Molecular Cloning of Ovomucoid Gene Sequences from Partially Purified Ovomucoid Messenger RNA†

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ABSTRACT: Preparation of milligram amounts of partially purified ovomucoid mRNA from hen oviduct was accomplished by a combination of precise sizing techniques and selective purification of the poly(adenylic acid)-containing RNA by affinity chromatography. Characterization of ovomucoid mRNA was performed by several independent methods. (1) The ovomucoid-enriched mRNA ($mRNA_{om}$) migrates as a single band during agarose–urea gel electrophoresis at pH 3.5. (2) Translation of this mRNA in a wheat germ cell-free translation assay revealed that 52% of the total peptides synthesized were specifically precipitable with an ovomucoid 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 major radioactive peak. (4) RNA excess hybridization to a labeled cDNA probe synthesized from the partially purified $mRNA_{om}$ demonstrated the existence of a predominant mRNA species with a $R_0t_{1/2}$ value of 2×10^{-3} that represented 40–50% of the total RNA in the preparation. Since these criteria demonstrated that the $mRNA_{om}$ was only about 50% pure after conventional techniques of RNA purification had been exhausted, we employed the technique of bacterial cloning to complete the purification of the coding portion of the ovomucoid gene. Double-stranded DNA was synthesized from the partially purified $mRNA_{om}$, and agarose gel electrophoresis was used to select essentially full-length dsDNA molecules. A preliminary restriction map of this $dsDNA_{om}$ was prepared to assist in removing the inserted DNA from amplified bacterial plasmids. After dATP tailing with terminal transferase, the dsDNA was annealed to a bacterial plasmid, pBR322; this chimeric plasmid was then used to transform *Escherichia coli* χ1776. Fifteen ampicillin-sensitive, tetracycline-resistant clones were obtained which hybridized to [^{32}P]cDNA synthesized from $mRNA_{om}$. In order to screen these clones for plasmids containing ovomucoid DNA sequences, a new, simple assay based on hybridization and translation analyses was developed. Plasmid DNA was purified from several candidate clones, digested with restriction endonuclease *Hha*I, denatured and bound to Millipore filters. Oviduct mRNA was hybridized to these DNA filters, and hybridizable mRNA species were removed from the DNA by heat denaturation. The mRNA was then translated in a wheat germ translation system, and the products were assayed for immunoprecipitable ovomucoid polypeptides. Several plasmid DNAs were shown to contain ovomucoid DNA by this procedure. One clone, pOM100, was partially sequenced by the method of Maxam & Gilbert (Maxam, A., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 560–564). The nucleotide sequence corresponds to the amino acid sequence of portions of two glycosylated ovomucoid peptides. Thus, the cloning of the ovomucoid DNA sequence from a partially purified preparation of ovomucoid mRNA has been accomplished and confirmed by two independent criteria.

The hen oviduct offers an attractive model system for studying the regulation of gene expression by steroid hormones. Administration of estrogen to the newborn chick stimulates oviduct growth and differentiation and results in the appearance of a number of new specific intracellular proteins (O'Malley et al., 1969; Palmiter, 1972, 1973; Chan et al., 1973; Harris et al., 1973, 1975; Sullivan et al., 1973; O'Malley & Means, 1974; Hynes et al., 1977). The synthesis of one of these proteins, ovalbumin, has been studied extensively. Ovalbumin mRNA has been purified (Rosen et al., 1975), and a full-length dsDNA copy synthesized (Monahan et al., 1976b) and cloned in a bacterial plasmid (McReynolds et al., 1977). More recently, ovalbumin genomic DNA sequences have been isolated from restriction enzyme digests of hen DNA and cloned (Woo et al., 1978). The other three major proteins under estrogenic control in the oviduct tubular gland cell, ovomucoid, conalbumin, and lysozyme, have been less extensively studied (Palmiter, 1972; Hynes et al., 1977). In order to study the mechanisms by which steroid hormones regulate expression of separate genes in this tissue, we are attempting to isolate other estrogen-induced genes from the chick oviduct. The purification of ovomucoid mRNA and cloning of its DNA sequence would permit a structural and functional comparison of this gene with the ovalbumin gene. Ovomucoid was chosen since it represents approximately 8% of the total synthesized protein in estrogen-stimulated oviduct (Palmiter, 1972). Furthermore, ovomucoid mRNA could easily be assayed in either a wheat germ or reticulocyte cell-free protein synthesizing system (Palmiter & Smith, 1973).

We now report the purification of the ovomucoid structural gene sequence. Conventional techniques of RNA purification were used to prepare an ovomucoid-enriched mRNA. Since translation and hybridization analyses demonstrated that this ovomucoid mRNA was at most 50% pure, the purification was completed by molecular cloning. dsDNA was synthesized from the partially purified ovomucoid mRNA, annealed to the bacterial plasmid pBR322, and used to transform *E. coli* χ1776. Several clones were isolated which were shown by hybridization analysis and DNA sequencing to contain an inserted ovomucoid DNA sequence.

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Materials and Methods

Materials. White leghorn laying hens were purchased from Rich-Glo Farm, La Grange, Texas; their oviducts were rinsed with cold 0.9% NaCl solution and frozen in liquid nitrogen immediately after dissection. Liquified phenol and reagent grade formamide were purchased from Fisher Scientific Co. Creatinine phosphate, creatine phosphokinase, and sodium dodecyl sulfate were from Sigma Chemical Co. Oligo(dT)-cellulose (T3) was purchased from Collaborative Research and Sepharose 4B from Pharmacia Fine Chemicals. Sucrose (ribonuclease-free), UltraPure urea, and [^{14}C]valine were purchased from Schwarz/Mann, and agarose (electrophoretic grade) was from Bio-Rad Laboratories. Deoxyribonucleoside triphosphates were obtained from P-L Biochemicals and radioactive nucleotides from ICN. [^{35}S]Methionine (755 Ci/mmol) and [α-^{32}P]dATP (200 Ci/mmol) were purchased from Amersham Corp. Restriction endonucleases *Pst*I and *Bam*HI were purchased from New England Biolabs and *Hha*I, *Alu*I, *Eco*RI, *Hin*dIII, and *Hae*III from Bethesda Research Labs. Nitrocellulose filter disks were obtained from Millipore Corp. Bacteriophage T4 polynucleotide kinase was purchased from Boehringer, and S_1 nuclease and poly(dA) were from Miles Laboratories. Goat anti-ovomucoid serum was purchased from Antibodies Incorp., Davis, Calif. An immunoglobulin G fraction of this serum was prepared by ammonium sulfate precipitation. This anti-ovomucoid IgG fraction showed no cross-reactivity with egg white proteins other than ovomucoid, as judged by Ouchterlony diffusion analysis and immunoelectrophoresis. This anti-ovomucoid preparation was used for all antibody precipitations.

Wheat germ was a gift from General Mills, Inc., Minneapolis, Minn. Avian myeloblastosis virus reverse transcriptase was supplied by Dr. J. W. Beard, Life Sciences, Inc., St. Petersburg, Fla. Calf thymus terminal deoxynucleotidyl transferase was a generous gift of Dr. R. Ratliff, University of California, Los Alamos, New Mexico. Plasmid pBR322, linearized with the restriction endonuclease *Pst*I and tailed with dTTP, was supplied by Dr. A. Dugaiczyk of this department.

Bacterial Strains and Growth Conditions. E. coli K12 strain RR1 containing plasmid pBR322 (Ap^r, Tc^r) was kindly provided by Dr. H. W. Boyer (University of California, San Francisco). For isolation of pBR322 (Bolivar et al., 1977), RR1 was grown in M9-glucose minimal medium. *E. coli* K12 strain χ1776 was the recipient strain for plasmid transformation experiments. This strain was obtained from Dr. R. Curtiss III (University of Alabama, School of Medicine, Birmingham). *E. coli* χ1776 was grown in L broth supplemented with diaminopimelic acid (DAP; C_f = 100 μg/mL) and thymidine (thd; C_f = 40 μg/mL) in the presence of naladixic acid (Nal; C_f = 50 μg/mL).

