The Ovalbumin Gene

PARTIAL PURIFICATION OF THE CODING STRAND*

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Purified ovalbumin messenger RNA was employed to selectively enrich the concentration of the gene coding for ovalbumin from total chick DNA by molecular hybridization. The coding strand of the ovalbumin gene was partially purified from sheared chick DNA by affinity column chromatography using ovalbumin mRNA immobilized on phosphocellulose. The concentrations of the ovalbumin DNA sequence in various DNA fractions were quantitated by measuring their rates of hybridization with \[^{32}P\] labeled ovalbumin mRNA. When apparent \(C_{60}\) values of these reactions were compared to the apparent \(C_{60}\) value obtained from the hybridization reaction between \[^{32}P\] ovalbumin mRNA and complementary DNA synthesized against ovalbumin mRNA using the enzyme reverse transcriptase, purification of the coding ovalbumin DNA strand over total chick DNA was estimated to be approximately 9,600-fold. There was no apparent degradation of the 4,000 nucleotide strands of chick DNA throughout the purification procedure. Since ovalbumin mRNA has a complexity of 1890 nucleotides, the resulting DNA was more than twice the length of ovalbumin mRNA and thus should contain DNA sequences adjacent to the structural portion of the ovalbumin gene.

Evidence accumulated recently has indicated that estrogen induces the synthesis of ovalbumin messenger RNA in the chick oviduct via a "transcriptional control" mechanism (1–12). To attempt definitive studies on the interaction between the ovalbumin gene and oviduct DNA-dependent RNA polymerase, hormone-receptor complexes and chromosomal proteins, the ovalbumin gene must eventually be purified. The study of these interactions and their effects on transcription of the isolated ovalbumin gene in vitro would provide further insight into the molecular mechanism of steroid hormone action as well as the general mechanism by which effector molecules regulate gene expression in eukaryotes. Such studies have been successfully carried out in several prokaryotic systems where purified operon DNAs were used to investigate the mechanism of interaction with various regulatory proteins. These studies have led to the revelation of the sequence, organization, and function of operator and promoter regions. Moreover, such investigation has greatly enhanced the understanding of regulatory aspects of transcription of these operons by repressors and inducers at the molecular level (13–16).

Owing to the enormous complexity of eukaryotic genomes, isolation of specific genes from a variety of these organisms has been restricted to a class of reiterated genes with \(G + C\) contents that were quite distinct from that of the bulk of nuclear DNA. Examples are those genes coding for ribosomal RNA, transfer RNAs, 5 S RNA, and histone messenger RNAs (17–28). The gene coding for fibroin messenger RNA also has an unusually high \(G + C\) content and is the only unique sequence eukaryotic gene which has been isolated to date (27, 28). Isolation of a unique eukaryotic gene with a density which is similar to total DNA, such as that for ovalbumin, requires methods of DNA fractionation based on parameters other than size or density alone. The availability of large quantities of ovalbumin messenger RNA purified to homogeneity with respect to various physical, chemical, and biological criteria (29, 30) allowed us to partially purify its DNA complement from total chick DNA by molecular hybridization. Purified ovalbumin mRNA was immobilized onto phosphocellulose and its DNA complement was enriched 9,600-fold from total chick DNA by affinity chromatography.

EXPERIMENTAL PROCEDURE

Materials—White Leghorn laying hens were purchased from Rich-Glo Farm, La Grange, Texas; oviducts and livers were quickly frozen in liquid nitrogen after dissection. Liquified phenol and reagent grade formamide (\(A_{260} < 0.25\)) were from Fisher Scientific Co., Fair Lawn, N. J.; phenol was redistilled before use. Wheat germ was a generous gift from General Mills, Inc., Minneapolis, Minn. Creatine phosphate, creatine phosphokinase, and sodium dodecyl sulfate were from Sigma Chemical Co. \([^{14}C]\) [valine (260 mCi/mmol), ultrapure urea, and sucrose (ribonuclease free) were from Schwarz/Mann, Orangeburg, N. Y. Pancreatic ribonuclease A and \(a\)-amylase were from Worthington. Pronase was from Calbiochem, Oligo(dT)-cellulose (T3) was from Collaborative Research, \[^{35}S\] carrier free \(> 300\) mCi/ml was from Amersham/Searle. Agarose (electrophoretic grade), Chelex 100, and Cellex P powder were from Bio-Rad Laboratories, Richmond, Calif. CsCl was from EM Laboratories, Inc., Elmsford, N. Y. 1,1′-Carbonyldimidazole was from Aldrich Chemical Co., Milwaukee, Wisc.
dine and N,N-dimethylformamide were from Matheson, Coleman and Bell, Norwood, Ohio. Avian myeloblastosis virus reverse transcriptase was a kind gift from Dr. J. W. Butel, Immunobiology Laboratory, University of Florida, College of Veterinary Medicine, Gainesville, Fla. Deoxyribonucleoside triphosphates were from Pabst, Milwaukee, and the radioactive nucleotides were from New England Nuclear Corporation.

