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Effect of estrogen on gene expression: Purification of vitellogenin messenger RNA

[immunoprecipitated polysomes/polysomal RNA/poly(U)-Sepharose chromatography/rabbit reticulocyte lysate]

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ABSTRACT **We report initial studies on estrogen-mediated regulation of egg yolk protein synthesis in the rooster. Egg yolk proteins are normally synthesized as a large precursor, vitellogenin, in the liver of the laying hen; roosters synthesize vitellogenin only when treated with estrogen. Polysomal RNA from the liver of estrogen-treated roosters was translated in a reticulocyte cell-free system, and the newly synthesized proteins were identified by a highly specific and sensitive indirect immunoprecipitation reaction. The messenger RNA that specifies vitellogenin has been purified more than 800-fold from rooster liver polysomal RNA by a combination of methods, including immunoprecipitation of polysomes and chromatography of RNA on poly(U)-Sepharose.**

In recent years an important approach has been developed for studying the molecular mechanisms by which hormones regulate gene expression in eukaryotic organisms. The goal of this approach is to study the regulatory effects of hormones *in vitro*, and involves the following steps: isolation of a specific messenger RNA (mRNA) from the target tissue after administration of hormone; identification of this mRNA by its ability to program the synthesis of a specific protein *in vitro*; purification of the specific mRNA to homogeneity; use of the pure mRNA as a template for synthesis of complementary DNA; and, finally, use of the complementary DNA as a probe for hybridization studies of *in vitro* transcripts from purified components with RNA polymerase and chromatin (1–14). Studies successfully utilizing this approach have been limited to specialized tissues in which the potential for gene expression is severely limited. We have recently begun a study along the general lines of this approach but have chosen to study specific regulation of gene expression in liver, a tissue with diverse genetic potential. Our studies involve an investigation of the induction of vitellogenin synthesis by estrogen in rooster liver.

Avian vitellogenin is a phosphoprotein that is normally synthesized in the liver of laying hens but is synthesized in roosters only after administration of estrogen. Vitellogenin was first identified in the plasma of estrogen-treated male *Xenopus laevis* (15, 16). After its synthesis in the liver, this phosphoprotein is transported through the plasma to the oviduct where it is deposited in the developing oocyte as lipovitellin and phosvitin, the two amphibian egg yolk proteins that are formed by cleavage of vitellogenin (16). Bergink *et al.* (17) have suggested that vitellogenin might also exist in the plasma of laying hens and estrogen-treated roosters. Deeley and coworkers* showed that vitellogenin can be isolated from the plasma of estrogen-treated roosters as a homogeneous protein and showed by several criteria that this molecule is the precursor of lipovitellin and phosvitin.

The use of the avian vitellogenin system to study the mechanism of the effect of hormone on gene expression offers several advantages: (*a*) the background level of vitellogenin in the plasma and in the liver of untreated roosters is essentially zero; (*b*) administration of estrogen results in a level of vitellogenin synthesis at which 10% of the liver polysomes are engaged in vitellogenin synthesis (K. P. Mullinix and R. G. Deeley, unpublished observation), resulting in at least a 200-fold increase in the concentration of this protein in the plasma; and (*c*) lipovitellin and phosvitin, the two components of the vitellogenin molecule, are well characterized and easily obtainable in large quantities from the egg. The work presented here describes our initial isolation of the messenger RNA that specifies vitellogenin, and the use of a cell-free system for protein synthesis *in vitro* as a means of following the purification of this mRNA.

MATERIALS AND METHODS

Preparation of Lipovitellin and Vitellogenin. Lipovitellin was prepared by the procedure of Bernardi and Cook (18, 19). Vitellogenin was isolated from the plasma of estrogen-treated roosters and was purified as described by Deeley and coworkers*.