Preparation of Total RNA. Extraction of RNA from the magnum portion of frozen hen oviducts was performed essentially as described previously (Rosen et al., 1975).

Purification of Poly(A)-Containing mRNA. An RNA fraction enriched in poly(A)-containing RNA was obtained by oligo(dT)-cellulose chromatography by the method of Aviv & Leder (1972).

Sepharose 4B Chromatography. Chromatography of the poly(A)-enriched RNA on a Sepharose 4B column was performed as described previously (Rosen et al., 1975), with the exception that the sample was first heated at 70 °C for 1 min and then quick-cooled in ice water to denature the RNA. The ovomucoid mRNA activity in individual fractions was assayed in a wheat germ translation system as described below.

dT-Cellulose Chromatography. Fractions from the Sepharose column containing ovomucoid mRNA activity were combined, heated at 70 °C for 1 min, quick-cooled, and diluted into 100 mL of 0.2 M KCl, 0.01 M Tris-HCl, pH 7.5, 1 mM EDTA. This sample was then applied to 5 g of oligo(dT)-cellulose packed in a 1.6 × 10 cm column. The column was washed with application buffer, and poly(A)-containing RNA was eluted with 0.01 M Tris-HCl, pH 7.5, 1 mM EDTA.

Sucrose Gradient Centrifugation. mRNA (200–300 μg) recovered from the second dT-cellulose chromatography step was heated at 70 °C for 1 min, quick-cooled, and applied to 12.4-mL linear gradients of 5–20% sucrose in 0.01 M sodium acetate pH 5.0, 1 mM EDTA, 0.1 M NaCl. The gradients were centrifuged at 38 000 rpm in an SW 40 rotor at 3 °C for 18–19 h. The gradients were fractionated into 0.5-mL samples. The RNA was precipitated with ethanol, redissolved in water, and assayed for $mRNA_{om}$ activity by the translation assay.

Translation Assay. The ability of an RNA preparation to direct ovomucoid synthesis was tested in a heterologous cell-free protein synthesizing system derived from wheat germ, the preparation of which was described previously (Rosen et al., 1975).

The conditions employed in the cell-free assay system were essentially as previously described (Rosen et al., 1975) except for the following modifications: 84 mM KCl, 60 μM spermine, and 8 μM [^{14}C]valine (260 Ci/mol) were used. Between 0.5 and 3.0 μg of RNA was translated per 0.1-mL reaction, depending upon the purity of the mRNA preparation being tested. Ovomucoid mRNA activity was determined by a specific immunoprecipitation procedure using monospecific antibody raised in goats against purified ovomucoid. The antibody–antigen reaction mixture contained 10 mM NaCl, 0.75% deoxycholate (DOC), and 1.25% Triton X-100 to minimize nonspecific trapping of other proteins. After a 45-min incubation at 25 °C, the reaction mixture was incubated at 4 °C for 5–15 h. The precipitate was collected by centrifugation at 7000*g* for 15 min, washed once in the detergent buffer, and collected on Millipore filters.

Product analysis of the total peptides synthesized in the wheat germ assay in response to exogenous mRNA was performed by $NaDodSO_4$[1] gel electrophoresis of the released polypeptide chains. Following a 2-h incubation, the ribosomes were removed by centrifugation at 105 000*g* for 1 h at 4 °C. 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 °C. Samples were then precipitated with Cl_3CCOOH as above. The pellets were washed once with acetone, once with ethanol, and then dried. The dried precipitates were suspended in 0.06 M Tris-HCl, pH 6.8, containing 10% glycerol, 5% 2-mercaptoethanol, and 3% $NaDodSO_4$. Analysis on 1.5-mm 12.5% polyacrylamide slab gels containing 0.1% $NaDodSO_4$ was performed as described by Laemmli (1970).

Enzymatic Synthesis of [^{3}H]DNA Complementary to $mRNA_{om}$. The procedure used for the preparation of a tritiated DNA complementary to the enriched ovomucoid mRNA ($mRNA_{om}$) was essentially as described previously (Woo et al., 1977). The final concentration of reverse transcriptase enzyme was 480 U/mL.

RNA Excess Hybridization to [^{3}H]cDNA. Tritiated DNA complementary to the purified ovomucoid mRNA ([^{3}H]-cDNA) was synthesized as described above. The specific activity was 1.21 × 10^7 cpm/μg. RNA excess hybridization ex-

[1] Abbreviation used: $NaDodSO_4$, sodium dodecyl sulfate.

periments with this [^{3}H]cDNA were carried out in 5-mL tapered, glass hybridization vials (Regis Chemical Co., Morton Grove, Ill.). The hybridization reaction was performed in a final 50-μL volume as described previously (Harris et al., 1975). [^{3}H]cDNA, 25 ng/mL (15 000 cpm/vial), and 1.2 μg/mL of mRNA were used.

Enzymatic Synthesis of Double-Stranded cDNA. [^{3}H]-dCTP (360 μCi) was lyophilized to dryness and resuspended in 3 mL of a solution containing 50 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 39.2 mM 2-mercaptoethanol, 1 mM dTTP, dGTP, dATP, and dCTP and 60 μg/mL of cDNA. Reverse transcriptase was added to a final concentration of 600 U/mL and the solution was quickly blended on a Vortex mixer and incubated for 90 min at 46 °C. The reaction mixture was then made 50 mM in EDTA and 1% in sodium sarcosyl and chromatographed on a Sephadex G-50 column. The excluded fraction was collected and precipitated with 2 volumes of ethanol in 0.1 M potassium acetate (pH 4.7).

S_1 Nuclease Nicking of dsDNA. The conditions for cleavage of the hairpin loop (Monahan et al., 1976b; Higuchi et al., 1976; Efstratiadis et al., 1976) in dsDNA were found to vary from preparation to preparation of the cDNA and S_1 nuclease (McReynolds et al., 1977). Furthermore, the kinetics of S_1 digestion of cDNA vary with the concentration of DNA in the solution (unpublished observations). Routinely, therefore, the kinetics of S_1 digestion of a cDNA preparation were measured immediately prior to its use for cleavage of the hairpin loops in dsDNA. The conditions chosen for the cleavage were those under which an identical amount of cDNA was completely degraded in the shortest possible time at 25 °C. In the experiments reported here, 20 units of S_1 nuclease per μg of DNA was incubated in 20 μL of 0.3 M sodium acetate, pH 4.5, 0.6 M NaCl, 4 mM $ZnCl_2$ for 75 min. The dsDNA was then extracted with chloroform and precipitated with ethanol.

Isolation of Long dsDNA for Sequence Studies and Cloning. S1-treated dsDNA of the appropriate size range was selected on a 2% agarose slab gel in 50 mM Tris (pH 8.4), 20 mM sodium acetate, 18 mM sodium chloride, and 2 mM EDTA. Electrophoresis was performed in the same buffer for 5 h at 70 V. The gel apparatus was made by Blair Craftline (Cold Spring Harbor, N.Y.). Visualization was accomplished under ultraviolet light after staining in 5 μg/mL ethidium bromide in the electrophoresis buffer, or by radioautography when [^{32}P]dATP was used during the synthesis of the cDNA.

DNA species were eluted from the gels using the procedure of Sharp et al. (1974).

Restriction Endonuclease Cleavage of dsDNA. Reaction conditions for restriction endonuclease cleavage of dsDNA varied with the particular enzyme and were essentially as suggested by the supplier. Enzymes were added at 1 unit/μg of DNA substrate and incubated 3 h at 37 °C. The reactions were stopped by heating at 68 °C for 5 min.

