Identification and Isolation of Ovalbumin-synthesizing Polysomes

I. SPECIFIC BINDING OF 125I-ANTI-OVALBUMIN TO POLYSOMES*

(Received for publication, November 3, 1971)

RAFAEL PALACIOS,‡ RICHARD D. PALMITER,§ AND ROBERT T. SCHIMKE

From the Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305

SUMMARY

Polysomes involved in ovalbumin synthesis were identified by the binding of 125I-anti-ovalbumin to hen oviduct polysomes. Techniques were developed for the isolation of undegraded hen oviduct polysomes and for the preparation of 125I-y-globulin free of ribonuclease activity. The distribution of 125I-anti-ovalbumin in the polysome profile is in accordance with the size of the polysomes that are expected to be synthesizing ovalbumin. Anti-ovalbumin does not react with polysomes extracted from tissues that do not synthesize ovalbumin, and antibodies directed against a protein that is not synthesized in the oviduct do not bind to hen oviduct polysomes. These criteria indicate that the reaction is specific for polysomes that synthesize ovalbumin. Polysomes that bind 125I-anti-ovalbumin can be precipitated by the addition of ovalbumin and an excess of specific antibody.

The avian oviduct is a system of choice for studying specific polysomes because (a) it synthesizes the majority of the egg white proteins (11, 12), one of which, ovalbumin, comprises as much as 60% of the total protein being synthesized; (b) there is an abundance of ribosomes (approximately 400 mg per hen oviduct magnum); and (c) the rate of protein synthesis is regulated by steroid hormones (13-15).

MATERIALS AND METHODS

Chemicals—Pancreatic RNase crystallized five times was from Worthington, and lactoperoxidase and crystallized bovine serum albumin were from Calbiochem. Sodium heparin was obtained from Fisher, sucrose (RNase-free) from Mann, and NCS solubilizer from Amersham Searle.

Animals—White leghorn laying hens were obtained from Kimber Farms, Fremont, California. Oviduct preparations were made within the first 3 hours after collecting the hens at Kimber Farms. To ensure that all of the hens were in an actively laying state, only those that had laid an egg or that had an egg in the oviduct the day the tissue was prepared were used.

Sterile Technique—in order to minimize the possibility of RNase contamination, all glassware was acid-washed and autoclaved, and all buffers and sucrose solutions with which polysomes were in contact were sterilized by autoclaving. All reactions with polysomes were carried out in sterile plastic tubes, and all solutions were handled with sterile plastic pipettes. Suspensions of DEAE and CM-cellulose and Sephadex G-100 in equilibration buffers were sterilized by autoclaving. Dialysis tubing was boiled in 5% NaHCO3 containing 0.1 mM EDTA, washed in distilled water, and autoclaved.

Oviduct Preparation—Hens were killed by decapitation, and the magnum portion of the oviduct was removed and placed on a Petri dish on ice. The oviduct was opened longitudinally with scissors and scraped with a scalpel, leaving behind the serous membrane. About 16 g of tissue were obtained from each oviduct. The oviduct preparation was either used immediately or divided into portions which were stored at -20°.

Homogenization—Homogenate was prepared with either fresh tissue or with tissue that had been frozen for up to 4 weeks at -20°.

* This work was supported in part by Research Grant GM 14931 from the National Institute of General Medical Sciences, Research Grant P-427C from the American Cancer Society, and a Public Health Service International Research Fellowship (F05 TW 1601).
‡ Permanent address, Departamento de Biologia Molecular, Instituto de Investigaciones Biomedicas, Universidad Nacional Autonoma de Mexico.
§ Present address, G. D. Searle Research Laboratory, Department of Biochemistry, High Wycombe, Buckinghamshire, England.