Purification of Antilipovitellin Antibody. Antibodies against lipovitellin were elicited in sheep by immunization with pure lipovitellin in complete Freund's adjuvant. A blood sample was obtained prior to immunization. The immunoglobulin fraction of this sample, referred to in the *text* as preimmune serum, was used to determine the nonspecific immunoprecipitation of the products of cell-free synthesis. The immunoglobulin fraction of antilipovitellin antiserum was prepared by ammonium sulfate fractionation of the serum. Antilipovitellin antibody was purified further by passing the immunoglobulin of the antiserum over a Sepharose column to which lipovitellin had been attached by the CNBr procedure (20). This step resulted in a high degree of purification of antilipovitellin antibody.

Isolation of Rabbit Anti-Sheep γ-Globulin Antibodies. Commercially available antisera against sheep γ-globulin (Meloy Laboratories; Miles Laboratories) were precipitated three times with ammonium sulfate at a saturation of 40% to isolate the rabbit anti-sheep immunoglobulin fraction, which was subsequently dialyzed against 0.15 M NaCl and stored at −20°.

Estrogen Treatment of Roosters. White Leghorn roosters

* R. G. Deeley, K. P. Mullinix, W. Wetekam, H. M. Kronenberg, J. D. Eldridge, M. Meyers, and R. F. Goldberger, manuscript submitted.

(9–10 weeks old) were given an intramuscular injection of 17-β estradiol (20 mg/kg), followed by a second injection 1 week later. Eighteen hours after the second injection, the livers were excised and processed for the isolation of polysomes.

Isolation of Polysomes. Polysomes were isolated from the livers of estrogen-treated and untreated roosters by the procedure of Palmiter (21).

Preparation of Polysomal RNA from Rooster Livers. Polysomal RNA was extracted from liver polysomes of estrogen-treated and untreated roosters and from immunoprecipitated polysomes by the chloroform/phenol procedure in the presence of heparin, as described by Palmiter (21).

Preparation of Immunoprecipitated Polysomes. The crude liver polysomes of estrogen-treated roosters were subjected to an indirect immunoprecipitation reaction according to Shapiro *et al.* (22). Polysomes synthesizing vitellogenin could be specifically precipitated from the crude polysome preparation by the use of purified antilipovitellin antibody.

Fractionation of Polysomal RNA. The oligo(dT)-cellulose procedure (23) and the poly(U)-Sepharose procedure (ref. 24; and Pharmacia manual) were applied to the fractionation of polysomal RNA from crude polysomes. As an example of a typical fractionation of polysomal RNA, the poly(U)-Sepharose procedure may be summarized as follows: Polysomes were resuspended in 1% sarcosine containing EDTA (0.03 M). A sample containing 280 A_{260} units was applied to a column containing 2.5 ml of poly(U)-Sepharose (Pharmacia), prepared and equilibrated according to the procedure of Lindberg *et al.* (24). After washing the column with 12 ml of application buffer, a RNA fraction containing 4.4 A_{260} units was eluted with buffer containing 90% formamide. This fraction was precipitated with ethanol and stored overnight at −20°. The precipitate was collected by centrifugation, washed, and dissolved in 0.02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.5. The fractionation of polysomal RNA from immunoprecipitated polysomes on poly(U)-Sepharose was identical to the procedure outlined above with one modification: immunoprecipitated polysomes were resuspended in 1% sarcosine containing sodium dodecyl sulfate (1%) and EDTA (0.03 M).

Preparation of Rabbit Reticulocyte Lysates. Isolation, washing, and lysis of reticulocytes from anemic New Zealand white rabbits were done according to Gilbert *et al.* (25) with slight modifications: Blood was collected into an equal volume of 0.1 M sodium citrate buffer, pH 7.0, to prevent clotting. The washing buffer for the reticulocytes was changed to 30 mM Hepes, pH 7.4, containing sodium acetate (100 mM), potassium acetate (50 mM), and magnesium acetate (5 mM). The cells were lysed by the addition of 2 volumes of 2 mM magnesium acetate containing dithiothreitol (1 mM). After centrifugation of the suspension for 20 min at 15,000 × *g*, the lysate (supernatant) was stored in aliquots at −80°.