Poly(dA) Addition. Terminal poly(dA) "tails" were added to the 3′ ends of S_1-treated dsDNA by terminal deoxynucleotidyl transferase. The final reaction volume of 50 μL contained 0.2 M potassium cacodylate, pH 7.2, 1.0 mM $CoCl_2$, 1.0 mM 2-mercaptoethanol, and 0.5 mM dATP. The reaction contained 144 μCi (0.97 mmol) of [^{32}P]dATP and 14 μg/mL dsDNA. Nucleic acid carrier (*E. coli* tRNA) was added to 40 μg/mL. Terminal transferase (190 units) was added for 140 min at 37 °C. The reaction was terminated by the addition of EDTA to 10 mM.

Annealing of dsDNA to pBR322. Chimeric plasmids were formed by hybridization of pBR322 (4.4 μg/mL) to the "tailed" dsDNA (1 μg/mL), as described previously (McReynolds et al., 1977).

Biosafety Precautions. All bacterial transformations were carried out according to the National Institutes of Health Guidelines (*Federal Register,* 1976). Transformation and the initial steps of plasmid purification were performed under approved P-3 physical containment. All bacterial transfers were carried out using approved vectors and hosts and were performed in a certified laminar flow hood in the P-3 containment facility.

Transformation of χ1776. Transformation of χ1776 was performed as previously described (McReynolds et al., 1977) with the following modifications. Incubation of the final cell suspension in $CaCl_2$ was for 3 min at 42 °C rather than 5 min at 37 °C. The mixture was diluted threefold with fresh growth medium and incubated for 3 h at 37 °C in a New Brunswick gyratory shaker. Finally, the cell suspension was spread onto tetracycline (Tc) L-agar plates (1% agar, 12.5 μg/mL Tc in L broth) supplemented with DAP (100 μg/mL), and incubated at 37 °C for 3 days. The tetracycline resistant (Tc^r) colonies were transferred with sterile wood applicators to a fresh Tc plate and an ampicillin (Ap; 40 μg/mL) agar plate. Recombinant clones were scored as the number of Tc^r/Ap^s clones on these plates.

Direct Colony Transfer and in Situ Hybridization. Tc^r clones were directly transferred to nitrocellulose filter disks. A filter was carefully laid over the surface of the agar plate and allowed to soak thoroughly. It was then carefully removed to avoid smearing of the colonies. All colonies on the plate were transferred onto the filter in this procedure and the residual cells were allowed to grow into colonies at 37 °C. This plate then served as a masterplate. The plate was returned to the 37 °C incubator for 47 h. The colonies on the replicate filters were lysed in situ by the method of Grunstein & Hogness (1975). The dried filters containing denatured DNA were treated with 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin (Denhardt, 1966; Botchan et al., 1976) at 68 °C. Washing and hybridization with [^{32}P]cDNA synthesized from partially purified $mRNA_{om}$ was performed as described by Botchan et al. (1976). Clones containing a complementary dsDNA insert were identified by autoradiography (McReynolds et al., 1976) and the positive clones on the master plate were streaked onto fresh Tc plates for further analysis.

Isolation of Recombinant Plasmid DNA. Recombinant plasmid DNA was prepared by amplification of pBR322-dsDNA recombinants in χ1776. Cells were grown in DAP-supplemented L broth at 37 °C to an optical density of 0.4 at 600 nm. Chloramphenicol was added to 25 μg/mL and the culture was incubated for an additional 5 h. Cleared lysates were prepared from these cells by the method of Katz et al. (1973) and chromatographed through a Sephadex G-100 column. Recombinant plasmid DNA was then purified by the method of Katz et al. (1977).

Slab Gel Electrophoresis. Digestion products of plasmid DNA were separated by agarose slab gel electrophoresis as described above. Detection of fragments which contained inserted DNA sequences was accomplished by Southern blotting (Southern, 1975) and hybridization to [^{32}P]cDNA synthesized from $mRNA_{om}$ (Botchan et al., 1976).

Electrophoresis on polyacrylamide slab gels was done in a Bio-Rad slab gel apparatus. Six percent polyacrylamide gels contained 5.68 g of acrylamide, 0.32 g of *N,N′*-methylenebisacrylamide, 60 μL of *N,N,N′,N′*-tetramethylethylenediamine (Temed), 0.6 mL of 10% ammonium persulfate in 50 mM Tris–borate, pH 8.3, 1 mM EDTA.

5′-End Labeling. Restriction fragments of recombinant plasmid DNAs (40 μg) were digested with snake venom

TABLE I: Purification of Ovomucoid mRNA.

	sp act.[a] (cpm/μg)	purification[b] (fold)	ovomucoid synthesized[c]/total protein synthesized (%)
total extract	100	1	3
dT-cellulose bound	1 900	19	12
Sepharose peak	8 000	80	24
dT-cellulose bound	10 500	105	35
sucrose gradient peak	27 000	270	52

[a] Defined as the total amount of ovomucoid polypeptides synthesized in the wheat germ translation assay (see Materials and Methods) in response to 1 μg of the various RNA preparations. [b] Purification of $mRNA_{om}$ over the total RNA in the preparation. [c] Determined as the ratio of immunoprecipitated ovomucoid in the wheat germ translation assay to the total Cl_3CCOOH-precipitated polypeptides.

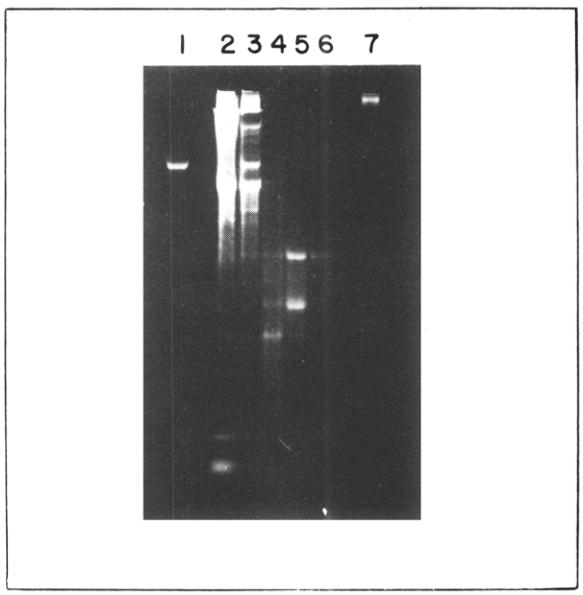

FIGURE 1: Analysis of $mRNA_{om}$ purification by agarose gel electrophoresis. A 2.25% agarose gel was cast in 0.025 M sodium citrate, pH 3.5, containing 6 M urea. RNA (4–20 μg), depending upon the number of RNA species in the preparation, was dissolved in 50 μL of 0.025 M sodium citrate, pH 3.5, containing 8 M urea, 20% sucrose, and 0.005% bromphenol blue. Electrophoresis was carried out at 50 V for 1 h, then 70 V for 7 h more. The nucleic acids were visualized by staining the gel in 50 mM Tris-HCl, pH 8.0, containing 5 μg/mL ethidium bromide for 30 min, and then photographing under UV light. From left to right, the seven slots contained: (1) ovalbumin mRNA; (2) total oviduct RNA extract; (3) oligo(dT)-cellulose bound RNA; (4) Sepharose 4B pooled RNA; (5) 2nd oligo(dT)-cellulose bound mRNA (low salt); (6) RNA pooled from 5% to 20% sucrose gradient; (7) globin mRNA.

phosphodiesterase (0.25 μg) and bacterial alkaline phosphatase (10 μg) in a reaction volume of 50 μL. The reaction buffer contained 0.01 M Tris-HCl, 0.01 M $MgCl_2$, pH 9.3. After incubation at 37 °C for 30 min, the mixture was extracted with an equal volume of phenol, mixed for 5 min on a vortex mixer, and precipitated with 3 volumes of 95% ethanol.

