Acknowledgments

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References

Bayliss, G. J., Marsden, H. S., and Hay, J. (1975), *Virology 68*, 124.
Ben-Porat, T., Kaplan, A. S., Stehn, B., and Rubenstein, A. S. (1976), *Virology 69*, 547.
Burgoyne, L. A., Waqar, M. A., and Atkinson, M. R. (1970), *Biochem. Biophys. Res. Commun. 39*, 254.
Francke, B. (1977), *Biochemistry 16* (preceding paper in this issue).
Gibson, W., and Roizman, B. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68*, 2818.
Gibson, W., and Roizman, B. (1972), *J. Virol. 10*, 1044.
Huang, R. C., and Huang, P. C. (1964), *J. Mol. Biol. 39*, 365.
Huang, W. M., and Lehman, I. R. (1972), *J. Biol. Chem. 247*, 3139.
Keir, H. M., and Gold, E. (1963), *Biochim. Biophys. Acta 72*, 263.
Kolber, A. R. (1975), *J. Virol. 15*, 322.
Morrison, J. M., and Keir, H. M. (1968), *J. Gen. Virol. 3*, 337.
Mosig, G., and Bock, S. (1976), *J. Virol. 17*, 756.
Murray, B. K., and Biswal, N. (1974), *Intervirology 4*, 14.
Shlomai, J., Friedmann, A., and Becker, Y. (1976), *Virology 69*, 647.
Stein, G., and Farber, J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69*, 2918.
Stein, J. L., Reed, K., and Stein, G. S. (1976), *Biochemistry 15*, 3291.

Ovalbumin Gene: Purification of the Coding Strand†

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ABSTRACT: Purified ovalbumin messenger RNA ($mRNA_{ov}$) was employed to isolate the gene coding for ovalbumin from total chick DNA by molecular hybridization. Using $mRNA_{ov}$ covalently linked onto phosphocellulose, the coding strand of the ovalbumin gene was enriched 10 000-fold from sheared chick DNA by affinity hybridization chromatography. This gene sequence was further purified by sulfhydryl-Sepharose column chromatography after hybridization with an excess of mercurated $mRNA_{ov}$. The concentration of the ovalbumin gene sequence in the DNA fraction eluted from the sulfhydryl-Sepharose column by 0.1 M 2-mercaptoethanol was quantitated by saturation hybridization with ^{125}I-labeled $mRNA_{ov}$. When the initial slope of this reaction was compared to that obtained from the reaction between [^{125}I]$mRNA_{ov}$ and full-length complementary DNA ($cDNA_{ov}$) synthesized against $mRNA_{ov}$ using reverse transcriptase, purification of the coding ovalbumin DNA strand from the $mRNA_{ov}$ affinity column purified DNA preparation was 18-fold. The overall purification of the ovalbumin gene from total chick DNA was thus 180 000-fold, and two out of every five DNA molecules in the final preparation contained sequences of the structural ovalbumin gene. There was no apparent degradation of the 5000 nucleotide strands of chick DNA throughout the purification procedure. Since $mRNA_{ov}$ has a complexity of 1890 nucleotides, the resulting DNA was more than twice the length of $mRAN_{ov}$ and should contain DNA sequences tandomly linked to the structural ovalbumin gene, which may play a regulatory role in its expression.

A variety of steroid hormones stimulate growth, differentiation, and other biologic functions of their respective target tissues by exerting their primary action at the transcriptional level (O'Malley and Means, 1974; Woo and O'Malley, 1975; Chan et al., 1973; Harris et al., 1973, 1975, 1976; Sullivan et al., 1973; Palmiter, 1973; Tsai et al., 1975a,b; Schwartz et al., 1975). This conclusion has led workers in the area of hormone action to focus their investigations on various regulatory aspects of specific eucaryotic gene expression. These investigations, however, are hampered seriously by the enormous genomic complexities of eucaryotes. Specific proteins inducible by hormones are generally encoded by unique DNA sequences which may constitute only one-millionth of the entire genome. Consequently, although transcriptional studies using unfractionated chromatin have contributed to our present understanding of eucaryotic gene expression, the exact molecular mechanism of reguation of specific eucaryotic gene expression remains unresolved. In order to perform definitive studies on the regulation on eucaryotic gene expression at the molecular level, the various components of the transcriptional machinery must eventually be purified so that the entire system can be reconstituted in vitro. Such studies require the development of methodology for gene isolation.