Preparation of Purified Ovalbumin mRNA—Ovalbumin mRNA was purified from hen oviduct total nucleic acid extracts of hen oviducts, as described previously (29, 30) with the following modifications: (a) nick translation was replaced by oligo(dT)-cellulose column chromatography, and (b) the RNA was heated at 70° for 1 min and quick cooled before chromatography on the Sephacryl 4B column and the second oligo(dT)-cellulose column. Better resolution and recovery of ovalbumin mRNA were obtained with these modifications. Throughout the purification procedure, ovalbumin mRNA and total mRNA activities were quantitated by the cell-free wheat germ translation assay (29), and the purity of the mRNA was monitored by acid-urea agarose gel electrophoresis (29). The purified ovalbumin mRNA was labeled with Na$^{32}$P to a specific radioactivity of $10^8$ cpm/pg as previously described (30). The labeled product was co-migrated with untreated ovalbumin mRNA in acid urea agarose gels.

Synthesis of DNA Complementary to Ovalbumin mRNA—DNA complementary to purified ovalbumin mRNA was synthesized to various specific radioactivities using reverse transcriptase isolated from avian myeloblastosis virus. (29). To obtain a specific radioactivity of $10^8$ cpm/pg, the cDNA$,m$ was synthesized as follows. 0.2 uCi each of [SH]dGTP, [SH]dATP, and [SH]dCTP were lyophilized to dryness and dissolved in 1 ml of a solution containing 50 mM Tris-HCl, pH 7.6, 1.0 mM MgCl$\text{2}$, 10 mM sodium acetate, pH 4.5, and the [SH]cDNA,, was again precipitated with alcohol and redissolved in 0.001 M Na,EDTA, pH 7.0. Two volumes of a solution of 80% ethanol were added to the DNA solution. This DNA solution was mixed and immediately incubated at 46° for 5 min. The reaction mixture was adjusted to contain 0.5% of sodium Sarkosyl, 10 mM Na$\text{2}$EDTA, and 100 pg/ml of sheared Escherichia coli DNA. The sample was passed through a Sephadex G-50 column at room temperature in 0.01 M Tris-HCl, pH 7.6, containing 0.1 M NaCl. Nucleic acid in the excluded fractions was precipitated with ethanol and redissolved in 0.5 ml of 0.1 M NaOH, containing 10 mM Na$\text{2}$EDTA, and heated at 60° for 30 min. The solution was chilled, adjusted to pH 5.0 with 2 M sodium acetate, pH 4.5, and the [SH]DNA,m was again precipitated with alcohol. Approximately 15 to 20% of the [SH]DNA,m represented complete complements of ovalbumin mRNA and greater than 50% revealed a complexity of 1000 nucleotides or more (31). These characteristics were determined by several methods including analysis by alkali sucrose gradient centrifugation, polyacrylamide gel electrophoresis in the presence of 88% formamide and washed through a gelatin-urea gradient previously described (31). To synthesize cDNA,m with very low specific radioactivities all ingredients in the synthetic reaction were identical to those described above, except only $[^{32}P]$GTP was used as label. Specific activity of the $[^{32}P]$cDNA,, in excess was carried out as previously described (30). $[^{32}P]$cDNA,m mRNA, $10^8$ cpm (10$^9$ cpm/µg), was mixed with 0.5 µM of $[^{32}P]$GTP, $[^{32}P]$ATP, and $[^{32}P]$CTP to a total concentration of 300 µM in hybridization buffer (0.01 M N-tris(hydroxymethyl)methyl-2-amino ethanesulfonic acid, 0.75 M Na,EDTA, and 25 µM/ml of purified mRNA.,). The final concentrations of the $[^{32}P]$GTP, $[^{32}P]$ATP, and $[^{32}P]$CTP were adjusted to 35 µM each. The mixture was chilled in ice for 5 min before reverse transcriptase was added to give a final concentration of 60 units/ml. This solution was mixed and immediately incubated at 46° for 5 min. The reaction mixture was adjusted to contain 0.5% of sodium Sarkosyl, 10 mM Na,EDTA, and heated at 60° for 30 min. The solution was chilled, adjusted to pH 5.0 with 2 M sodium acetate, pH 4.5, and the [SH]DNA,m was again precipitated with alcohol. Approximately 15 to 20% of the [SH]DNA,m represented complete complements of ovalbumin mRNA and greater than 50% revealed a complexity of 1000 nucleotides or more (31). These characteristics were determined by several methods including analysis by alkali sucrose gradient centrifugation, polyacrylamide gel electrophoresis in the presence of 88% formamide and washed through a gelatin-urea gradient previously described (31). To synthesize cDNA,m with very low specific radioactivities all ingredients in the synthetic reaction were identical to those described above, except only $[^{32}P]$GTP was used as label. Specific activity of the $[^{32}P]$cDNA,, in excess was carried out as previously described (30).