The pellet was redissolved in 20 μL of 50 mM glycine–NaOH, pH 9.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 25% glycerol. The DNA solution was added to 200 μCi of dried [α-^{32}P]dATP (3500 Ci/mmol), and 4 μL of T4 polynucleotide kinase (4000 units/mL) was added. The reaction was incubated at 37 °C for 30 min. The end-labeled fragment was chromatographed on Sephadex G-100 to remove excess [^{32}P]dATP. The DNA was dried under vacuum in a desiccator and then redissolved in the appropriate enzyme buffer (100-μL reaction volume). The separation of labeled 5′ ends was accomplished by recutting the fragments with a second restriction enzyme. After restriction enzyme digestion, the two end-labeled fragments were separated by electrophoresis through a 4% polyacrylamide gel and eluted. Fragments were eluted from the gel by the method of Maxam & Gilbert (1977).

DNA Sequencing. Sequencing of end-labeled DNA fragments was accomplished by the method of Maxam & Gilbert (1977).

After chemical modification of the fragments, the reaction products were separated on 8% and 20% polyacrylamide thin gels (20 cm × 40 cm × 0.4 mm) containing 7 M urea at 25 mA (Sanger & Coulson, 1978).

Results

Partial Purification of Ovomucoid mRNA. Sequential chromatography of a total hen oviduct RNA extract on oligo(dT)-cellulose, Sepharose 4B, which substantially separated the ovalbumin mRNA activity from ovomucoid and lysozyme mRNA activities, and a second oligo(dT)-cellulose column, yielded an mRNA preparation which consisted primarily of two different size classes of RNA, as shown in Figure 1. To separate these two size classes, the mRNA preparation was denatured and applied to 12.2-mL linear 5–20% sucrose gradients. Although the RNA banded in a rather broad peak, judicious collection of fractions from the leading edge of the peak resulted in the removal of the bulk of the smaller contaminating mRNA. The final purified mRNA (as recovered from the sucrose gradient) migrated as a single band on the gel. Using globin mRNA and ovalbumin mRNA as standards, the size of the purified mRNA ($mRNA_{om}$) was estimated to be 800 nucleotides (Figure 1).

Translation Analysis of Ovomucoid mRNA Purification. The enrichment of $mRNA_{om}$ relative to other mRNA species during the purification was assessed by translation of the RNA preparations in a cell-free translation system derived from wheat germ. Ovomucoid mRNA activity was determined by specific immunoprecipitation of ovomucoid peptides using a partially purified goat anti-ovomucoid immunoglobin G. The measurement of radioactivity incorporated into Cl_3CCOOH-insoluble material was used as an indication of total mRNA activity.

The purification of ovomucoid mRNA as followed by translation is shown in Table I. The combination of purification steps listed resulted in a 270-fold increase in the specific activity of ovomuocid mRNA compared with the total RNA extract. The percentage of immunoprecipitable ovomucoid in the wheat germ postribosomal supernatant fraction compared with the total peptides synthesized during purification increased from 3% to 52%.

An analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis of the peptides synthesized in the wheat germ cell-free translation assay in response to added $mRNA_{om}$ was performed. The labeled, anti-ovomucoid precipitable product

synthesized in the wheat germ system had a R_f of 0.42, considerably larger than the ovomucoid standard R_f of 0.30. This could represent a premature termination of translation, but more likely is due to a lack of glycosylation in vitro of the ovomucoid polypeptide.

RNA Excess Hybridization to [^{3}H]cDNA. Although the purified RNA was essentially one band on agarose gels and was translated primarily into one species of polypeptide, only 52% of the total peptides synthesized in the translation assay were precipitable with anti-ovomucoid. In order to determine whether the antibody precipitation of the translated peptides was at fault, or whether the $mRNA_{om}$ was indeed only 52% pure, an RNA complexity analysis was performed. An aliquot of the [^{3}H]cDNA synthesized from $mRNA_{om}$ was characterized on a 5–20% alkaline sucrose gradient. Using full-length globin cDNA and ovalbumin cDNA as standards, the majority of the [^{3}H]cDNA was shown to represent an essentially complete copy of the $mRNA_{om}$ (data not shown). RNA excess hybridization was carried out using the ovomucoid-enriched RNA recovered from the sucrose density gradient at a RNA/DNA ratio of approximately 48. Analysis of the data indicated that a hybridization curve containing three different RNA classes provided a statistical best fit for the data (Figure 2). The equivalent $R_0t_{1/2}$ values for the most abundant, intermediate, and least abundant classes are, respectively, 5×10^{-3}, 1.5×10^{-1}, and 4.1. If a correction is made for the fact that each component represents only a fraction of the total RNA population, the $R_0t_{1/2pure}$ for the most abundant component is 2.0×10^{-3}. This value is intermediate between the $R_0t_{1/2}$ values for pure ovalbumin mRNA (Monahan et al., 1976a) and globin mRNA (Honjo et al., 1974) and is therefore consistent with the complexity of a purified ovomucoid mRNA. The fact that this component represents ~40% of the mRNA sequence present is consistent with the translation data, in which 50% of the mRNA was determined to be $mRNA_{om}$.

Preparation of dsDNA from $mRNA_{om}$. Since it was clear that $mRNA_{om}$ was not a pure species, and conventional methods of RNA purification had been exhausted, we decided to complete the purification, and isolate the coding ovomucoid DNA sequence at the same time by molecular cloning. To this end, dsDNA was prepared from the partially purified $mRNA_{om}$ and the hairpin loop cut with S_1 nuclease as described in Materials and Methods. The size of the S_1-treated dsDNA was estimated on 2% agarose gels to be 800–850 nucleotide pairs, although small amounts of dsDNA of heterogeneous size (from 1100 bp to 300 bp) were also observed. To obtain only full-length dsDNAs for restriction analysis and cloning, a preparative agarose gel was run using the same conditions. After excision of a 0.6-cm band which should contain dsDNAs of about 775–900 base pairs, the gel was stained in ethidium bromide and photographed under UV light to ensure that the distinct DNA band had been excised. We recovered approximately 40% of a particular DNA band from an agarose gel by the extraction method used (Sharp et al., 1974).

Preparation of Chimeric Plasmids. For molecular cloning experiments, the dsDNA was "tailed" with [α-^{32}P]dATP to an average length of 51 dAs per 3′ terminus by terminal transferase. About 20 000 dpm of this material (negligible mass) was subjected to electrophoresis on a 2% agarose gel at pH 8.4. After visualizing the SV40 *Hae*III standard with ethidium bromide staining, autoradiography revealed a discrete ^{32}P-labeled species of length 900–1000 base pairs.

Chimeric plasmids were formed by annealing this [^{32}P]-dsDNA to *Pst*1-cut pBR322. Ligation of the hybrid molecule as well as repair of any gaps which might occur due to the difference in length of the poly(dA) and poly(dT) regions occurred in vivo after transformation (Chang & Cohen, 1977).

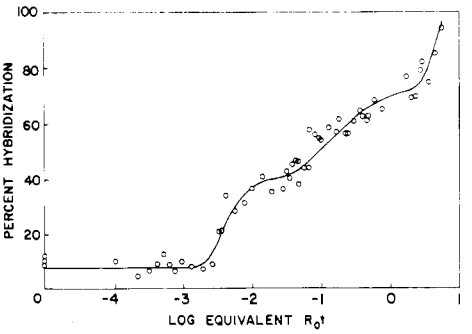

FIGURE 2: RNA excess hybridization to [^{3}H]cDNA. The hybridization reactions were carried out at 68 °C in 0.01 M Tris-HCl, pH 7.0, containing 0.6 M NaCl, 0.002 M EDTA, 25 ng/mL of [^{3}H]cDNA (specific radioactivity 1.2×10^5 cpm/μg), and 1.2 μg/mL $mRNA_{om}$ in a final volume of 50 μL. The reaction time ranged from 5 s to 71 h. The reaction mixtures were frozen in dry ice–acetone at various time points and assayed for hybrid formation as detailed in Materials and Methods.