Ovalbumin is an egg-white protein synthesized in the chick oviduct in response to estrogen stimulation (O'Malley et al., 1967; Palmiter et al., 1971), and the ovalbumin gene is a unique DNA sequence in the chick genome (Harris et al., 1973; Sullivan et al., 1973). Since this gene possesses identical physical-chemical properties to the bulk of chick DNA (Woo

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et al., 1975) and cannot be purified by conventional methods of DNA fractionation, we have taken advantage of the only unique chemical feature of this gene, i.e., its nucleotide sequence, for its isolation. We have previously reported the partial purification of the coding DNA strand of the ovalbumin gene by affinity hybridization (Woo et al., 1976). We now wish to report further purification of this gene sequence by sulfhydryl-Sepharose column chromatography.

Materials and Methods

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 ($A_{270} < 0.25$) were from Fischer Scientific Co., Fair Lawn, N.J.; phenol was redistilled before use. Oligo(dT)-cellulose (T3) was from Collaborative Research. $Na^{125}I$ (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. Avian myeloblastosis virus reverse transcriptase was a kind gift from Dr. J. W. Beard, Life Sciences, Inc., St. Petersburg, Fla. Deoxyribonucleoside triphosphates were from P-L Biochemicals, Milwaukee, and the radioactive nucleotides were from New England Nuclear Corp. Calf thymus deoxynucleotidyl terminal transferase was from Dr. Winston Salser, Biology Department, UCLA. Sulfhydryl-Sepharose was a kind gift from Dr. Ming-Jer Tsai, Department of Cell Biology, Baylor College of Medicine.

Preparation of Purified Ovalbumin mRNA. Ovalbumin mRNA was purified from total nucleic acid extracts of hen oviducts as described previously (Woo et al., 1975) with the following modifications: (a) nitrocellulose filtration was replaced by oligo(dT)-cellulose column chromatography, and (b) the RNA was heated at 70 °C for 1 min and quick cooled in an ice-water bath before chromatography on the Sepharose 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, and the purity of the mRNA was monitored by acid-urea-agarose gel electrophoresis (Rosen et al., 1975). The purified ovalbumin mRNA was labeled with $Na^{125}I$ to a specific radioactivity of 10^7 cpm/μg as previously described (Woo et al., 1975). The labeled product 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 using reverse transcriptase isolated from avian myeloblastosis virus by a slight modification of a previously reported procedure (Monahan et al., 1976). The conditions employed were the following: 100 μg/mL purified ovalbumin mRNA was incubated at 46 °C for 30 min with 50 mM Tris-HCl,[1] pH 8.3, 50 mM KCl, 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 25 μg/mL oligo(dT_{12-18}), 40 μg/mL actinomycin D, 1 mM each of the four deoxynucleoside triphosphates with one radioactively labeled, and 600 units/mL reverse transcriptase. The majority of the cDNA product synthesized under these conditions was complete complements of the mRNA. A mass yield of 50% of the mRNA used was obtained.

[1] Abbreviations used are: Tris, 2-amino-2-hydroxylmethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

Affinity Chromatography. Ovalbumin mRNA was covalently coupled to phosphocellulose as described previously (Woo et al., 1976). The resin was used to purify the coding strand of ovalbumin DNA by hybridization of 5000 nucleotide fragments of chick DNA. Three cycles through such a column results in a 10 000-fold purification as determined by hybridization of the specifically bound DNA to ^{125}I-labeled mRNA (Woo et al., 1976).