The radioactive content was determined by liquid scintillation spectrometry in a *spectrofluorotoluene* scintillation fluid (32). Per cent correction for quenching was determined by *spectrofluorotoluene* scintillation fluid (32). Per cent correction for quenching was determined by the internal standard method (33).

Preparation of Sheared Chick DNA—Chick liver DNA, free of protein and RNA, was prepared by a modification of the procedure of Marmur (34) as described previously (34). The DNA was adjusted to 1 mg/ml in 0.1 M sodium chloride/0.01 M sodium citrate, pH 7.0, containing 0.2 M NaCl and 0.005 M Na$\text{2}$EDTA and sheared in a French Press to a mean length of 4000 nucleotides. The length of the sheared DNA was estimated from sedimentation in alkaline sucrose gradients. Sheared DNA solutions were further treated with 50 µl/ml of pronase for 1 hour at 37° and extracted twice with the same buffer (phenol/sodium dodecyl sulfate buffer, pH 8.0) used for total nucleic acid extraction. The DNA was finally precipitated from solution with alcohol and redissolved in 0.01 M Na$\text{2}$EDTA, pH 7.0. Two volumes of a buffer (0.15 M Na$\text{2}$ tris(hydroxymethyl)methyl-2-amino ethanesulfonic acid, 0.0075 M Na$\text{2}$EDTA, 1.125 M NaCl, and 75% formamide, pH 7.0) was added to the DNA solution. This DNA solution was passed through a Chase 100 column before use for hybridization reactions.

Coupling Purified Ovalbumin mRNA to Phosphocellulose—Purified ovalbumin mRNA was chemically linked to Cellex P powder using a method based on that described by Saxinger et al. (35) and by Shi and Martin (36). Cellex P powder, 10 g, was repeatedly suspended in water and decanted until no particles remained. The powder was washed on a Buchner funnel and washed successively with large volumes of water, 0.25 M NaCl in 0.25 M HCl, 0.25 M Na$\text{2}$EDTA, and finally H2O. The powder was resuspended in 400 ml of 0.1 M tributylamine hydrochloride, pH 0.0, and filtered. This procedure was repeated four times and the powder was then dried by suction. It was then dried in vacuo over P2O$_5$/KOH for 7 to 10 days, and activated by gently shaking for 10 minutes at $0^\circ$C in 100 ml of N,N-dicyclohexylcarbdiamide dissolved in anhydrous dimethylformamide (100 mg/ml). The activated powder was collected on a Buchner funnel, washed extensively with anhydrous dimethylformamide followed by acetone, and finally dried in vacuo over Drierite overnight. Purified ovalbumin mRNA, 0.7 mg, and 1 µg of ovalbumin mRNA ($10^8$ cpm/µg) in 1 ml of water were added to 2 g of the activated phosphocellulose powder using a Pasteur pipette. The mixture was stirred with a glass rod for 5 min and again dried in vacuo over Drierite overnight. The coupling reaction was carried out by shaking the mixture gently in 100 ml of anhydrous pyridine at $50^\circ$ for 24 hours. The resin was collected by centrifugation at 3,000 × g for 15 min and the supernatant fluid was removed. The excess activated phospho groups were inactivated by gently mixing with 100 µl of 15% NH$_4$OH in an ice-water bath for 20 min. The mixture was again centrifuged and the supernatant fluid was removed. The pellet was again washed with 100 ml of 10% NH$_4$OH, and once with 50% formamide and five times with water in a similar manner. About 30% of the radioactivity was solubilized from the resin during the ammonium hydroxide treatment, but all of the other washing combined contributed no more than 10% of the total input radioactive ovalbumin mRNA. Very little radioactivity could be eluted from the resin by hybridization buffer at 70°, and the binding capacity of the resin did not diminish appreciably after 2 months of continuous use.