1 R. D. Palmiter, manuscript in preparation.

‡ The abbreviations used are: CM-cellulose, carboxymethyl-cellulose; anti-Ov, anti-ovalbumin; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.
for these tissues. These modifications were made in order to avoid polysome break-
min at 27,000 x g in a Sorvall refrigerated centrifuge, and the 5% sodium deoxycholate and 5% Triton X-100 were added; two volumes of a freshly prepared detergent solution containing
sodium heparin per ml. The tubes were centrifuged at 4° for 1 and 3 hours at 41,000 rpm (283,000 x g) in the SH 283 rotor (International Equipment). After centrifugation, an opales-
cent polysome band was present in the boundary of the two su-
crose layers. This band was extracted in about 0.7 ml of sucrose solution by puncturing the side of the tube with a sterile syringe approximately 5 mm below the band. The polysomes were dialyzed at least 6 hours against polysome buffer in an ice water
bath. After dialysis the preparation was centrifuged for 5 min at 27,000 x g and stored at 0°. In these conditions, polysomes showed no signs of degradation for at least 24 hours as determined by sucrose gradient sedimentation. Analysis of polysome pro-
files was performed as described previously (13).

Isolation of Polysomes—Aliquots of the supernatant (ti ml) were layered over a discontinuous sucrose gradient containing 2 ml of 2.5 m sucrose and 4 ml of 1.0 m sucrose (both sucrose
solutions were prepared in polysome buffer containing 40 μg of sodium heparin per ml). The tubes were centrifuged at 4° for 1 and 3 hours at 41,000 rpm (283,000 x g) in the SH 283 rotor. After centrifugation, an opales-
cent polysome band was present in the boundary of the two su-
crose layers. This band was extracted in about 0.7 ml of sucrose solution by puncturing the side of the tube with a sterile syringe approximately 5 mm below the band. The polysomes were dialyzed at least 6 hours against polysome buffer in an ice water
bath. After dialysis the preparation was centrifuged for 5 min at 27,000 x g and stored at 0°. These modifications were made in order to avoid polysome break-
down that was observed when the original technique was used for these tissues.

Purification of γ-Globulin—Rabbits were immunized with antigens that had been purified to electrophoretic homogeneity, and the γ-globulin fraction of serum was partially purified by ammonium sulfate precipitation as previously described (14). After dialysis against 10 m sodium phosphate, 15 m NaCl, pH 7.2, the γ-globulin (0.1 to 0.3 g) was applied to a 1.5-cm diameter column containing 5 cm of DEAE-cellulose overlaid with 5 cm of CM-cellulose, both equilibrated with the same
buffer as used for dialysis. The elution of γ-globulin from the column was monitored by measuring A280; the peak fractions were pooled and frozen.

Iodination of γ-Globulin—For every 10 ml of purified γ-globulin (8 to 12 mg per ml in the buffer described above but with pH adjusted to 7.5 with NaOH), 0.5 ml of lactoperoxidase (0.8 mg per ml in the same pH 7.8 buffer) and 0.1 ml of K131 (New England Nuclear, 10 mCi per ml, 2.4 μmoles per mCi) were added. The reaction was started by addition of 0.5 ml H2O2 (90 μl, freshly prepared) and continued for 15 min at room temperature with occasional shaking. The reaction products were then washed through an 0.8-cm diameter disposable column containing 1 cm of DEAE-cellulose overlaid with 1 cm of CM-cellulose prepared as above. The γ-globulin was concentrated by addition of ammonium sulfate to 40% saturation. The resultant precipitate was collected by centrifugation and dissolved in 10

mm sodium phosphate-0.1 mm EDTA, pH 7.8. The protein (approximately 30 mg in 2 ml) was applied to a Sephadex G-100 column (2.5 x 90 cm) equilibrated at 4° with the same buffer. Radioactivity migrated in a single peak. Radioactive fractions were pooled and frozen.

Characterization of Iodinated γ-Globulin—After the chromatography procedures described above, the γ-globulin was free to RNase, lactoperoxidase, and free iodine (see ”Results”). When treated electrophoretically on SDS acrylamide gels, the γ-globulin migrated as two peaks with molecular weights similar to the heavy and light chain of γ-globulin (Fig. 1). The final concentration of γ-globulin was 1 to 2 mg per ml and the specific activity ranged from 4 x 106 to 10 x 106 cpm per mg or protein (50% counting efficiency). The percentage of iodinated γ-globulin which is precipitated by ovalbumin was determined as before (14) and was found to be between 35 and 40%.