Addition of Poly(dC) to DNA. Forty micrograms of affinity column purified ovalbumin DNA was incubated at 25 °C for 15 min with 100 units of calf thymus terminal deoxynucleotidyl transferase in the presence of 0.2 M potassium cacodylate, pH 7.2, 1 mM $CoCl_2$, 1 mM dithiothreitol, and 50 μM dCTP containing 250 μCi of [^{3}H]dCTP in a total volume of 0.5 mL (Chang and Bollum, 1971). The reaction was terminated by the addition of EDTA to 10 mM and sodium sarkosyl to 1%. The DNA was deproteinized by phenol extraction and recovered from the aqueous phase by alcohol precipitation. From the amount of nucleotides incorporated, it was estimated that approximately 10 mol of dCMP had been added per mol of 3′-terminus. The DNA was labeled to a specific activity of 40 000 cpm/μg and the amount of poly(dC)-containing DNA recovered was 30 μg.

Mercuration of Ovalbumin mRNA. Ovalbumin mRNA was mercuated by the procedure of Dale et al. (1975) and Dale and Ward (1975). Purified ovalbumin mRNA (150 μg) was incubated at 50 °C for 6 h in the presence of 5 mM sodium acetate, pH 6.0, containing 4 mM mercuric acetate in a final volume of 1 mL. The reaction was terminated by the addition of 1 volume of 50 mM Tris-HCl, pH 7.6-0.1 M NaCl-10 mM EDTA. Excess mercuric acetate was removed by Sephadex G-50 column chromatography and the RNA eluted at the void volume was precipitated with ethanol. The RNA was redissolved in 1 mL of 50 mM Tris-HCl, pH 7.6-0.1 M NaCl-1 mM EDTA and applied to a sulfhydryl-Sepharose column (0.6 × 10 cm) at room temperature and a flow rate of 5 mL/h. After extensive washing, the column-bound mercurated nucleic acid was eluted with the same buffer containing 0.1 M 2-mercaptoethanol, precipitated with ethanol, and redissolved in water.

Purification of the Coding Ovalbumin DNA by Sulfhydryl-Sepharose Column Chromatography. Twenty-five micrograms of mercurated RNA was mixed with 30 μg of the poly(dC)-containing chick DNA enriched for the coding ovalbumin DNA strand, precipitated with ethanol, and redissolved in 0.5 mL of the formamide-containing hybridization buffer. The solution was heated at 70 °C for 10 min and incubated at 55 °C for another 10 min. The solution was chilled and diluted tenfold with 4.5 mL of ice-cold water. The diluted solution was then applied to a sulfhydryl-Sepharose column at room temperature. To minimize further nucleic acid hybridization during this time period, the bulk of nucleic acid solution was chilled in ice and applied to the column in small aliquots. The bound nucleic acid was subsequently eluted with 0.1 M 2-mercaptoethanol as described above. The mercaptoethanol eluate was heated at 37 °C for 16 h after adjusting to contain 0.3 N NaOH and 10 mM EDTA. The solution was subsequently neutralized with 2 M sodium actate, pH 4.5, and the purified coding ovalbumin DNA sequences precipitated with alcohol after adding yeast tRNA to 20 μg/mL as carrier.

Quantitation of Coding Ovalbumin DNA Strand by Saturation Hybridization with [^{125}I]Ovalbumin mRNA. The saturation hybridization conditions were essentially the same as previously reported (Harris et al., 1976). Various amounts of DNA (0.2-2000 ng per reaction point) were incubated at