Amplification of Chimeric Plasmids. In order to obtain large amounts of chimeric plasmids for screening, the plasmids formed in vitro were mixed with $CaCl_2$-treated χ1776 recipient cells. Transformation of χ1776 to tetracycline resistance was detected by plating the transformation mixture on L-agar plates supplemented with DAP (100 μg/mL) in the presence of tetracycline (Tc; 12.5 μg/mL). A total of 34 Tc^r clones were selected. Portions of these clones were transferred to fresh Tc and Ap (ampicillin; 40 μg/mL) plates. This procedure provides a counter selection for loss of Ap^r by insertion of DNA into the *Pst* site of pBR322 (Bolivar et al., 1977). Twenty-nine Tc^r clones were also Ap^s.

The remainder of each Tc^r clone on the original Tc plate was transferred directly to nitrocellulose filters. The filters were immediately processed for hybridization by in situ lysis (Grunstein & Hogness, 1975), and treatment with Denhardt's solution (Denhardt, 1966; Botchan et al., 1976). A replica of each filter was kept by incubating the original Tc plate at 37 °C for 48 h. Relatively thick agar layers (30–40 mL) must be used in the original plating of transformants to allow for dehydration during long incubation. This technique saves the time required for laborious transfer of individual clones to filters. The growth of colonies on the filters (Grunstein & Hogness, 1975) prior to lysis was not required.

In this study, 15 of the 29 Tc^r/Ap^s clones contained inserted DNA complementary to [^{32}P]cDNA synthesized from $mRNA_{om}$. The clones shown not to contain hybridizable cDNA inserts may contain very small inserted sequences sufficient to destroy Ap^r function in pBR322. The method of direct transfer and hybridization allowed quick identification of cloned DNAs of hybridizable length. The screening for specific hybridization as well as minimum size made this procedure more reliable for the purposes of this study than merely selection by antibiotic resistance. Direct transfer of colonies saves 2–3 days in the preparation of χ1776 clones for in situ hybridization and leaves an exact replica of the lysed clones for further study.

Plasmid DNA was prepared from positive clones as described in Materials and Methods. Routinely, 100–200 μg of purified, covalently closed circular DNA was obtained per L of cell culture.

Determination of Lengths of Inserted DNA in Recombinant Plasmids. Cleared lysates from the 15 positive clones were prepared from 50-mL cultures. These crude DNA preparations

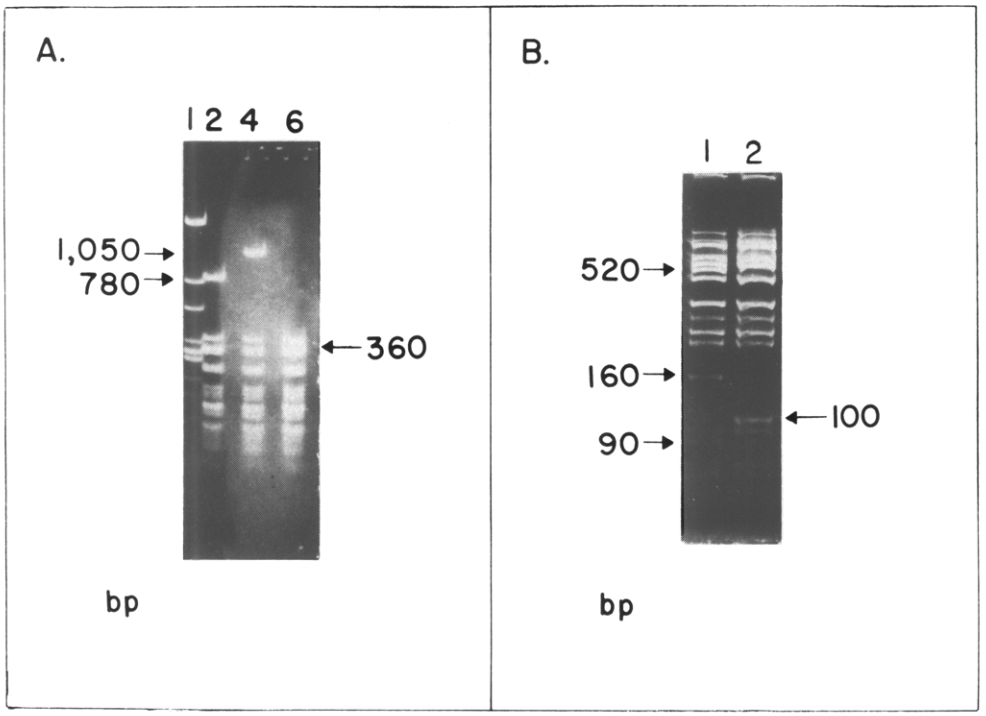

FIGURE 3: Electrophoresis of *Hha*I and *Alu*I digests of plasmid DNAs. (A) *Hha*I digests analyzed on a 2% agarose slab gel. Electrophoresis was in 50 mM Tris (pH 8.4), 20 mM sodium acetate, 18 mM sodium chloride, and 2 mM EDTA for 5 h at 70 V. After the gel had been stained in ethidium bromide (5 μg/mL) for 30 min, the DNA was visualized under ultraviolet light. The samples shown are: (1) *Hae*III cut SV40 DNA standard; (2) *Hha*I digest of pOM111; (4) *Hha*I digest of pOM100; (6) *Hha*I digest of pBR322. The size of the unique bands in the pOM100 and pOM111 digests, and the *Hha*I fragment surrounding the *Pst*I cloning site in pBR322, are indicated in base pairs. (B) *Alu*I digests of pOM100 and pBR322 on a 6% acrylamide slab gel. Six percent polyacrylamide gels contained 5.68 g of acrylamide, 0.32 g of *N,N'*-methylenebisacrylamide, 60 μL of *N,N,N',N'*-tetramethylethylenediamine (Temed), 0.6 mL of 10% ammonium persulfate in 50 mM Tris–borate, pH 8.3, 1 mM EDTA. The *Alu*I fragments of pOM100 (1) show three unique bands (indicated by arrows) when compared with the *Alu*I digest of pBR322 (2), which contains one band (arrow) not seen in 1. The sizes are given in base pairs.

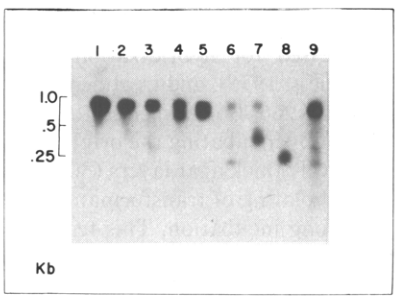

FIGURE 4: The action of restriction enzymes on ^{32}P-labeled ovomucoid-enriched dsDNA (dsDNA$_{om}$). [^{32}P]dsDNA$_{om}$ (20 000 cpm) and 1 μg of DNA were incubated with 1 unit of various restriction endonucleases for 3 h at 37 °C. Electrophoresis of the digests was performed on a 2.5% agarose gel as in Figure 3. The gel was placed on Kodak X-omat R film for 24 h at −80 °C to produce the autoradiograph. The restriction endonucleases used were: (1) none; (2) *Hin*dIII; (3) *Hha*I; (4) *Eco*RI; (5) *Bam*HI; (6) *Pst*I; (7) *Hae*III; (8) *Hin*fI; (9) *Alu*I. The sizes of the fragments were estimated by comparison with *Hae*III cut SV40 DNA visualized with ethidium bromide.

were subjected to electrophoresis on a 1% agarose slab gel. All except four contained plasmids which were larger than the parent plasmid pBR322. The four showed no visible plasmid band, possibly due to low cell densities in these cultures or incomplete lysis during preparation.