Measuring Radioactivity—Samples were precipitated with 5% trichloroacetic acid with 400 μg of carrier BSA, collected on Whatman GF/C filters, dissolved in NCS, and counted in a toluene-based scintillator fluid (14). For measuring radioac-
tivity in the pellets of the sucrose gradients, the bottom of the tube was cut and treated the same as the filters.

RESULTS

Relative Rates of Synthesis of Egg White Proteins in Hen Ovi-
duct—The relative rate of synthesis of the egg white proteins,
ovalbumin, conalbumin, ovomucoid, and lysozyme, was measured in the oviduct of actively laying hens. For this purpose, explants of oviduct were incubated in the presence of radioactive amino acids and then the radioactivity precipitated by antibodies directed against the specific proteins was compared to the total acid-precipitable radioactivity (Fig. 2). The results are similar to those obtained in chick oviduct after stimulation with steroid hormones1 and indicate that the major protein synthesized is ovalbumin (64%) and that ovalbumin, conalbumin, ovomucoid, and lysozyme account for more than 85% of the total protein synthesis.

Analysis and Isolation of Hen Oviduct Polysomes—A typical polysome sedimentation pattern from hen oviduct is presented in Fig. 3 (broken line). The profile is similar to that found in chick oviduct after estrogen stimulation (14). Most of the ribosomes (approximately 70%) migrate in the polysome region with the highest concentration in the region that corresponds to aggregates of about 10 to 14 ribosomes. When hen oviduct was incubated for 5 min with radioactive amino acids, the radioactivity was predominantly in the polysome region (10). The absence of a radioactive peak in the monosome region is an indication that polysomes are not degraded extensively during isolation and analysis. The yield of ribosomes (including monosomes) has varied in different preparations from 10 to 18 mg per g of hen oviduct. The extraction procedure described gives about 95% recovery of ribosomes.3 Polysomes isolated from oviduct frozen at -20° have the same sedimentation properties as those isolated from fresh tissue; hence, oviduct tissue was frequently frozen before use.

To obtain undegraded polysomes separated from the soluble components of the cell and in reasonably high concentrations, we used a method which does not involve pelleting the ribosomes (see "Materials and Methods"). Whenever we tried to resuspend pelleted polysomes they were significantly degraded, as inferred from their sedimentation characteristics. The method described here gives a polysomal profile which is nearly identical with the original profile except that fewer small polysomes and monosomes are present (Fig. 3, solid line). Approximately 60% of the polysomes were recovered by this method (36% of total ribosomes) and the concentration varied from 1.5 to 2.5 mg per g of hen oviduct. The extraction procedure described gives about 95% recovery of ribosomes.

Fig. 2. SDS-acrylamide gel electrophoresis of proteins synthesized by explants of laying hen oviduct in culture. The explants were incubated for 1 hour with a radioactive amino acid mixture (50 μCi per ml) in Medium 199 as previously described (14). The tissue was homogenized as in the preparation of polysomes (see "Materials and Methods") and centrifuged for 1 1/2 hours at 40,000 g. An aliquot (150 μg of protein) of the supernatant was precipitated and washed with trichloroacetic acid, dissolved in SDS, and treated electrophoretically as described (14). The migration of marker proteins is indicated by C, conalbumin; O, ovalbumin; and L, lysozyme. Ovomucoid migrates the same as ovalbumin.3

Inset, the relative rate of synthesis of egg white proteins. Aliquots of the supernatant were precipitated with specific antibodies against each of the proteins (14)? and the percentage of the total trichloroacetic acid-precipitable radioactivity which was immunoprecipitable is indicated. In a control experiment, BSA was added to an aliquot and precipitated with anti-BSA; 0.3% of the total radioactivity was precipitated and this number has been subtracted from the values above. All precipitations were performed in duplicate with an excess of antibody as previously described (14).