Reticulocyte Ribosome Preparation. Reticulocyte ribosomes for the reconstituted reticulocyte cell-free system were isolated by the procedure of Schreier and Staehelin (26), with slight modifications. The rabbit reticulocyte lysate was centrifuged for 1 hr at 100,000 × *g* to sediment the ribosomes and polysomes. The post-microsomal supernatant was withdrawn for isolation of rabbit reticulocyte "pH-enzymes" (see below). The walls of the tube were rinsed with buffer R: 20 mM Hepes, pH 7.6, containing potassium acetate (50 mM), magnesium acetate (1 mM), and dithiothreitol (1 mM). The pellet was then resuspended in buffer R (2 ml for ribosomes from 10 ml of rabbit reticulocyte lysate), immediately frozen, and kept at −120°.

Extraction of "pH-Enzymes" from the Post-Microsomal Supernatant of Rabbit Reticulocyte Lysates. The isolation procedure for the "pH-enzyme" fraction from rabbit reticulocytes is described by Falvey and Staehelin (27) and was applied in its general outline with some minor modifications. Ten milliliters of the post-microsomal supernatant were adjusted to pH 5.0 with 1 M acetic acid, and the precipitate that formed was immediately centrifuged for 10 min at 10,000 × *g*. The precipitate was resuspended in 2 ml of buffer R adjusted to pH 8.2, and kept frozen at −120°. The final pH of the resuspended "pH-enzymes" ranged between 7.5 and 7.7.

Cell-Free Protein Synthesis Incubations. Cell-free protein synthesis was generally carried out at 28° for the times indicated in the tables and figures. Incubation mixtures with a total volume of 100 μl contained: 20 mM Hepes, pH 7.6; potassium acetate (25 mM); either 0.5 μCi of [^{14}C]leucine (specific activity 280 mCi/mmol in the assay) or 5.0 μCi of [^{3}H]leucine (specific activity 2.5 Ci/mmol in the assay); GTP (0.5 mM); ATP (1 mM); creatine-phosphate (20 mM); 0.4 units of phosphocreatine kinase; 0.5 μg of deacylated calf liver tRNA; hemin (10 μM); and 50 μl of rabbit reticulocyte lysate. For the reconstituted system, 25 μl of "pH-enzymes" plus 10 μl of rabbit reticulocyte ribosomes were used instead of the lysate. The amounts of polysomal RNA or mRNA after chromatography on poly(U)-Sepharose are given individually in the legends of figures and in the tables.

Preparation of Wheat Germ Extract. The preparation of, and the cell-free synthesis with, wheat germ extracts were identical to the procedure described (28).

Indirect Immunoprecipitation Assays of Cell-Free Synthesis Products. Cell-free synthesis was terminated by the addition of 20 μg of ribonuclease A. To identify newly synthesized polypeptides, 50 μl of a cell-free incubation was mixed with 250 μl of an immunoprecipitation reaction mixture containing in the final immunoprecipitation: 10 mM [^{12}C]leucine; NaCl (0.15 M); sodium-phosphate buffer (10 mM), pH 7.2; deoxycholate (2%); Triton X-100 (1.3%); and 20 μg of purified antilipovitellin antibody, which was enough to precipitate quantitatively 2 μg of vitellogenin. The reaction mixture was allowed to stand for 1 hr at room temperature before the addition of rabbit anti-sheep γ-globulin at a concentration sufficient to precipitate quantitatively the antilipovitellin antibody. After 1.5 hr, the visible precipitate was centrifuged for 90 sec in 400-μl polypropylene tubes in the Beckman microfuge model B. The immunoprecipitate was washed three times with 200 μl of phosphate-buffered saline: 10 mM sodium-phosphate buffer, pH 7.2, containing NaCl (0.15 M) and [^{12}C]leucine (10 mM). It was then dissolved in 0.1 ml of 1 M NaOH and subsequently neutralized with an equal volume of 1 M HCl in 50 mM sodium phosphate buffer, pH 6.8. Radioactivity was determined in 10 ml of Aquasol in the Beckman LS-355 scintillation spectrometer.