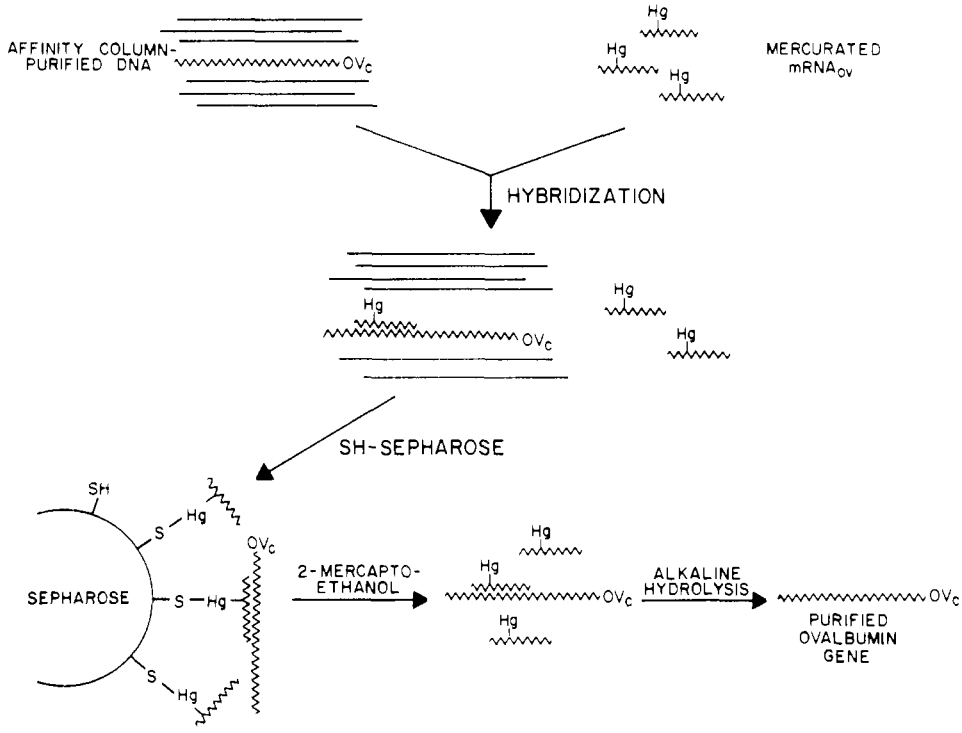

FIGURE 1: Schematic representation of the purification of coding the ovalbumin DNA strand (OVc) from affinity column-purified DNA by sulfhydryl-Sepharose column chromatography after hybridization with mercurated ovalbumin mRNA.

68 °C for 40 h with 3 ng of [^{125}I]mRNA$_{ov}$ in the presence of 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 0.6 M NaCl, 2 mM EDTA, and 20 μg of *Escherichia coli* tRNA in a final volume of 50 μL. At the end of the incubation period, the percentage of [^{125}I]mRNA$_{ov}$ hybridization was determined after ribonuclease digestion as described previously (Woo et al., 1976).

Results

Mercuration of Ovalbumin Messenger RNA and Sulfhydryl-Sepharose Column Chromatography. We have previously reported the purification of the coding ovalbumin DNA strand 10 000-fold from total chick DNA by affinity hybridization (Woo et al., 1976). Because the ovalbumin messenger RNA was immobilized in the affinity hybridization procedure, the rate of hybridization between the messenger RNA and the coding DNA strand was greatly reduced, and an extended period of incubation time was necessary to carry the reaction to 80% completion. During this time period, hybridization between homologous chick DNA sequences was also taking place and would eventually lead to contamination of the final ovalbumin gene preparation by nonovalbumin chick DNA sequences. However, the hybridization between the homologous chick DNA sequences could theoretically be prevented if the hybridization reaction could be carried out for short time periods in the presence of a large excess of ovalbumin messenger RNA in liquid medium. The only remaining problem would then be the recovery of the mRNA/DNA hybrid from the bulk of DNA. This step could be accomplished by column chromatography on sulfhydryl-Sepharose if the mRNA was mercurated as depicted in Figure 1.