Fig. 3. Polysome profiles of hen oviduct supernatant (---) and of a purified polysome preparation (---). One milliliter of 10% hen oviduct supernatant or 1.0 ml of the purified polysome preparation containing 10 A260 units per ml was layered over a 11.4-ml continuous sucrose gradient (0.5 to 1.5 M) and centrifuged for 1 and ½ hours at 283,000 gmsx in the SB 283 rotor (International Equipment). The optical density profile was measured with a 2-mm path length cell and then multiplied by 5 (13).
shifts the sedimentation to that of monosomes, and dialysis molecules are specific for ovalbumin). A significant amount of natant, the polysome region, and the pellet. Most of the radioactivity is in the supernatant region and represents unreacted with 125I..anti-Ov, radioactivity is distributed among the super-y-globulin molecules (recall that only 40% of the y-globulin Fig. 4A shows that after incubation of hen oviduct polysomes the antibody preparations used in this work contained no RNase activity when analyzed with polysomes as substrate. Preparation of 125I-y-Globulin Free of RNase Activity—To study the binding of antibodies to polysomes, it was essential to have a y-globulin preparation free of RNase activity. RNase activity of different antibody preparations was tested by incubating polysomes in the presence of large amounts of the y-globulin fraction, and the polysome profiles obtained were compared with those of control polysomes. This represents a very sensitive assay for RNase activity. Crude y-globulin fractions were freed of RNase activity by DEAE- and CM-cellulose chromatography. During the iodination procedures RNase activity reappeared. It was found that the lactoperoxidase preparation completely degrades polysomes. A combination of DEAE- and CM-cellulose and Sephadex G-100 chromatography was used to free the iodinated y-globulin preparation of RNase activity and other contaminants (see "Materials and Methods"). All of the antibody preparations used in this work contained no RNase activity when analyzed with polysomes as substrate.

Binding of 125I-Anti-Ov Antibody to Hen Oviduct Polysomes— Fig. 4A shows that after incubation of hen oviduct polysomes with 125I-anti-Ov, radioactivity is distributed among the supernatant, the polysome region, and the pellet. Most of the radioactivity is in the supernatant region and represents unreacted y-globulin molecules (recall that only 40% of the y-globulin molecules are specific for ovalbumin). A significant amount of label is present in the polysome region. An important characteristic of this binding is that it is not uniformly distributed along the polysome profile but is associated largely with the region that corresponds to large polysome (indicated by the specific activity measurements of Fig. 4A, histogram).

Fig. 4B shows that sites available for binding anti-Ov can be saturated. When the polysome preparation was incubated with a large amount of unlabeled anti-Ov before adding 125I-anti-Ov, the binding of 125I was inhibited. That the sites are specific for anti-Ov is indicated by the experiment in Fig. 4C, where the polysomes were incubated with unlabeled anti-BSA before adding the 125I-anti-Ov. In this case, the nonspecific antibody failed to prevent the binding reaction. Moreover, Fig. 4D indicates that 125I-anti-BSA does not bind to hen oviduct polysomes.

To investigate further the specificity of the binding reaction of anti-Ov to polysomes, we studied hen liver and brain polysomes prepared by methods similar to those used for oviduct polysomes, introducing some minor modifications that were necessary to prevent polysome breakdown (see "Materials and Methods"). Fig. 5, A and B shows that neither brain nor liver polysomes bound 125I-anti-Ov. Moreover, 125I-anti-Ov does not bind to liver polysomes even when oviduct supernatant is mixed with liver homogenate prior to isolating liver polysomes (Fig. 5C). This indicates that there is no nonspecific association of ovalbumin with polysomes to account for the binding of the 125I-anti-Ov.

