Ovalbumin Messenger RNA of Chick Oviduct: Partial Characterization, Estrogen Dependence, and Translation In Vitro

(poly(A) sequence/estrogen action/protein synthesis/ sucrose gradient centrifugation)

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ABSTRACT A rapidly-labeled RNA fraction can be isolated from hen oviduct polysomes that has characteristics of the messenger RNA (mRNA) for the cell-specific protein, ovalbumin. This RNA, which sediments in the 8-17S region of sucrose gradients, possesses properties suggestive of the presence of a polyadenylate acid sequence and can be translated with fidelity in a cell-free protein synthesizing system derived from rabbit reticulocytes. The identity of the protein product as ovalbumin is confirmed by three methods, and translation of ovalbumin mRNA is shown to be dependent both on amount of exogenous mRNA and incubation time. Both rate and extent of ovalbumin synthesis is enhanced by the addition of a protein extract from polysomes that contains peptide chain initiation factors. Finally, the presence of this specific mRNA is shown to be estrogen-dependent: it is induced by estrogen administration to immature chicks, disappears upon cessation of estrogen treatment, and can be reinduced by a single injection of estrogen to chicks that have been pretreated with estrogen and then withdrawn from the hormone.

The cell-specific synthesis of ovalbumin serves as a characteristic biochemical marker for the estrogen-dependent differentiation of the chick oviduct (1-3). Studies to date have shown that estrogen induces changes in the pattern of synthesis of nuclear RNA before appearance of ovalbumin in the tissue (3-5). These observations are consistent with a hormone-regulated expression of the ovalbumin gene. However, the only direct assessment of messenger RNA (mRNA) is the ability of an RNA fraction to support the de novo synthesis of a specific protein in an in vitro translation system. Recently, rabbit reticulocyte lysate has been used successfully for this purpose by several laboratories, including our own (6-8). We now report data demonstrating that an 8-17S fraction of RNA isolated from oviduct polysomes contains the mRNA for ovalbumin that can be translated with fidelity in the reticulocyte system. Synthesis of ovalbumin in vitro shows a linear dependence on the amount of exogenous mRNA and is stimulated by addition of translation factors required for initiation of protein synthesis. Finally, the presence of the ovalbumin mRNA will be demonstrated to be directly dependent upon the degree of estrogen stimulation of the oviduct.

MATERIALS AND METHODS

Animals. Immature chicks used in this study were Rhode Island Reds and were 7-days old at the beginning of the experiments. Young laying hens (5-6 months) were either Rhode Island Red or White Leghorns. For hormone experiments, immature chicks received daily subcutaneous injections of diethylstilbestrol (5 mg) in sesame oil. Precise injection protocols are described in the text.

Isolation of Polysomes. Polyribosomes were isolated from chick oviduct as described. Fractionation of isolated polysomes on sucrose gradient has also been described (9, 10). For experiments with rapidly labeled polysomal RNA, oviduct was incubated in Eagle's minimal medium (1 ml/g) that contained [3H]cytidine (50 μCi/ml; 24.0 Ci/mmol) and [3H]-adenosine (50 μCi/ml; 27.4 Ci/mmol). Incubation was performed at 37° for 1 hr. Flasks were shaken under an atmosphere of 95% O2-5% CO2 throughout the incubation period. Polysomes were then isolated from the oviduct tissue (9, 10).

Isolation of Polysomal RNA. Oviduct polysomes were suspended in a buffer containing 5 mM Tris-HCl (pH 7.6), 5 mM MgCl2, and 0.5% sodium dodecyl sulfate (SDS) at a final concentration of 150 A260/ml. Polysomal RNA fractions were then extracted and isolated by sucrose gradient centrifugation (11). Only three fractions of RNA were collected: <8 S, 8-17 S, and 18-30 S. RNA was precipitated at -20° with 95% ethanol containing 0.2 M NaCl Precipitates were washed twice with 95% ethanol, and dissolved in water at a final concentration of 150 A260/ml. For estimation of RNA mass, 1 A260/ml was assumed to equal 40 μg. RNA samples were stored at -196° with no demonstrable loss of activity for 4 months.

Protein Synthesizing System. A 1:4 (v/v) lysate of rabbit reticulocytes was generously supplied by Dr. W. French Anderson of the NIH (12). Ribosomes and 0.5 M KCl ribosomal wash (reticulocyte ribosomal fraction I) were prepared from the lysate as described by Gilbert and Anderson (12). The protein synthesizing system contained, in a final volume of 0.5 ml: 0.2 ml lysate, 1.0 mM ATP, 0.2 mM GTP, 7.5 mM phosphoenol pyruvate, 1.5 enzyme units pyruvate kinase, 2.0 mM MgCl2, 20 mM Tris-HCl (pH 7.4 at 23°), 1 mM KCl; 20 μM each of 19 amino acids (minus valine), 10 μM [14C]valine (1.0 μCi; 572 dpm/pmol), and various amounts of RNA preparations. Incubation was performed at 37°. Aliquots (25 μl) were removed from each tube and acid precipitable material was prepared for counting as described (10).