In order to obtain materials which would allow such a purification procedure to be employed, we have mercurated purified ovalbumin messenger RNA according to the procedure of Dale et al. (1975). During this mercuration procedure, however, the mRNA$_{ov}$ was found to have undergone partial degradation as indicated by gel electrophoresis under denaturing conditions. Since the rate of hybridization is a function of chain length of the reacting nucleic acid species (Wetmur and Davidson, 1968), it was necessary to determine the optimal hybridization conditions so that the mercurated mRNA$_{ov}$ could be used for the purification of the ovalbumin gene. Mercurated mRNA$_{ov}$ was thus allowed to hybridize to various R_0t values with [^{3}H]cDNA$_{ov}$ synthesized from purified mRNA$_{ov}$ using reverse transcriptase. Under the hybridization conditions employed, it was observed that the reaction had proceeded to 90% completion with an apparent $R_0t_{1/2}$ value of 4×10^{-3} (Figure 2A). This $R_0t_{1/2}$ value was only 10% greater than that of a reference hybridization curve using intact mRNA$_{ov}$ in an identical hybridization medium (Figure 2A). Thus, although partial degradation of the mRNA$_{ov}$ had occurred during the mercuration period, our data suggested that the mercurated RNA was still capable of forming stable hybrids with complementary nucleic acid sequences at a rate similar to that of intact mRNA.

Furthermore, since RNA/DNA hybrids uaually have greater thermal stability than DNA/DNA hybrids (Bishop, 1972), the hybridization conditions can be modified to favor the formation of RNA/DNA hybrids while minimizing homologous chick DNA reannealing. The hybridization between [^{3}H]cDNA$_{ov}$ and mercurated mRNA$_{ov}$ was thus performed in the same medium to various R_0t values at elevated temperatures. It was observed that the rates of hybridization were identical at 45 and 50 °C with an apparent $R_0t_{1/2}$ value of 4×10^{-3} while the reaction proceeded very slowly at 65 °C (Figure 2B). At 55 °C, however, the apparent $R_0t_{1/2}$ value has increased to 6×10^{-3}, indicating that the rate of hybridization had merely been reduced by 50% (Figure 2B). Since 55 °C is only 7 °C below the melting temperature of 62 °C for total chick DNA under these conditions, the reannealing between homologous chick DNA sequences would be much slower under these conditions (Britten et al., 1974). Thus, greater purification of the coding ovalbumin DNA strand could be achieved by performing the hybridization with mercurated