Materials. All reagents for the cell-free protein synthesis were from Sigma Chemical Co. L-[^{12}C]Aminoacid mixture was from Schwarz/Mann. Radioactive amino acids were from New England Nuclear Corp. Rabbit anti-sheep γ-globulin antiserum was obtained from Meloy or Miles Laboratories. Ribonuclease A (RNase A) was from Worthington Biochemical Corp. Poly(U)-Sepharose was from Pharmacia. Oligo(dT)-cellulose was kindly supplied by Dr. Philip Leder.

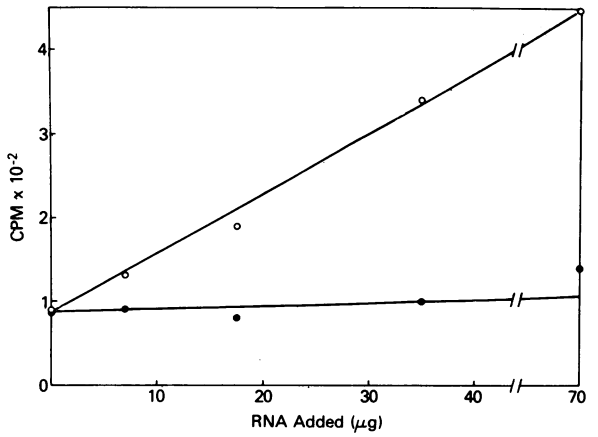

FIG. 1. Polysomal RNA activity in a reconstituted rabbit reticulocyte cell-free system. After 60 min of incubation of the cell-free system in the presence of polysomal RNA from estrogen-treated (○) or untreated (●) roosters at 28°, the newly synthesized peptides were identified by indirect immunoprecipitation, as described in *Materials and Methods*. The counts represent the specific immunoprecipitates from 50 μl of the reaction mixtures after subtraction of about 350–400 cpm for a nonspecific immunoprecipitate, in which the antilipovitellin antibody was replaced by preimmune serum. A control cell-free incubation (without any RNA added) gave 90 cpm over the nonspecific immunoprecipitate in the specific immunoprecipitation with antilipovitellin antibodies.

RESULTS

mRNA activity of polysomal rooster liver RNA

The ability of crude polysomal RNA of rooster liver to stimulate protein synthesis could be tested in a mRNA-dependent protein-synthesis system prepared from wheat germ, by measuring radioactive leucine incorporation into hot trichloroacetic acid-insoluble protein (Table 1). The wheat germ system provides a convenient means for quickly assessing the mRNA activity of a RNA preparation. The results revealed a 2- to 3-fold increase in the activity of polysomal liver RNA from estrogen-treated roosters over that from untreated roosters. This difference was not due to variations among different preparations of RNA, but was routinely reproducible.

Characterization of the rabbit reticulocyte cell-free system

Studies on translation of specific mRNA species in reticulocyte cell-free systems require careful characterization of the system for each specific RNA preparation. It has been well established (9, 29–35) that numerous preparations of polysomal RNA and viral RNA inhibit the endogenous synthetic activity of rabbit reticulocyte lysates and reconstituted systems from rabbit reticulocytes. We therefore examined synthesis of a specific protein in the cell-free reticulocyte reconstituted system, and monitored synthesis over a wide range of polysomal RNA concentrations by a highly specific immunoprecipitation procedure, using purified antilipovitellin antibody (see *Materials and Methods*). Under the appropriate conditions, the system showed a RNA concentration-dependent reaction (Fig. 1). The radioactivity in immunoprecipitates increased linearly with from 7 to 35 μg of polysomal liver RNA from estrogen-treated roosters per 50 μl of incubation and sloped off moderately at higher RNA concentrations. This indication of "saturation" may reflect the inhibitory effect of ribosomal RNA superimposed upon the stimulatory effect of the mRNA in the polysomal RNA preparation. Polysomal liver RNA from unstimulated roosters showed almost no activity increase in the immunoprecipitation reaction, indicating that vitellogenin-specific mRNA is not synthesized in the normal rooster liver. The values in Fig. 1 have been corrected for a nonspecific precipitation with preimmune serum.