The binding characteristics with three different quantities of 125I-anti-Ov are shown in Fig. 6. The distribution of radioactivity along the profile shows the same qualitative pattern at different concentrations of anti-Ov. The percentage of y-globulin bound decreases as the concentration of y-globulin present in the reaction increases, suggesting saturation of the available binding sites. The amount of radioactivity in the pellet is a constant proportion (~40%) of the total polysome bound radioactivity. When liver and brain polysomes were used for the binding reaction (Fig. 5), radioactivity was not found in the pellet, indicating that it is not due to aggregation of unreacted anti-Ov. Furthermore, RNase treatment of polysomes after the binding reaction prevents the pelleting of this radioactivity, suggesting its association with polysomes (10). With the centrifugation conditions used in this study, approximately 10% of the ribosomes normally are recovered in the pellet. Hence, the radioactivity found in the pellet could be due to (a) the association of 125I-anti-Ov with rapidly sedimenting polysomes, or (b) the immunological cross-linking of two or more polysomes that normally sediment at lower rates. To distinguish between these possibilities, polysomes recovered in the pellet after sucrose gradient centrifugation (without addition of y-globulin) were re...
The main emphasis of this paper is to demonstrate the specificity of this binding reaction. Several lines of evidence support the proposal that the $^{125}$I-anti-Ov is reacting with those polysomes that synthesize ovalbumin. The $^{125}$I radioactivity in polysomes is not distributed randomly, but, rather, it is associated mainly with large polysomes (Fig. 4) which is in accordance with the size of the polysomes that are expected to synthesize ovalbumin (13). The binding of $^{125}$I-anti-Ov to hen oviduct polysomes can be saturated, indicating that the antibody is reacting with specific sites (Figs. 4 and 6). An antibody prepared against a protein that is not synthesized in the oviduct does not bind to hen oviduct polysomes (Fig. 4), and, conversely, $^{125}$I-anti-Ov does not react with polysomes extracted from tissues that are not engaged in ovalbumin synthesis (Fig. 5). When oviduct supernatant is mixed with liver before the isolation of polysomes, the $^{125}$I anti-Ov does not react with the liver polysomes (Fig. 5C), indicating that the binding is not due to a nonspecific association of ovalbumin with ribosomes. Moreover, when polysomes were incubated with radioactive ovalbumin no radioactivity was found in the polysome region (10).

Theoretically, the method of binding iodinated antibodies to polysomes has sufficient sensitivity to detect nascent chains which were incubated with added ovalbumin (to obtain 1 mole of ovalbumin per mole of the specific antibody) and then an excess of cold anti-Ov $\gamma$-globulin was added (to obtain 6 molecules of specific antibody per molecule of ovalbumin), the radioactivity incorporated in the polysome region pelleted with about 30% of the $A_{260}$ of the gradient (Fig. 5). The radioactivity recovered in the pellet was about 40% of the total $^{125}$I-anti-Ov added to the preparation, which is the percentage expected if all of the specific anti-Ov antibody was precipitated by ovalbumin. The most outstanding characteristic of the precipitation reaction is that it affected mainly large polysomes, as can be concluded by comparing the polysome profiles before and after precipitation (Fig. 7B). This is in accordance with the distribution of $^{125}$I-anti-Ov across the polysome profile (Fig. 6). That the polysome precipitation is not due to nonspecific trapping of polysomes in the antigen-antibody precipitate is demonstrated in an experiment in which the same steps were used to form a precipitate between BSA and its corresponding antibody in the presence of polysomes with bound $^{125}$I-anti-Ov (Fig. 7C).

The characteristics of the polysome profile were the same as those of the control with almost no decrease (about 3%) in the total $A_{260}$ recovered in the polysome region. The quantification of this precipitation reaction and the isolation of RNA and nascent polypeptide chains from the immunoprecipitated polysomes will be presented in a companion paper (10).

**FIG. 6.** Binding of different concentrations of $^{125}$I-anti-Ov to hen oviduct polysomes. Polysomes (10 $A_{260}$ units in 1 ml) were incubated 60 min at 4° with (A) 0 $\mu$g, (B) 50 $\mu$g, and (C) 150 $\mu$g of $^{125}$I-anti-Ov. After the reaction, polysome profiles and radioactivity were determined as described in Fig. 4.