Affinity Chromatography—The procedure was carried out as described by Shih and Martin (37). Two grams of phosphocellulose-ovalbumin mRNA matrix was packed in a jacketed column (2 × 15 cm). The column was connected in series with a peristaltic pump, a reservoir, and a second jacketed column (2 × 15 cm) which was back-connected to the column containing the affinity resin in a closed system. The second column was maintained at 70° for 30 min in the absence of T$\text{4}$C of DNA so that all DNA molecules would be denatured before coming into contact with the ovalbumin mRNA affinity resin in the first column which was regulated at 48°. Six thousand A$\text{260}$ units of sheared chick DNA in 500 ml of hybridization buffer was recycled through this system for 30 min at a flow rate of 100 ml/min. After the sample was thoroughly mixed with 50 ml of pronase for 1 hour at 37°, the resin was collected by centrifugation at 3,000 × g for 15 min and the two columns were disconnected. The resin was washed with fresh hybridization buffer until no further A$\text{260}$-absorbing material could be eluted. The column temperature was then raised to 55° for 15 min and the resin was again washed with fresh buffer. The hybridization buffer was added to the hybridization buffer at 70°. About 30% of the radioactivity was solubilized from the resin with fresh hybridization buffer at 70°, and the binding capacity of the resin did not diminish appreciably after 2 months of continuous use.

1The abbreviations used are: cDNA,m, enzymatically synthesized DNA complementary to ovalbumin mRNA; mRNA,m, ovalbumin mRNA.
pooled and diluted to 500 ml with hybridization buffer. The diluted DNA solution was recycled through the affinity resin column for 70 hours in a similar manner for a second time. At the end of the hybridization period, the affinity resin was washed with hybridization buffer successively at 46, 55, and finally at 70°. The 70° eluate from the second affinity chromatography step was diluted to 300 ml with hybridization buffer and recycled through the affinity resin for a third time. After 70 hours of hybridization, the bound nucleic acid was again eluted at 70°.

**Chick DNA/[125I]-Ovalbumin mRNA Hybridization—**Ovalbumin DNA sequence present in various chick DNA fractions was detected by hybridization with [125I]-ovalbumin mRNA as described above. The DNA fractions were treated with 0.1 M NaOH at 65° for 1 hour, chilled, neutralized with 2 M sodium acetate, pH 4.5, and precipitated with alcohol. The precipitate was dissolved in hybridization buffer. The DNA samples were treated with 0.1 N NaOH at 62° for 1 hour, chilled, neutralized with 2 M sodium acetate, pH 4.5, and precipitated with alcohol. The precipitate was dissolved in hybridization buffer.

**RESULTS**

**Enrichment of Ovalbumin DNA Sequence by Affinity Chromatography**—The coding ovalbumin DNA strand was enriched from total chick DNA after hybridization with ovalbumin mRNA that was coupled to the inert phosphocellulose matrix. The matrix was washed free of unhybridized DNA and the ovalbumin DNA could then be eluted from the matrix by raising the temperature sufficiently to allow the hybrids to dissociate. To characterize the affinity column, 130,000 cpm of [32P]cDNA, 107 cpm/μg, in the presence of excess chick DNA was allowed to recycle through the resin at 46° in a total volume of 60 ml. Aliquots of 1 ml were taken at various times of hybridization for radioactivity determination. Radioactivity content in the hybridization medium diminished gradually to a level below 35% of the initial radioactivity after 70 hours of hybridization (Table IA). After extensive washing at 46°, bound nucleic acid was eluted at 70° and the radioactivity recovered in the eluate was 80,000 cpm, corresponding to 61.5% of the starting radioactivity (Table IB). During the course of the reaction, 6 A260 units of DNA were recovered from a total of 750 A260 units, representing a mass yield of 0.8%. The purification of [32P]cDNA, by this procedure was therefore 78.8-fold.