Two of the plasmids, designated pOM100 and pOM111, were purified from larger volumes of cells and digested with several restriction enzymes. *Hha*I cleaves native pBR322 on either side of the *Pst*I cloning site creating a fragment of approximately 340 bp which contains the *Pst*I site. *Hha*I does not cleave cDNA synthesized from mRNA$_{om}$. Digests of pOM100 and pOM111 with *Hha*I produced unique bands of 1050 bp and 780 bp, respectively (Figure 3A). These data indicated that pOM100 contained approximately 700 bp and pOM111 400 bp of inserted DNA. Thus, pOM100 could contain enough DNA to code for the entire ovomucoid protein (Kato et al., 1977).

The size of the pOM100 insert was also determined by *Alu*I digestion and electrophoresis on a 6% polyacrylamide slab gel (Figure 3B). The *Alu*I fragment of pBR322 which contains the *Pst*I site is 100 bp in length. Comparison of *Alu*I digests of pBR322 and pOM100 showed three unique bands in the pOM100 digest. These bands correspond to fragments of 520, 160, and 90 bp in length. The 100 bp *Alu*I fragment of pBR322 is absent in the pOM100 digest. This estimate of 670 bp of inserted DNA agrees well with the data from the *Hha*I digestion.

Restriction Mapping of Ovomucoid-Enriched dsDNA and Recombinant Plasmids. For these studies, dsDNA was synthesized from the mRNA$_{om}$ using [^{32}P]dGTP in the second strand. This labeled dsDNA was then digested with several restriction endonucleases, in order to obtain a restriction map for the later sequence analysis of cloned dsDNA. The radioautography results of the digestion with eight different endonucleases are shown in Figure 4. None or very little of the enriched dsDNA$_{om}$ was cut by the restriction endonucleases *Hha*I and *Hin*dIII. Both *Eco*RI and *Bam*H1 digestion resulted in two equally intense, labeled species; one is apparently uncut dsDNA and the other is only slightly smaller. *Hae*III also leaves some uncut material and produces predominantly a species of ~350 nucleotides as well as much smaller material. *Alu*I digestion produced predominantly a species only slightly

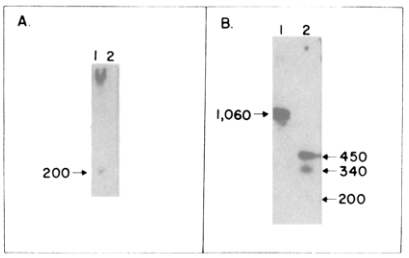

FIGURE 5: Electrophoresis of *Pst*I, *Hha*I, and *Pst*I + *Hha*I digests of pOM100 on 2% agarose slab gels. (A) *Pst*I digests of (1) pOM100 and (2) pBR322. After electrophoresis under the conditions of Figure 3, the DNA fragments were transferred to filter paper by the techniques of Southern (1975). The transferred DNA was hybridized to [^{32}P]cDNA prepared as described in Materials and Methods, and the dried filter paper exposed to Kodak X-omat R film at −80 °C with a Polaroid enhancing screen. (B) *Hha*I (1) and *Pst*I + *Hha*I (2) digests of pOM100. The electrophoresis and Southern transfer were performed as in A. The fragment sizes are listed in base pairs.

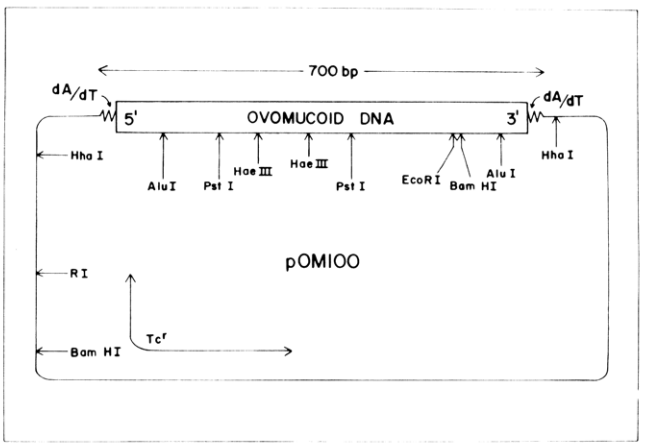

FIGURE 6: A partial restriction map of pOM100. The map shows the inserted ovomucoid DNA fragment (on an expanded scale) and its orientation in the pBR322 plasmid DNA. The approximate cleavage sites for the restriction endonucleases *Hha*I, *Pst*I, AluI, *Hae*III, *Eco*RI, and *Bam*HI are indicated. The ovomucoid DNA is inserted at the *Pst*I site of pBR322, and therefore interrupts the ampicillin resistance gene.

smaller than the uncut dsDNA; however, distinct fragments of 275 bp and 225 bp and also two fragments less than 150 bp were produced. *Hin*fI apparently cut all of the dsDNA, producing only one discernible species of ~250 nucleotide pairs. *Pst*I digestion left a small portion of the material uncut and produced a very distinct labeled species of ~200 nucleotide pairs, as well as some larger material. Since a majority of the dsDNA species should be dsDNA synthesized from ovomucoid mRNA, the distinct 200 bp *Pst*I fragment would presumably be cut from this ovomucoid dsDNA ($dsDNA_{om}$). Therefore, any recombinant plasmid that yielded this 200 bp *Pst* fragment would be likely to contain an ovomucoid DNA insert.

Indeed, the *Pst* digestion of pOM100 did result in two bands on agarose gels, and one of them was 200 bp in length. The second fragment migrated close to linear pBR322. This result suggested that the 200-bp fragment resulted from two internal cuts in the inserted DNA sequence, and that the rest of the DNA insert (~500 bp) was still attached to pBR322. This was confirmed by a Southern blot of a 2% agarose gel containing a *Pst*I digest of pOM100 as shown in Figure 5A. The autoradiogram from this experiment revealed that both *Pst* fragments of pOM100 specifically bound [^{32}P]cDNA synthesized from $mRNA_{om}$. In addition, a double digestion of pOM100 by *Pst*I and *Hha*I followed by blotting and hybridization showed that the *Hha*I fragment, which contains the entire cloned DNA sequence, is cut into three hybridizable bands with *Pst*I (Figure 5B). The 200-bp fragment, which is diffuse and barely visible at this short exposure, is an internal fragment of the cloned DNA resulting from two *Pst* cuts. The 445- and 340-bp fragments then would represent the two ends of the cloned DNA sequence, with some flanking pBR322 sequences attached.

Several other chimeric plasmids prepared in this study were found to contain the identical 200-bp *Pst*I fragment which was detected by Southern blotting and hybridization. These data were suggestive that these plasmids all contain ovomucoid DNA since $mRNA_{om}$ was the predominant messenger RNA in the initial preparation.

Other restriction endonucleases which cleaved the pOM100 insert are: *Bam*HI, *Hae*III, *Eco*RI, and *Hin*fI. A partial restriction map of pOM100 appears in Figure 6. Both *Eco*RI and *Bam*HI cut the cloned DNA sequence once, at sites very close to each other and at an end of the cloned DNA sequence distal from the single *Eco*RI and *Bam*HI sites in pBR322. Furthermore, both enzymes cut the 340-bp *Hha*I + *Pst*I fragment, indicating that this fragment is attached to the plasmid away from the *Eco*RI site of pBR322, while the 450-bp *Hha*I + *Pst*I fragment is attached near the *Eco*RI site (Bolivar et al., 1977).

The approximate positions of two *Alu*I and *Hae*III sites are also indicated in Figure 6. Besides *Hha*I, *Hin*dIII also did not cleave the pOM100 insert. The 5′ to 3′ orientation of the cloned DNA was determined by partial sequencing (described below).

Demonstration of Clones Containing an Inserted Ovomucoid DNA Sequence. The 15 "positive" clones that resulted from the cloning of dsDNA made from the purified $mRNA_{om}$ were shown to hybridize to a [^{32}P]cDNA probe made to this same mRNA preparation. Since the $mRNA_{om}$ was far from homogeneous, however, the probe should also represent many sequences. Thus it was necessary to identify a clone unambiguously as containing an ovomucoid insert.