Antiovalbumin Assay. The remainder of the reactions (475 μl) was used for the formation of an antiovalbumin-
ovalbumin complex. Tubes were prepared as follows: 100 µl antiovalbumin serum was first added, followed by 200 µl 5% sodium deoxycholate (w/v), 130 µl 4% Triton X-100 (v/v), the 475-µl sample, 10 µg (10 µl) ovalbumin carrier, and 40 µl of a buffer containing 10 mM NaPO4 (pH 7.5) and 15 mM NaCl. Reactions were allowed to stand at room temperature for 30 min. 1 ml Of wash buffer (10 mM NaPO4, pH 7.5; 150 mM NaCl; 3% Triton X-100; and 10 mM [3H]adenosine) was then added, and the tubes were thoroughly mixed on a Vortex mixer. The sides of the tubes were rinsed with 1 ml of 0.9% saline, and samples were centrifuged for 5 min at 10,000 X g. The supernatant fluid was aspirated, and precipitates were collected on 0.45-µ Millipore filters. Samples were then washed with 20 ml of buffer. Filters were dried 10 min under an infrared lamp and counted in Spectrofluor-toluene. Counting efficiency for 14C was 80-86%. In each experiment, several control tubes were included. Some controls contained liver or brain RNA and were prepared exactly as described above. Other tubes contained oviduct 8-17 S RNA but antibovine serum albumin was used instead of antiovalbumin. These controls, which ranged from 140-350 cpm, were subtracted from all experimental values.

Polyacrylamide Gel Electrophoresis. After incubation in the lysate, samples were centrifuged at 150,000 X g (RA2) for 2 hr. The supernatant fluid was removed and dialyzed overnight against 20 mM KPO4 (pH 7.0). Electrophoresis on 7.5% polyacrylamide gels and the manner of determining radioactive activity has been described (9).

Acrylamide-SDS Gel Electrophoresis. The antibody-antigen pellet was dissolved in 50 µl of a solution containing 10 mM dithiothreitol and 1% (w/v) SDS. Authentic [3H]ovalbumin (100 µg) prepared in our laboratory (13) was also added. We solubilized this mixture by heating to 70° for 2 min. After addition of 25 µl of 50% sucrose containing 1% bromphenol blue, electrophoresis was performed on SDS-acrylamide gels at pH 7.0 as described by Weber and Osborn (14). Gels were sliced at 1-mm intervals and radioactivity was determined (9).

Carboxymethyl cellulose Chromatography. The antibody-antigen precipitates from five reaction tubes were pooled, and 5.0 mg ovalbumin (Pentex) was added. Pellets were dissolved in 2 ml of 5 M NaCl for 1 hr at 22°. This mixture was then dialyzed overnight against three changes of 0.1 M NH4OAc buffer (pH 3.8). The sample was then applied to a carboxymethyl cellulose column equilibrated with the buffer at pH 3.8 and eluted with stepwise increases in the pH of the NH4OAc buffer as described by Rhodes et al. (13). 5-ml Fractions were collected and 2 ml of each was counted in Spectrofluor-toluene:Triton X-100 (2:1, v/v).

RESULTS

A typical polysome profile obtained from hen oviduct is shown in Fig. 1A. Several peaks of absorbancy at 254 nm can be seen, each representing a particular species of polysome (9, 10). When oviduct is incubated with [3H]cytidine and [3H]adenosine for 1 hr before polysome isolation, the radioactivity is found to be associated primarily with the heavier region of the polysome gradient (Fig. 1A). Extraction of these polysomes with SDS (11), followed by centrifugation on a 10-30% sucrose gradient, reveals that most of the radioactivity migrates on the light side of the 18S ribosomal RNA peak (Fig. 1B). Fig. 1C shows the radioactivity profile when the 8-17S fraction of RNA collected from the gradients (see Fig. 1B) is again centrifuged on 5-20% sucrose gradients. The radioactivity sediments as a single broad peak between the 4S and 18S marker RNAs.

Most of the radioactivity present in the 8-17S RNA fraction of the oviduct is retained on Millipore filters (Table 1). On the other hand little L-cell ribosomal RNA or oviduct 4S RNA is retained. When these filters are treated with RNase and then thoroughly washed, about 25% of the radioactivity in the 8-17S RNA fraction remains on the filter. Extraction of the RNase-resistant material with SDS and centrifugation on 5-20% sucrose-SDS gradients reveals that the radioactivity now exists in the greater than 4S region of the gradients (Fig. 1D). This material can be compared with the 8-17S fraction eluted from filters but not treated with RNase. Binding and subsequent elution from the filter does not alter the sedimentation characteristics of the 8-17S RNA fraction (see Fig. 1C and D). These data are consistent with the presence in the 8-17S RNA fraction of a sequence of polyadenylic acid (15).