Six thousand A260 units of total chick DNA, sheared to a mean length of 4,000 nucleotides, was thus allowed to recycle through the ovalbumin mRNA affinity column. An additional washing of the resin at 55° was employed before elution at 70°. Twenty-six and 16.5 A260 units of DNA were eluted at 55° and 70°, respectively. Aliquots of these fractions were assayed for ovalbumin DNA sequences by hybridization with [125I]-ovalbumin mRNA. Total chick DNA reacted very slowly, and the phosphocellulose-unbound DNA reacted at a further reduced rate (Fig. 1). On the other hand, DNA eluted at 70° hybridized with the mRNA to a level of 60% and at a much higher rate with an apparent C50 value of 40. The extent of hybridization did not reach completion because the ovalbumin DNA to [125I]-ovalbumin mRNA ratio in the reaction might not have been sufficient (39), since only a small aliquot of the DNA solution was used in the experiment. Although the 55° eluate also reacted with [125I]-ovalbumin mRNA faster than total chick DNA, its apparent C50 value was approximately 9 times greater than that of the 70° eluate (Fig. 1).

Sixty-five A260 units of the DNA recovered from the affinity column was rechromatographed through the same resin. Approximately 2.4 A260 units of DNA were recovered in the subsequent 70° eluate. This time the DNA content in the 55° wash was not significant. The unbound DNA and the 70° eluate

**DNA was originally sheared randomly and the DNA/[125I]-mRNA hybrids might contain single strand [125I]-mRNA tails. Since we were assaying hybrid formation by ribonuclease digestion, the tails would be degraded and escape scoring. This effect would contribute to the observation that the extent of hybridization was less than complete. Furthermore, these tails were capable of hybridizing with additional DNA molecules. But the rate of this reaction was slower than the original hybridization reaction and would cause the curves to deviate from ideal C50 curves (40).**

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**Table 1**

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Affinity chromatography of [125I]-cDNA&lt;sub&gt;ov&lt;/sub&gt;</th>
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<tbody>
<tr>
<td><strong>A.</strong></td>
<td>Recycling time</td>
</tr>
<tr>
<td></td>
<td>hours</td>
</tr>
<tr>
<td>0</td>
<td>130,000</td>
</tr>
<tr>
<td>20</td>
<td>100,000</td>
</tr>
<tr>
<td>40</td>
<td>70,000</td>
</tr>
<tr>
<td>70</td>
<td>50,000</td>
</tr>
<tr>
<td><strong>B.</strong></td>
<td>DNA samples</td>
</tr>
<tr>
<td></td>
<td>cpm</td>
</tr>
<tr>
<td>Initial hybridization</td>
<td>130,000</td>
</tr>
<tr>
<td>medium</td>
<td>80,000</td>
</tr>
</tbody>
</table>

**Fig. 1.** Hybridization of [125I]-ovalbumin mRNA to various DNA fractions obtained by affinity column chromatography using ovalbumin mRNA coupled to phosphocellulose. Each reaction point contained 10,000 cpm of [125I]-ovalbumin mRNA (10<sup>7</sup> cpm/μg). Conditions of hybridization were as described under "Experimental Procedure," Duration of hybridization ranged from 30 s to 100 hours. Hybrid formation was assayed using ribonuclease as described under "Experimental Procedure," and the data points were corrected by a 5% background ribonuclease resistance as determined by treating an aliquot of the reaction mixture in an identical manner except that the mixture was not incubated. □—□, total chick DNA; ---, DNA fraction not bound to the ovalbumin mRNA affinity resin; •—•, DNA fraction eluted from the affinity resin at 55°; ○—○, DNA fraction eluted from the affinity resin at 70°.
were again treated with alkali and assayed for ovalbumin DNA sequence by hybridization with 125I-labeled ovalbumin mRNA. The unbound DNA was inactive whereas the 70° eluate reacted at a further accelerated rate with an apparent C_{ot} value of 2.3 (Fig. 2). The extent of hybridization was 70% and the apparent reduction of the C_{ot} value was 17.4-fold when compared to that of the 70° eluate from the first affinity column. When chromatographed through the affinity column for a third time, the 70° eluate hybridized with 125I-ovalbumin mRNA with an apparent C_{ot} value of 1.25 (Fig. 3). Apparent purification was therefore 1.84-fold over the second affinity column step and the DNA mass recovered was 0.9 A_{260} units.