**FIG. 7.** Precipitation of hen oviduct polysomes that bind $^{125}$I-anti-Ov. Polysomes (10 $A_{260}$ units in 1 ml) were incubated at 4° as follows: A, 150 $\mu$g of $^{125}$I-anti-Ov for 30 min; B, 150 $\mu$g of $^{125}$I-anti-Ov for 30 min, then 15 $\mu$g of purified ovalbumin for 30 min, and finally 625 $\mu$g of unlabeled anti-Ov for 30 min; C, 150 $\mu$g of $^{125}$I-anti-Ov and 150 $\mu$g of unlabeled anti-BSA for 30 min, followed by 15 $\mu$g of purified BSA for 30 min, and then 625 $\mu$g of unlabeled anti-BSA. The polysome profile of experiment A is represented as a broken line in B. After the reaction, polysome profiles and radioactivity were determined as described in Fig. 4.

The results show that $^{125}$I-anti-Ov binds to hen oviduct polysomes. The importance of preventing RNase activity cannot be overemphasized. Both crude $\gamma$-globulin fractions and the lactoperoxidase used in the iodination procedures had substantial RNase activity. By using sterile techniques and DEAE- and CM-cellulose and Sephadex G-100 chromatography, this activity can be removed from the $\gamma$-globulin, and the polysomes are completely stable when incubated with these preparations. Another potential problem is the contamination of polysomes with supernatant proteins. Small amounts of ovalbumin (0.1 to 1.0 $\mu$g per 10 $A_{260}$ units of polysomes) can obscure the binding of antibody to polysomes and lead to uninterpretable results.
constitute as little as 1% of the total population. We are aware of only one previous study of iodinated antibody binding to nascent chains. Warren and Peters (3) used 125I-anti-albumin in an attempt to localize albumin-synthesizing polysomes isolated from rat liver. Their results are difficult to interpret due to non-specific binding of control antibodies to polysomes and the lack of evidence that the polysomes were undegraded and uncontaminated by soluble antigen. We have investigated the binding of 125I-anti-conalbumin and 125I-anti-lysozyme to hen oviduct polysomes. Preliminary experiments suggest that anti-conalbumin binds specifically; however, the characteristics of the binding of anti-lysozyme are peculiar and to date uninterpretable. Thus, the binding of immunoglobulins to polysomes may be quite variable and the reaction must be studied carefully for each combination of antibody and polysomes.

In addition to identifying specific polysomes, the binding of antibodies to polysomes has important consequences such as: (a) the possibility of quantitating the relative number of specific nascent chains in a polysomal preparation and hence indirectly measuring the amount of translatable mRNA, and (b) the precipitation of specific polysomes, either directly with antigen or indirectly with anti-immunoglobulins. Previous studies have shown that ribosomes from liver, muscle, and plasma cells can be precipitated with antibodies to albumin (9), myosin (8), and immunoglobulins (6, 7), respectively. However, the specificity of the precipitation reaction has not been demonstrated completely in most of these studies. Recently Holme et al. (16) have reported the precipitation of rabbit reticulocyte ribosomes using antisera against rabbit hemoglobin. When complete γ-globulin molecules were used, specific and control antibodies precipitated the same proportion of polysomes, but if the antibodies were digested with pepsin to remove the Fe portion of the γ-globulin, the precipitation with the control antibody was avoided. Another potential approach for isolating specific polysomes is the use of affinity chromatography in which the nascent polypeptide chains may react with specific ligands bound to a column (17).

Fig. 7 shows that those polysomes which bind 125I-anti-Ov can be precipitated by adding ovalbumin and an excess of unlabeled anti-Ov. The characteristics and the specificity of this precipitation reaction are described in a companion paper (10). The ability to isolate undegraded polysomes synthesizing a single polypeptide chain will be a valuable tool for isolating and characterizing the components (mRNA and mRNA-specific proteins) involved in the synthesis of specific proteins.

Acknowledgments—We thank F. Dice for determining optimal conditions for iodinating proteins and Dr. R. Rhoads for critically reading this manuscript.

REFERENCES