Therefore, an assay based on mRNA hybridization and translation was developed. Recombinant plasmid DNA was isolated, as described above, from several positive clones. Plasmid DNA (100 μg) was then digested with the restriction endonuclease *Hha*I, heat denatured, and bound to Millipore filters as described in Figure 7. Either pure ovalbumin mRNA ($mRNA_{ov}$) or the enriched $mRNA_{om}$ was then hybridized to the DNA filters for 12 h at 42 °C in 600 μL of 50% formamide buffer. An equal amount of poly(dA) was added to prevent hybridization of the poly(A) tract of noncomplementary mRNAs to the linkers present in the filter-bound DNA. After removing the buffer, and washing the filters several times to remove nonspecifically adsorbed RNA, any hybridized RNA was eluted by denaturing in 0.01 M Tris-HCl, pH 7.4, 3 mM EDTA for 2 min at 90 °C. Carrier tRNA was added (10 μg/mL) and the RNA precipitated in ethanol. The recovered hybridizable RNA was translated in the wheat germ system, as described in Materials and Methods, and the amounts of ovalbumin- and ovomucoid-specific products were compared (Figure 7). Clone pOV230, a clone that contains the full-length ovalbumin structural gene sequence, was used to test the validity of the assay. The RNA that hybridized to this plasmid DNA was translated into ovalbumin, and not ovomucoid (Figure 7). This indicated that filter-bound recombinant plasmid DNA containing a known structural gene DNA sequence could selectively hybridize to a complementary mRNA species, and that this RNA could be removed from the filter and translated into antibody-precipitable peptides in the wheat germ system. The other two plasmids tested in Figure 7 both resulted from the transformation with $dsDNA_{om}$ reported in

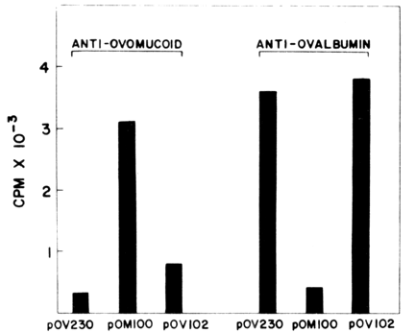

FIGURE 7: Hybridization-translation assays of cloned DNAs. Plasmid DNA (100 μg; either pOV230, pOM100, or pOV102) was digested with 30 units of *Hha*I at 37 °C for 16 h, followed by phenol extraction. After denaturing each restricted DNA in a small volume by heating at 100 °C for 10 min and quick cooling, each was diluted into 6 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$ in 4 × SSC to a final concentration of 10 μg/mL and passed twice through a Millipore filter at 4 °C. About 20% to 30% of the DNA bound to the filters. The filters were then baked at 70 °C in a vacuum oven to fix the DNA. One-half of each filter was then hybridized to pure $mRNA_{ov}$ and the other half to the enriched $mRNA_{om}$ in 70 mM Tris-HCl, pH 7.7, 7.2 mM EDTA, 0.42 M NaCl, 50% in formamide. After denaturing the RNA/DNA hybrids, this hybridized nucleic acid was recovered and translated in the wheat germ system (see Materials and Methods). The polypeptides synthesized were precipitated with either anti-ovomucoid or anti-ovalbumin. The radioactive peptides precipitated with anti-ovomucoid are shown for all three plasmid DNAs on the left, and the precipitates with anti-ovalbumin on the right. No correction factor for nonspecific trapping of label was applied to the antibody precipitations.

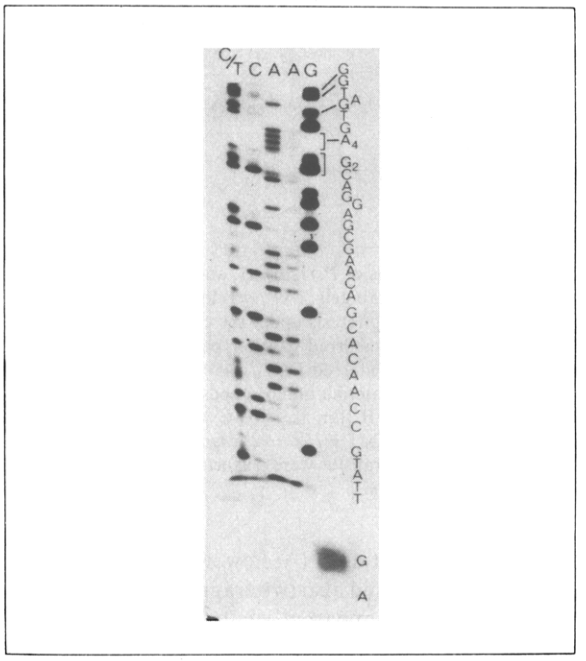

FIGURE 8: Autoradiograph of the nucleotide sequence derived from *Pst*I fragment Pl of pOM100. Plasmid DNA was cleaved with *Pst*I and the 5′ ends were dephosphorylated and labeled with [γ-^{32}P]dATP. Fragment P1 was isolated and cleaved with *Hin*fI. The sequence shown corresponds to the coding (mRNA) strand near the left *Pst*I site toward the *Hin*fI site in Pl (see map, Figure 6). Band doubling in the C + T and C lanes resulted in some anomalous bands. This may be due to incomplete β elimination. The nucleotide sequence matches the peptide sequence data determined by Beeley (1976) as reported in Results.

this manuscript. Translation of the mRNA that hybridized to the pOM100 DNA filter yielded immunoprecipitable ovomucoid. The clone from which pOM100 DNA was derived must therefore contain an inserted ovomucoid DNA sequence. The other plasmid DNA tested in Figure 7, pOV102, hybridized only to $mRNA_{ov}$. The fact that this plasmid contained an ovalbumin DNA sequence was not surprising since about 50% of the dsDNA used in the transformation represented non-ovomucoid sequences. Since the mRNA used to synthesize the dsDNA was considerably shorter than native ovalbumin mRNA, the mRNA preparation must contain fragments of about 800 base pairs derived from the 3′ end of ovalbumin mRNA.

Confirmation of an Ovomucoid Insert by DNA Sequencing. In order to confirm the identity of the cloned DNA of pOM100 as ovomucoid cDNA, a partial DNA sequence was determined. The amino acid sequence of four glycosylated peptides of ovomucoid has been reported (Beeley, 1976).

Recombinant plasmid pOM100 was cleaved with *Pst*I and digested with snake venom phosphodiesterase (McReynolds et al., 1978). This treatment enabled labeling of the 5′ termini with bacterial alkaline phosphatase and T4 polynucleotide kinase (Maxam & Gilbert, 1977). The 200-bp *Pst*I fragment (designated P1) was isolated on a 12% polyacrylamide slab gel. The fragment was eluted from the gel with 90% efficiency in recovery of label.

The purified fragment was incubated with *Hin*fI which cleaves P1 into two fragments of 116 and 84 bp in length. The DNA sequence of fragment P1 of pOM100, shown in Figure 8, codes for portions of two glycoslyated ovomucoid peptides whose amino acid sequence was determined by Beeley (1976). The matched sequence included a thirteen amino acid peptide, as shown in Diagram I. This sequence match is well beyond random probability and proved that the inserted DNA of pOM 100 contained ovomucoid sequences.