Estimation of Extent of Enrichment of Ovalbumin DNA

Although partially purified DNA fractions hybridized with 125I ovalbumin mRNA much faster than did chick DNA, the precise extent of enrichment and the recovery of ovalbumin DNA sequence from total chick DNA could not be readily determined. This is because the C_{ot} value of the DNA-excess hybridization reaction between total chick DNA and 125I-ovalbumin mRNA could not be accurately assessed (Fig. 1). These parameters, however, could be determined by comparison of the apparent C_{ot} values obtained from the hybridization reactions using the partially purified DNA fractions and that using enzymatically synthesized cDNA, which should hybridize with 125I-ovalbumin mRNA at the highest rate. Under identical hybridization conditions, the reaction was essentially complete at a C_{ot} value of 1.0 with an apparent C_{ot} value of approximately 0.011 (Fig. 3). Moreover, identical C_{ot} curves were obtained at three different cDNA, to mRNA ratios. Using this number as a reference point, the C_{ot} value in the total chick DNA/125I-ovalbumin mRNA hybridization reaction was calculated to be approximately 12,000. Thus the enrichment of ovalbumin DNA from total chick DNA was approximately 9,600-fold by affinity chromatography (Table II).

Characterization of Ovalbumin DNA/125I-Ovalbumin mRNA Hybrid—Although the DNA purified by affinity chromatography hybridized with 125I-ovalbumin mRNA at a rate about 9,600-fold faster than that of total chick DNA, the fidelity of the hybrid must be verified. Fig. 4 shows the melting profile of the hybrid obtained using hydroxyapatite column chromatography. The melting profile of a control hybrid containing enzymatically synthesized cDNA, and 125I-ovalbumin mRNA was also obtained at the same time. Under the conditions employed, the T_m values of the two hybrids were 85 and 86°, respectively. The lack of substantial melting of the hybrids at lower temperatures strongly indicates that the 125I-ovalbumin mRNA was indeed in a true hybrid form since the mRNA by itself does not bind to hydroxyapatite at 60° in the presence of 0.14 M sodium phosphate, pH 7.0.

The hybrid was also characterized with respect to density by equilibrium sedimentation in CsCl gradients (Fig. 5). The hybrid banded at a density which ranged from 1.7050 to 1.7650 and was lower than that of the hybrid formed between [3P]cDNA, and ovalbumin mRNA (p = 1.7720). Native chick DNA and [3P]cDNA, banded at densities of 1.6960 and 1.7000, respectively, while 125I-ovalbumin mRNA banded at 1.8100. The relative broadness of the radioactivity peak suggests that the size of the single-stranded DNA region relative to the size of the DNA/RNA double-stranded region in the hybrid was polydisperse. These observations would be expected since

### Table II

**Purification of ovalbumin gene from total chick DNA**

<table>
<thead>
<tr>
<th>Material</th>
<th>DNA content</th>
<th>Apparent C_{ot}</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total DNA</td>
<td>24,000</td>
<td>12,000*</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DNA bound to 1st mRNA, affinity column</td>
<td>65</td>
<td>40*</td>
<td>300</td>
<td>81</td>
</tr>
<tr>
<td>DNA bound to 2nd mRNA, affinity column</td>
<td>2.4</td>
<td>2.3*</td>
<td>5200</td>
<td>52</td>
</tr>
<tr>
<td>DNA bound to 3rd mRNA, affinity column</td>
<td>0.9</td>
<td>1.25*</td>
<td>9600</td>
<td>36</td>
</tr>
</tbody>
</table>

*a This is an approximate value calculated from the apparent C_{ot} value of the cDNA,/125I-mRNA, hybridization reaction: Apparent C_{ot} (total chick DNA) = ((2 x 10^9)/(1890 - 620)) x 0.011 where 2 x 10^9 is the number of DNA nucleotides present in haploid chick genome, 1890 is the complexity of ovalbumin mRNA, 82 is the number average chain length of poly(A) within the mRNA, molecule, and 0.011 is the apparent C_{ot} value of the cDNA,/125I-mRNA, reaction (Fig. 5).

* Taken From Fig. 1.

* Taken From Fig. 2.

* Taken From Fig. 3.
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The DNA was originally sheared to more than twice the length of ovalbumin mRNA.

The purified DNA, although 9,600-fold enriched in the DNA sequence that is complementary to ovalbumin mRNA, is useful for future studies only if the DNA has not been degraded during the purification process. The size of the partially purified DNA preparation was thus analyzed on an alkaline sucrose gradient and compared to total chick DNA sheared to a mean length of 4,000 nucleotides. Since the migration of the two DNA peaks in gradients were identical (Fig. 6), the mean length of the partially purified DNA was still more than twice that of the ovalbumin mRNA. Thus, no significant degradation of DNA occurred during the isolation procedure.