Table 1. Stimulation of protein synthesis in wheat germ extract

RNA*	Cell-free protein synthesis† (cpm)
None	1,250
Unstimulated polysomal RNA	6,500
Stimulated polysomal RNA	18,600

* The concentration of the polysomal RNA from the livers of untreated roosters (unstimulated polysomal RNA) and estrogen-treated roosters (stimulated polysomal RNA) in the cell-free incubation was 44 μg/0.1 ml of incubation.

† The incubation was for 40 min at 28°C and contained 0.5 μCi of [^{14}C]leucine (specific activity 280 mCi/mmol). Total trichloroacetic acid-insoluble radioactivity was determined by a hot trichloroacetic acid precipitation procedure (28).

Sucrose density gradient centrifugation of liver polysomes revealed that estrogen treatment of the rooster resulted in a change in the size distribution of liver polysomes towards larger polysomes. The ability to program the synthesis of vitellogenin in a reticulocyte cell-free system was found in the RNA extracted from only the largest polysomes (hexasomes and larger) (data not shown here).

The kinetics of protein synthesis directed by polysomal liver RNA in the rabbit reticulocyte lysate, as measured by the specific immunoprecipitation reaction, suggest that the initial rate of synthesis was considerably faster than the rate established after 20 min and maintained for an additional 70 min (Fig. 2).

Purification of mRNA

The high degree of specificity and sensitivity achieved by utilizing the indirect immunoprecipitation reaction to detect specific protein synthesized in the cell-free reticulocyte system allowed us to use the system to monitor purification of the mRNA that specifies vitellogenin. Purification by poly(U)-Sepharose after immunoprecipitation of polysomes involved in vitellogenin synthesis (approximately 10% of total polysomes) was as described in *Materials and Methods*. The mRNA fraction obtained by poly(U)-Sepharose chromatography directed the synthesis of a protein in the reconstituted reticulocyte system that reacted specifically with purified antilipovitellin antibody. The specific synthetic capacity of this mRNA, expressed as counts per 1 μg of RNA, was more than 800 times higher than that of crude polysomal RNA from the liver of estrogen-treated roosters (Table 2).

The recovery of vitellogenin-specific mRNA from the theoretically expected fractions of oligo(dT)-cellulose and poly(U)-Sepharose chromatography (23, 24) varied with individual polysomal RNA preparations. In all experiments, however, poly(U)-Sepharose proved to be substantially superior to oligo(dT)-cellulose in retaining vitellogenin-specific mRNA, a finding also reported for ovalbumin mRNA by Shapiro and Schimke (36).

Identification of the products of cell-free synthesis

Characterization of the products of cell-free synthesis has been extended beyond specific immunoprecipitation with

Table 2. Purification of vitellogenin-specific mRNA

RNA†	Cell-free protein synthesis* (cpm/100 μg of RNA)	Increase in specificity
Unstimulated polysomal RNA	143	—
Stimulated polysomal RNA	1,020	1
mRNA [poly(U)-Sepharose fraction]	819,000	800

* Cell-free synthesis in a reconstituted rabbit reticulocyte system containing [^{14}C]leucine was carried out in 50-μl assays for 60 min at 28° with 100 μg of various RNA preparations. The newly synthesized proteins were precipitated by the indirect immunoprecipitation procedure outlined in *Materials and Methods* with antilipovitellin antibody or preimmune serum, and the radioactivity was corrected for the nonspecific precipitation with preimmune serum.