Discussion

Although the synthesis of ovomucoid is induced in the estrogen-stimulated chick oviduct, it still comprises only about 8% of the total protein synthesized in the fully stimulated hen oviduct (Palmiter & Smith, 1973). We have shown that several conventional techniques of mRNA purification yielded an ovomucoid-enriched mRNA preparation that contained only about 50% $mRNA_{om}$ by translation analysis. Although these techniques were capable of yielding large amounts of purified mRNA, most were based on size differences and consequently were not expected to yield pure $mRNA_{om}$. This is in agreement with the recent results of Buell et al. (1978) who used similar techniques to obtain an mRNA preparation that contained about 33% $mRNA_{om}$. The purification of $mRNA_{om}$ has been reported recently by Graner et al. (1977) using a specific immunoadsorption of ovomucoid polysomes. This method yields microgram quantities of mRNA, however, and is not suitable for the large-scale isolation of a pure mRNA species that we desired. Instead, molecular cloning was utilized to isolate the ovomucoid coding sequence from the preparation of ovomucoid-enriched mRNA. A dsDNA copy was synthesized from this mRNA and cloned in *E. coli* χ1776 using the plasmid vector pBR322. Recombinant transformants were selected by direct colony transfer followed by in situ hybridization. This method proved to be as fast as and more reliable than antibiotic selection for identification of inserts of sufficient length to

DIAGRAM I

^{72}SER TYR ALA ASN THR THR SER GLU ASP GLY LYS VAL MET^{84}

$_{5'}$AGT TAT GCC AAC ACG ACA AGC GAG GAC GGA AAA GTG ATG$_{3'}$

hybridize to a labeled cDNA probe. Fifteen "positive" clones were identified by this method. An assay was developed to screen these cloned DNAs, using hybridization of an impure mRNA preparation to the plasmid DNA and translation of the hybridizable mRNA species. Several clones containing ovomucoid DNA sequences were identified by this technique.

The purity of the $mRNA_{om}$ proved difficult to assess accurately for several reasons. Ovomucoid is a glycoprotein, and about 25% of the molecular weight is due to carbohydrate moieties (Feeney, 1971). In the wheat germ translation system used for routine mRNA translations, glycoslyation of the apoovomucoid could not be expected to occur. Since the anti-ovomucoid antiserum was produced against the native, glycosylated ovomucoid, a less than optimum antibody/antigen recognition was possible. Also, and probably for the same reason, the polypeptide produced by translation of the purified $mRNA_{om}$ had a different mobility than the ovomucoid standard on 12.5% acrylamide-$NaDodSO_4$ gels. Furthermore, hybridization analysis indicated that the cDNA population contained at least three different components (Figure 2). The most abundant class represented about 40% of the cDNA sequences and hybridized with a $R_0t_{1/2}$ value (2×10^{-3}) close to that expected for a pure mRNA (Monahan et al., 1976; Honjo et al., 1974). The intermediate class probably represents fragments of the 3′ end of ovalbumin mRNA which are the same size as the ovomucoid mRNA. Indeed, some of the 15 clones obtained after transformation with this $dsDNA_{om}$ have since been shown to contain truncated ovalbumin DNA inserts (Figure 7 and unpublished observations). The least abundant class may represent the many species of mRNA of the approximate size of $mRNA_{om}$ that may code for "housekeeping" enzymes or structural proteins. Since it was clear, therefore, that $mRNA_{om}$ was not pure, but did represent the predominant mRNA species, bacterial cloning procedures were used to complete the purification of the ovomucoid gene sequence.

Several examples of the cloning of cDNAs from impure mRNAs have been reported. In two instances, the cloning of the human globin gene sequences by Wilson et al. (1978) and of the mouse immunoglobin light-chain gene sequence by Seidman et al. (1978), pure probes existed which were used to screen for the proper recombinant plasmids. The other examples involved the cloning of the structural genes for the peptide hormones rat insulin (Ullrich et al., 1977), rat growth hormone (Seeburg et al., 1977), and human chorionic somatomammotropin (Shine et al., 1977). In each of these cases the proper recombinant clones were identified by sequencing the inserted DNA after cutting it out of the plasmid, and comparing the nucleotide sequence with the known amino acid sequence of the hormones. In the isolation reported here, however, we had neither a pure probe for hybridization analysis nor could we use DNA sequencing to screen a large number of clones.

Thus, in order to screen the 15 "positive" clones and identify one as containing an ovomucoid DNA insert, a different assay was needed. Coupled transcription-translation of SV40 genes and cloned *Drosophila* histone genes has been reported in *Xenopus* oocytes (DeRobertis & Mertz, 1977) and of SV40 genes and calf preproparathyroid gene in a wheat germ cell-free system (Roberts et al., 1975; Kronenberg et al., 1977). This method, however, requires an insert that is a full-length copy of the mRNA sequence. Instead, we have adapted a hybridization method used to identify *Adeno* 2 mRNA sequences (Gelinas & Roberts, 1977), and designed and demonstrated the utility of an alternative, simplified assay. Plasmid DNA was isolated and cut with the restriction endonuclease *Hha*I which did not cut the $dsDNA_{om}$. This digest was then denatured and bound to Millipore filters. mRNAs were hybridized to the DNA filters in 50% formamide at 42 °C. Removal of the supernatant and several rinses sufficed to remove nonhybridizable RNA; any complementary RNA species should hybridize to the cloned DNA on the filter. The use of immobilized DNA circumvents the problem of separating the DNA from the hybridizable mRNA. After denaturation of DNA/RNA hybrids, the recovered mRNA was then translated in the wheat germ cell-free system and the amount of immunoprecipitable ovomucoid and ovalbumin synthesized was compared (Figure 7). This method does not require a pure mRNA probe, or any form of transcription or sequencing techniques and should be applicable to any cloned gene sequence which codes for a protein to which antibodies have been prepared. Furthermore, it has an advantage over the recently employed hybrid-arrested cell-free translation (Paterson et al., 1977) in that a positive identification of the cell-free translation product is obtained rather than the absence of the cell-free product.

Based on the results of this assay, one particular clone, pOM100, was identified that contained DNA coding for ovomucoid. This plasmid contains approximately 650 bases of the ovomucoid mRNA sequence as shown by digestion of pOM100 with *Hha*I and *Alu*I (Figure 3). A partial restriction map of this cloned DNA is shown in Figure 6. Direct detection of the ovomucoid coding sequence was obtained and the 5′ → 3′ orientation of the ovomucoid insert was determined by matching amino acid and nucleotide sequence data which span the left *Pst*I site. As shown in the map, the 5′ end of the sense (mRNA) strand was attached to the plasmid proximal to the single pBR322 *Eco*RI site. It can be estimated, therefore, that the cloned fragment contains sufficient material to code for all but about 16 amino acids at the N terminus and the signal peptide (Palmiter et al., 1978). There is also enough DNA in the insert to complete the coding sequence plus approximately 150 base pairs of noncoding sequence at the 3′ terminus.

The successful cloning of the chick ovomucoid coding sequence makes possible the preparation of a pure ovomucoid DNA probe, which will be used to study the in vivo hormonal control of ovomucoid mRNA transcription, and also to purify and clone the native ovomucoid gene from chick DNA. Several eucaryotic genes have recently been shown to contain intervening DNA sequences (Brack & Tonegawa, 1977; Breathnach et al., 1977; Lai et al., 1978; Jeffreys & Flavell, 1977; Tilghman et al., 1978; Goodman et al., 1977; Valenzuela et al., 1978) and the restriction enzyme data reported in this study will be used to determine whether the ovomucoid gene also contains these sequences. Ultimately, the cloning of the genomic ovomucoid DNA sequence will permit the functional and structural comparison of two estrogen-inducible genes (ovomucoid and ovalbumin) within the same tissue.

After this manuscript had been submitted, a report detailing a similar assay was published (Harpold, M. M., Dobner, P. R., Evans, R. M., & Bancroft, F. C. (1978) *Nucleic Acids Res.* *5*, 2039–2053). These authors reported the cloning of a cDNA sequence copied from a partially pure rat pre-growth hormone mRNA. To identify the cDNA sequence in the recombinant clone, they developed a similar filter hybridization/translation assay utilizing Millipore filter bound plasmid DNA and partially purified pregrowth hormone mRNA. Conclusive evidence that one recombinant plasmid DNA selectively hybridized pre-growth hormone mRNA was provided.

Acknowledgments

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