Inubation of the reticulocyte lysate with [3H]ovalbumin, removal of the ribosomes, and electrophoresis of the supernatant fluid on 7.5% polyacrylamide gels yields a single major radioactive peak (Fig. 2A). Moreover, this peak is coincident with authentic hemoglobin marker. On the other
hand, when the reaction is performed in the presence of 100 μg of the 8–17S oviduct polysomal RNA, a second and much smaller peak appears (Fig. 2B). This peak, which comprises about 7% of the released acid-precipitable radioactivity, migrates on the gel to a position identical with ovalbumin standard.

In order to confirm the identity of the new protein product resulting from addition of oviduct 8–17S RNA as ovalbumin, we solubilized the antibody–antigen precipitate by heating in dithiothreitol after addition of authentic [3H]ovalbumin. This sample was subjected to electrophoresis on SDS-acrylamide gels, and the radioactivity profiles are shown in Fig. 2C. The 3H and 14C counts migrate as a single peak. Moreover, the radioactivity present in the [3H]ovalbumin and the 14C-labeled protein of the cell-free system coincide, indicating that the major product present in the antiovalbumin complex is ovalbumin.

Finally the antiovalbumin–[14C]antigen complex was solubilized in 5 M NaCl and, after the addition of ovalbumin standard, it was subjected to chromatography on a carboxymethyl cellulose column. Fig. 2D demonstrates the bulk of the 14C is eluted from the column at pH 4.8 and is thus coincident with ovalbumin A11 as described by Rhodes et al. (13). Moreover, it was possible to quantitatively precipitate the radioactive material recovered in the A11 peak with antiovalbumin serum.

The time course for the synthesis of oviduct mRNA-directed ovalbumin synthesis in the reticulocyte lysate is shown in Fig. 3. The rate of synthesis is linear for 30 min, at which point a much slower rate is reached. However, upon the addition of fraction I, prepared by extracting reticulocyte ribosomes with 0.5 M KCl, EDTA, and dithiothreitol (12), there is a marked stimulation in the rate of ovalbumin synthesis. Significant synthesis of ovalbumin can still be demonstrated after 3 hr of incubation.

The cell-free synthesis of ovalbumin is dependent upon the amount of added oviduct 8–17S polysomal RNA. Fig. 4 illustrates a linear increase in synthesis up to 90 μg of mRNA per reaction tube. At this point the [14C]ovalbumin comprises about 2–3% of the total protein synthesized (8). Additional amounts of the RNA fraction above 100 μg do not result in increased ovalbumin synthesis.

The preceding data have all dealt with the 8–17S RNA isolated from hen oviduct polysomes. Fig. 5 shows that hen RNA (H) can direct the synthesis of ovalbumin in the cell-free system. On the other hand, a similar amount of RNA obtained from polysomes of immature unstimulated chicks (U) does not possess ovalbumin mRNA activity. However, administration of estrogen to immature chicks for 16 days (16) results in appearance of ovalbumin mRNA activity. This RNA is about 60% as effective as an identical amount of hen oviduct mRNA. Again this ovalbumin mRNA activity is lost when the
TABLE 1. Characteristics of the rapidly-labeled polysomal RNA from chick oviduct: evidence for the presence of a poly(A) sequence

<table>
<thead>
<tr>
<th>Source of RNA</th>
<th>Untreated Acid-insoluble (cpm)</th>
<th>Untreated Bound to Millipore (cpm)</th>
<th>Untreated Bound to Millipore (%)</th>
<th>RNase-treated Bound to Millipore (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cell</td>
<td>5318</td>
<td>298</td>
<td>5</td>
<td>248</td>
</tr>
<tr>
<td>Oviduct 4 S</td>
<td>5514</td>
<td>1152</td>
<td>21</td>
<td>518</td>
</tr>
<tr>
<td>Oviduct 8-17 S</td>
<td>4589</td>
<td>4060</td>
<td>88</td>
<td>965</td>
</tr>
</tbody>
</table>

Standard [3H]ribosomal RNA was prepared from mouse L-cells by phenol extraction (specific activity, 3 × 10⁶ cpm/µg). Hen oviduct polysomes labeled with [3H]adenosine and [3H]cytidine were separated on sucrose gradients (9, 10), and RNA was extracted with SDS (11). The 4S and 8-17S RNA fractions from six sucrose gradients were pooled. Millipore binding and RNase treatment were as described by Lee et al. (15).

16-day treated chicks are withdrawn from estrogen (W). Finally, administration of estrogen to these withdrawn animals for 1, 2, and 4 days results in a progressive increase in the ability of the 8-17S RNA fraction to direct the synthesis of ovalbumin in vitro.