† The three preparations of RNA were: (*i*) polysomal RNA from livers of untreated roosters (unstimulated polysomal RNA); (*ii*) polysomal RNA from the livers of estrogen-treated roosters (stimulated polysomal RNA); and (*iii*) mRNA obtained by poly(U)-Sepharose chromatography of the polysomal RNA from immunoprecipitated polysomes of livers of estrogen-treated roosters (mRNA [poly(U)-Sepharose fraction]).

purified antilipovitellin antibody. A more specific way to test the similarities between protein newly synthesized *in vitro* and vitellogenin, the plasma precursor of lipovitellin and phosvitin, is to study competition of the indirect immunoprecipitation by authentic vitellogenin. Data from such an experiment are shown in Fig. 3. Increasing amounts of unlabeled purified vitellogenin, added to the indirect immunoprecipitation reaction, competed with the antigenically related product of cell-free synthesis for antibody binding. This competition was found only in those cases in which the cell-free synthesis was programmed by RNA from estrogen-treated roosters; RNA from control roosters did not stimulate synthesis of any protein antigenically related to vitellogenin. The values in Fig. 3 have not been corrected for nonspecific precipitation of product from the cell-free system without any RNA added in order to indicate clearly the background level of nonspecific synthesis (1400 cpm). This high background amounted to 40% of the total immunoprecipitated radioactivity, found in the absence of any competition, and was due to the very high specific activity of the [^{3}H]leucine (see *Materials and Methods*). The linear response of specific competition ranged from 0.8 μg to 10 μg of vitellogenin, which is in the expected range for the binding capacity of the purified antilipovitellin antibody in the immunoprecipitation assay.

DISCUSSION

Vitellogenin has been identified by Deeley and coworkers* as the precursor molecule of the two hen egg yolk phosphoproteins, lipovitellin and phosvitin. These workers showed that vitellogenin can be precipitated quantitatively by antibody against lipovitellin from the plasma of estrogen-treated roosters. Thus, the antigenic determinants present in the lipovitellin molecule are also present in vitellogenin. It therefore seemed reasonable to utilize antilipovitellin antibody to precipitate polysomes associated with vitellogenin-specific mRNA from a crude polysome preparation from the liver of estrogen-treated roosters. We show here that the mRNA extracted from such immunoprecipitated polysomes directs the synthesis, *in vitro*, of a protein with antigenic determinants in common with vitellogenin. We are not yet able to determine whether the protein synthesized *in vitro* from this RNA constitutes all parts of the vitellogenin molecule. The use of the purified antilipovitellin antibody for the immunoprecipitation reactions indicates, however, that the mRNA from estrogen-treated rooster liver polysomes specifies at least part of the lipovitellin portion of vitellogenin.

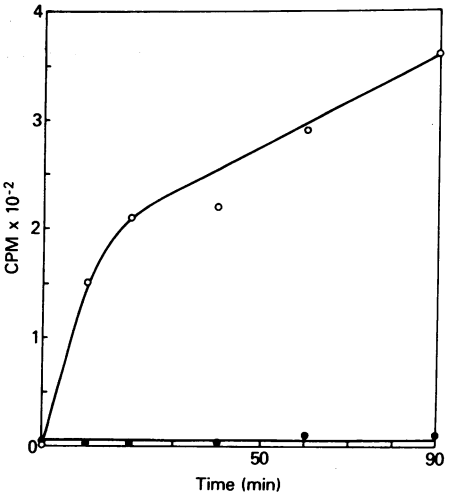

FIG. 2. Immunoprecipitation reactions with rabbit reticulocyte lysates programmed by polysomal liver RNA from untreated (●) and estrogen-treated (○) roosters. The incubation mixtures contained 40 μg of polysomal RNA in 50 μl. Incubation was terminated, at the times indicated, by the addition of 10 μg of RNase A. About 200 cpm have been subtracted from all points to correct the unspecific precipitation with preimmune serum.

