRNA concentration. Therefore, to avoid these difficulties we have applied three separate criteria to determine the purity of ovalbumin mRNA. (1) Analysis of the final RNA product in two different gel electrophoretic systems under denaturing conditions revealed a single band migrating at 21 S. Moreover, when this material was analyzed on formamide-containing sucrose gradients a single peak of absorbance at 16 S containing all of the ovalbumin mRNA activity was found. (2) A comparison of ovalbumin mRNA activity with total mRNA activity in the wheat germ assay revealed that 92% of the peptides synthesized were specifically immunoprecipitated with ovalbumin antiserum. (3) Analysis of the total peptides synthesized in the wheat germ system by SDS gel electrophoresis demonstrated a single peak of radioactivity exactly corresponding to a radioactive ovalbumin standard. Preliminary evidence from our laboratory (S. L. C. Woo *et al.*, in preparation) has also suggested that ^{125}I-labeled ovalbumin mRNA has distinct pancreatic RNase and T_1 fingerprints compared to ^{125}I-labeled chick

18S and 28S rRNAs. Thus, these independent lines of evidence all suggest that the ovalbumin mRNA is at least greater than 95% pure. Detection of minor contaminants, such as other 21S mRNAs or 21S rRNA fragments is obviously limited by the sensitivity of present techniques. However, these results suggest that the ovalbumin mRNA is of sufficient purity to be used for base sequence and secondary structure analysis as well as in gene isolation experiments. These types of experiments require reasonably large amounts of mRNA. The purified ovalbumin mRNA may also be used as a template for viral RNA directed polymerase to synthesize a complementary [^{3}H]cDNA probe for ovalbumin mRNA sequences (S. E. Harris *et al.*, in preparation). The preparative techniques discussed in this manuscript may have general applicability to the isolation of other eukaryotic mRNAs.

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