**DISCUSSION**

Although ovalbumin comprises more than 50% of the proteins synthesized in the estrogen-stimulated chick oviduct, the protein is coded by a unique sequence gene which is not amplified during estrogen stimulation (5, 6, 30, 31). Since there are $2 \times 10^9$ nucleotides in the DNA of a haploid chick genome and the complexity of the ovalbumin mRNA is approximately 1800 nucleotides, only ($1800/2 \times 10^9$) of the chick DNA is represented by the coding strand of the structural ovalbumin gene. Thus, the isolation of this DNA strand to homogeneity requires a purification of greater than 1-million-fold. The ovalbumin gene, as indicated by the base composition studies of purified ovalbumin mRNA (30), does not have a G + C content substantially different from that of total DNA and therefore cannot be isolated by equilibrium centrifugation alone. Molecular hybridization was thus employed as a tool to effect a partial purification of this gene sequence.

Initially, we allowed total chick DNA to hybridize with excess ovalbumin mRNA in liquid medium. The majority of the complementary DNA strand was hybridized to the mRNA instead of its DNA complement since the mRNA was in large excess (39). The DNA/mRNA hybrid was next removed from the bulk of DNA by an oligo(dT)-cellulose column. The fractionation was effective due to the presence of a polyadenylic acid tract on the 3' terminus of the mRNA. Although a 35-fold purification of the coding ovalbumin DNA strand was achieved in this manner, the yield was relatively poor because fractionation of the hybrid from total DNA depended greatly on the integrity of the mRNA at the end of the hybridization reaction. Should there be a slight degradation of the mRNA during hybridization, only those DNA strands that were hybridized to the 3' terminus of the mRNA would be co-purified with the mRNA in the oligo(dT)-cellulose column chromatography step. To overcome this difficulty, we next covalently immobilized ovalbumin mRNA onto an inert matrix and this
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Fig. 6. Alkaline sucrose gradient centrifugation of chick DNA sheared to a mean length of 4,000 nucleotides (Panel A), and DNA eluted from the third affinity column chromatography step (Panel B). The migration of the open (16.1 S) and closed (18.4 S) forms of φX174 DNA in parallel gradients are indicated by the arrows a and b, respectively.

matrix was incubated with a solution of denatured chick DNA. The matrix of choice was phosphocellulose because of its higher coupling efficiency with RNA as compared to neutral cellulose (36) and the stability of this affinity resin over a long period of time. Indeed, SV40 DNA was purified from large quantities of non-specific DNAs using SV40 RNA coupled to phosphocellulose (36). For the present study, this approach offers the advantage of providing a high yield of ovalbumin gene sequences because we did not have much difficulty in fractionating the hybrid from the starting material at the end of hybridization. Nevertheless, a long incubation time was required since the mRNA was immobilized.

A major difficulty encountered in our attempts to isolate the ovalbumin gene from sheared chick DNA by affinity chromatography using immobilized ovalbumin mRNA was the tendency of the denatured chick DNA to form DNA hyperpolymers during the prolonged incubation time. A hyperpolymer is a multistranded structure of reassociated DNA (41, 42). The formation of these hyperpolymers probably occurs through the reassociation of the middle repetitive sequences which constitute about 30% of the chick genome (41-43). When reassociated to high C<sub>ot</sub> values, the hyperpolymers formed were so large that they formed DNA gels and could be readily removed from solution by low speed centrifugation. Thus, subsequent fractionation of a DNA/RNA hybrid from the bulk of chick DNA would be less difficult if the formation of these hyperpolymers could be prevented. Since a hyperpolymer is formed by the reassociation of many DNA strands, its rate of formation should be slower than the rate of a simple repeated sequence reassociation where only two DNA strands are involved. The formation of DNA hyperpolymers could therefore be controlled by limiting the C<sub>ot</sub> values attained during the reassociation.

To determine the rate of hyperpolymer formation between total chick DNA sheared to 4,000 nucleotides in length, the denatured DNA was allowed to reassocate to various C<sub>ot</sub> values, followed by centrifugation and analysis of the DNA content of the pellets. Indeed it was observed that the formation of hyperpolymers was minimal if, under the hybridization conditions employed, the C<sub>ot</sub> value did not reach 100. It was thus possible to isolate the ovalbumin gene from sheared chick DNA using purified ovalbumin mRNA as long as the DNA reassociation C<sub>ot</sub> values during hybridization could be limited to less than 100.