The kinetics of *in vitro* synthesis of proteins immunologically related to vitellogenin showed two clearly distinct rates (see Fig. 2). The accumulation of an inhibitor of protein synthesis, such as "Q" (8), during the incubation, seems a more reasonable explanation for this phenomenon than does the possibility that two distinct but antigenically related proteins are synthesized.

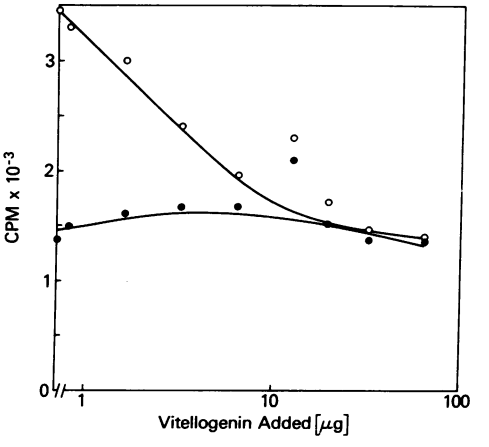

FIG. 3. Competition between labeled products of cell-free synthesis and unlabeled vitellogenin in the immunoprecipitation reaction. Synthesis was carried out for 90 min at 28° with a rabbit reticulocyte lysate and 40 μg of polysomal liver RNA from untreated (●) or estrogen-treated (○) roosters. 2.5 μCi of [^{3}H]leucine were added to each 50-μl incubation mixture. After the incubation, newly synthesized proteins were subjected to an indirect immunoprecipitation reaction, as described in *Materials and Methods*, with the indicated amounts of unlabeled vitellogenin as competitor. Incubations with preimmune serum instead of antilipovitellin antibody were not subtracted from the immunoprecipitated radioactivity.

The cell-free system and the indirect immunoprecipitation assay are sensitive enough to allow detection of newly synthesized protein from 5 μg of polysomal RNA or from 0.03 μg of the mRNA fraction obtained by poly(U)-Sepharose chromatography of the RNA from immunoprecipitated polysomes. This sensitivity will be useful for further purification of the mRNA specifying vitellogenin and might prove to be sensitive enough for studying the very first steps of RNA induction by estrogen in the rooster liver.

Chromatography of various preparations of polysomal RNA on oligo(dT)-cellulose gave results that differed from those described for globin mRNA (23). The reduced affinity of vitellogenin-specific mRNA for oligo(dT)-cellulose and poly(U)-Sepharose may be a reflection of a short poly(A) sequence. It is known that the poly(A) sequences in eukaryotic mRNA may vary in length as a result of aging (37). Such variations do not seem to impair the fidelity of translation, as shown for rabbit globin mRNA (38, 39). It is possible that the unusually large size of the vitellogenin-specific mRNA may make binding to affinity columns less favorable, even if its poly(A) length is similar to that of other species of mRNA (36).

Jost and coworkers (40) have recently described their attempts to purify the messenger RNA specifying the so-called heavy subunit of avian β-lipovitellin (41). Our studies indicate that the primary gene product is the messenger RNA specifying the precursor molecule, vitellogenin, and that the "heavy subunit of β-lipovitellin" is never synthesized as such in the avian liver, but is synthesized only as a part of the vitellogenin molecule.

The findings presented here demonstrate that estrogen treatment of the rooster results in the synthesis in the liver of a species of mRNA specifying a protein immunologically related to vitellogenin, the protein that has been identified as precursor for the egg yolk phosphoproteins, lipovitellin and phosvitin. This mRNA has been partially purified and its activity assessed in cell-free systems for protein synthesis *in vitro*. The protein synthesized *in vitro* has been identified by indirect immunoprecipitation with specific antibody and by competition of this immunoprecipitation by pure vitellogenin. Thus, a workable system has been developed for further studies on the mechanism of the effect of estrogen on regulation of gene expression in cells with a wide range of metabolic activities and diverse genetic potential.

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