DISCUSSION

These studies demonstrate that rapidly-labeled, heterogeneous polysomal RNA from differentiated oviduct contains the mRNA that codes for the cell-specific protein, ovalbumin. The active RNA fraction, when released from polysomes with SDS, sediments on sucrose gradients between 8 and 17 S.

Recent reports have demonstrated that in rabbit reticulocytes the mRNA for globin chains contains a sequence of polyadenylic acid at the 3' terminal end (16, 17). This poly(A) region may be a property of all mRNAs and allows these RNA fractions to bind to membrane filters, whereas other types of RNA will not bind (15, 18, 19). Moreover, the poly(A) sequence is resistant to RNase and, upon treatment with this enzyme, sediments to the 4-8S region of sucrose gradients. The 8-17S fraction of oviduct polysomal RNA appears to possess many of these same characteristics. Thus, when it is labeled with adenosine, a significant portion binds to Millipore filters.

Fig. 3. Time course of synthesis of ovalbumin in the ovalbumin mRNA-directed reticulocyte lysate system. Assays were performed in duplicate, and each tube contained 100 µg of 8-17S polysomal RNA of hen oviduct. Radioactivity represents only those cpm which were precipitable with anti-ovalbumin (see Methods). Reticulocyte ribosomal fraction I, when added, was present at 40 µg protein (10 µl) per tube. Solid line: control; dotted line: plus fraction I.

Fig. 4. The relation between the amount of oviduct mRNA (8-17S) added to the lysate system and the amount of ovalbumin synthesized. Incubations in the complete protein synthesizing system were at 37°C for 30 min (see Methods). All reactions were performed in duplicate, and duplicate values agreed within 30 cpm.

Fig. 5. The effect of estrogen on ovalbumin mRNA activity. Incubation with 100 µg RNA was done at 37°C for 30 min. Values represent the average of duplicates. Source of RNA: (H) hen oviduct; (U) unstimulated oviduct from 7-day old chicks; (I) oviduct from unstimulated chicks that received 16 daily injections of estrogen; (W) immature chicks that received estrogen for 16 days and then were withdrawn from hormone treatment for an additional 16 days; (w+1, w+2, and w+4) W chicks to which estrogen was readministered for 1, 2, and 4 days, respectively.
filters and is RNase-resistant (Table 1). Furthermore, the RNase-resistant material sediments on sucrose gradients as a >4 S molecule. These data then suggest that the oviduct polysomal 8-17 S fraction of RNA may also contain a sequence of poly(A).

The rabbit reticulocyte lysate has been used by Stavnezer and Huang to synthesize immunoglobulin from a mRNA fraction isolated from a mouse plasma cell tumor (6). Rhoads et al. (7) and our own laboratory (8) have used such a system to synthesize ovalbumin from RNA extracted from hen oviduct and estrogen-stimulated chick oviduct, respectively. The identity of ovalbumin as the product was confirmed by Rhoads et al. (7), using SDS-gel electrophoresis and Dowex column profile of a trypic digest of the anti-ovalbumin precipitated reaction product. In the present studies we have used three methods to demonstrate that ovalbumin is synthesized in response to the addition of 8-17 S polysomal RNA from hen oviduct: (i) polyacrylamide gel electrophoresis of the peptides synthesized and released during incubation of the lysate; (ii) SDS-gel electrophoresis of the solubilized product of the antiovalbumin precipitin reaction; and (iii) cochromatography on a carboxymethyl cellulose column. These data then leave little doubt as to the authenticity of the product.

The oviduct mRNA-directed synthesis of ovalbumin is dependent both upon time of incubation and amount of message. Moreover the addition of an aliquot of a fraction (fraction I) prepared from reticulocyte ribosomes which contain peptide chain initiation factors (12) stimulates both the rate and the extent of ovalbumin synthesis. At the end of a 60-min incubation, about 95% of the globin chains are free in the supernatant fraction whereas only about 50% of the ovalbumin chains have been released. In studies to be reported elsewhere it will be demonstrated that addition of fraction I results in an increased proportion of ovalbumin molecules completed.

The activity of ovalbumin mRNA is clearly dependent upon the hormonal state of the animal. Estrogen induces activity when administered to immature chicks and cessation of estrogen injections results in a disappearance of ovalbumin mRNA. Readministration of this hormone to animals withdrawn from estrogen causes, after a single injection, reappearance of the ovalbumin message as assayed by the cell-free synthesis of ovalbumin. The appearance of mRNA for ovalbumin is consistent with the other existing data concerning the sequence of events initiated by estrogen in the chick oviduct (1-5, 9). Moreover, these data then support the much abused hypothesis that steroid hormones can, in fact, act in target tissues to promote the accumulation of specific mRNA.