The closed recycling system described by Shih and Martin (37) was thus employed to enrich for the ovalbumin DNA sequences. Chick DNA was denatured in a second column immediately prior to re-exposure to the immobilized mRNA. Since the denatured DNA solution only remained in the hybridization column for a short time, the extent of hyperpolymer formation was limited and prolonged hybridization times were possible. After 3 days of recycling, greater than 90% of the ovalbumin DNA sequence was bound to the resin (Tables I B and II). When this procedure was repeated (three times), the bound DNA reacted with 125<sup>I</sup>-ovalbumin mRNA with an apparent C<sub>ot</sub> of 1.25 (Fig. 3). The extent of enrichment of ovalbumin gene over total chick DNA, however, was difficult to estimate because the C<sub>ot</sub> value of the reaction using total chick DNA cannot be accurately measured in the same assay. In order to achieve a C<sub>ot</sub> of 10,000 the DNA concentration must be greater than 10 mg/ml in order to prevent the hybridization time from exceeding 100 hours. At 10 mg/ml, the 4,000-nucleotide chick DNA fragments are quite viscous even in the presence of 50% formamide. When hybridization was carried out to a C<sub>ot</sub> of greater than 10,000, the viscosity of the DNA hyperpolymer formed was such that hybrid formation could not be accurately measured. This technical difficulty could be overcome if the C<sub>ot</sub> value of the reaction using pure ovalbumin gene and 125<sup>I</sup>-ovalbumin mRNA could be determined under the same hybridization conditions.

Complementary DNA generated against ovalbumin mRNA using reverse transcriptase is part of the ovalbumin gene. Our capability to generate a complete complementary DNA transcript of ovalbumin mRNA in large quantities (31) makes this approach feasible. The complementary DNA preparation was thus allowed to hybridize in large excess with 125<sup>I</sup>-ovalbumin mRNA and the C<sub>ot</sub> value obtained was 0.011 using three concentrations of cDNA<sub>ov</sub> in the hybridization medium (Fig. 3). Although comparison of this value with that obtained from the hybridization reaction between the final DNA preparation and 125<sup>I</sup>-ovalbumin mRNA (C<sub>ot</sub> = 1.25) is not quantitative, an approximate estimation of enrichment of the ovalbumin coding strand with respect to total chick DNA by repeated affinity chromatography should be (0.011/1.25) x ((2 x 10<sup>4</sup>)/ (1890 - 62)) = 9600-fold, where 2 x 10<sup>4</sup> is the number of DNA nucleotides present in haploid chick genome, 1890 is the complexity of ovalbumin mRNA and 62 is the number average chain length of polyadenylic acid at the 3' terminus of the mRNA (30). A greater degree of precision was difficult to obtain because the DNA/125<sup>I</sup>-ovalbumin mRNA reactions did not reach completion.

The fidelity of the partially purified ovalbumin DNA sequence was indicated by its ability to form stable hybrids with 125<sup>I</sup>-ovalbumin mRNA. The density and melting temperature of the hybrid composed of this DNA fraction and 125<sup>I</sup>-oval-
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bunin mRNA were quite similar to those of the hybrid formed between cDNA and ovalbumin mRNA (Figs. 4 and 5). Furthermore, the size distribution of the partially purified DNA was identical to that of the starting DNA (Fig. 6), indicating that no DNA degradation had occurred during the purification process. Since chick DNA was originally sheared to a mean length of 4,000 nucleotides, the partially purified DNA preparation described in this communication should contain some DNA sequences that are adjacent to the structural gene coding for ovalbumin, the complexity of which should be identical to that of the ovalbumin mRNA (189-62 nucleotides). Whether these “extra” DNA sequences contain the regulatory elements of the structural gene coding for ovalbumin is not known at the present time. However, DNA fragments much longer than 4,000 nucleotides containing the structure gene coding for ovalbumin can be partially purified in the same manner if necessary.

Additional purification of the ovalbumin gene (both coding and anticoding strands) followed by amplification in bacterial plasmids (44-46) are logical extensions of the present study. Only in this manner can we hope to obtain sufficient quantities of specific DNA sequences to study interactions of steroid hormone-receptor complexes and other regulatory proteins at a specific transcriptional locus.

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