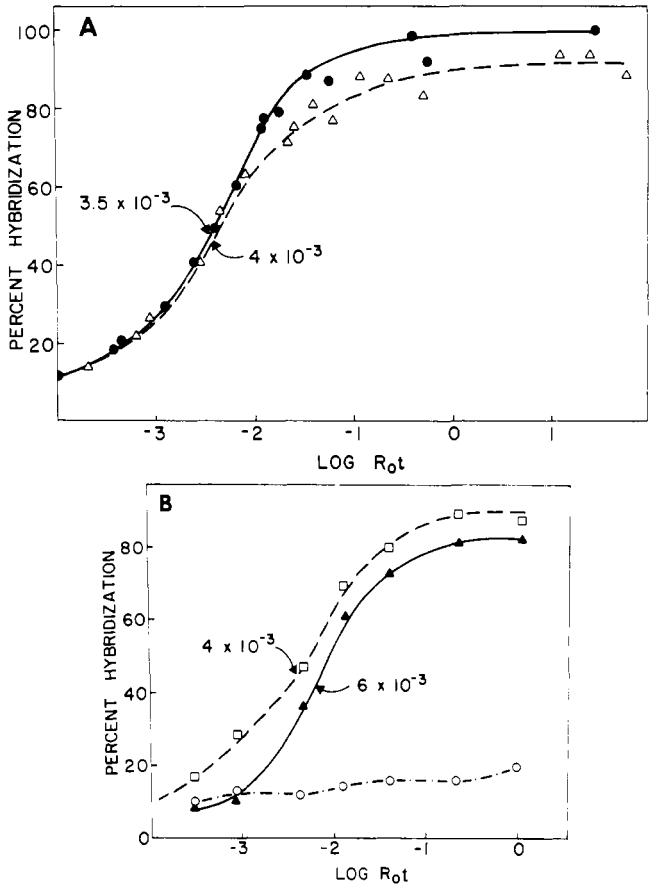

FIGURE 2: (A) Hybridization between [^{3}H]cDNA$_{ov}$ and intact mRNA$_{ov}$ or mercurated mRNA$_{ov}$. The hybridization reactions were carried out at 45 °C in 0.01 M *N*-Tris-methyl-2-aminoethanesulfuric acid, pH 7.0, containing 0.75 M NaCl, 5 mM EDTA, 50% formamide, 2×20^5 cpm/mL [^{3}H]cDNA$_{ov}$ (specific radioactivity 10^7 cpm/μg), and 4 μg/mL intact mRNA$_{ov}$ or mercurated mRNA$_{ov}$ in a final volume of 50 μL. The reaction time ranged from 1 min to 24 h. The reaction mixtures were frozen in dry ice at various time points. The reaction mixtures were thawed in ice, adjusted to 0.2 M sodium acetate, pH 4.5, containing 0.4 M NaCl, 2.5 mM zinc acetate, and 1600 units of S1 nuclease in a total volume of 0.4 mL, and incubated at 37 °C for 90 min. The mixtures were subsequently chilled and mixed with 50 μg of bovine serum albumin and 20% trichloroacetic acid. Precipitates were collected on Millipore filters, washed with 10% trichloroacetic acid, and dried. Hybrid formation was determined by liquid scintillation counting: (●-●) intact mRNA$_{ov}$ and (△-△) mercurated mRNA$_{ov}$. (B) Hybridization between [^{3}H]cDNA$_{ov}$ and mercurated mRNA$_{ov}$ at various temperatures. Conditions of hybridization and assay of hybrid formation were identical to those described above except for the hybridization temperature: (□-□) 50 °C; (▲-▲) 55 °C; and (O- - -O) 65 °C.

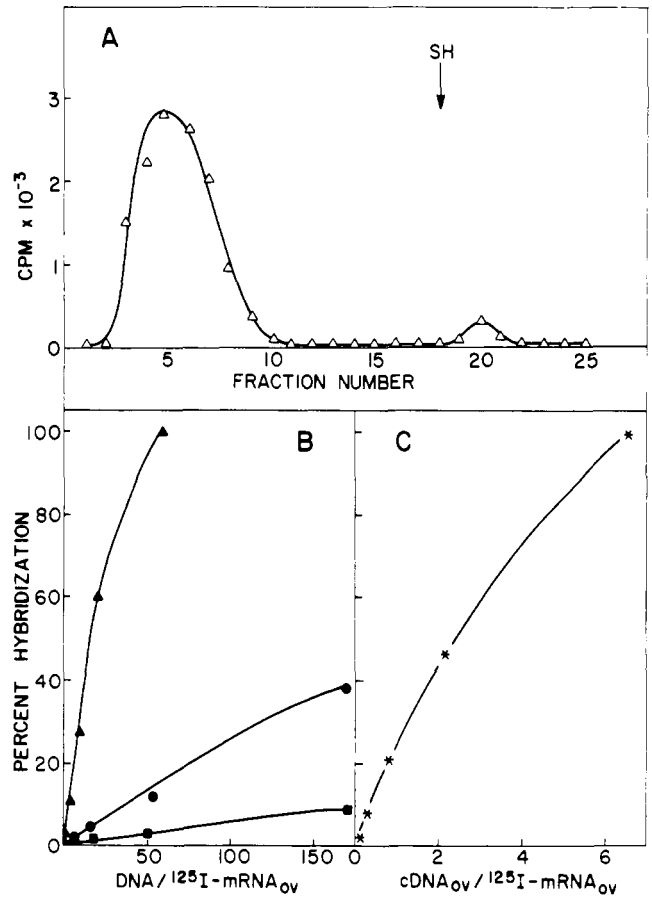

FIGURE 3: Purification of the coding ovalbumin DNA strand from affinity column purified DNA by sulfhydryl-Sepharose column chromatography after hybridization with mercurated mRNA$_{ov}$ (A, △-△). Conditions of hybridization and sulfhydryl-Sepharose column chromatography were carried out as described in the legend of Figure 2. The column flow-through fractions and the bound fractions were pooled separately, adjusted to 0.3 N NaOH, 10 mM EDTA, and incubated at 37 °C for 16 h to hydrolyze all RNA contents. The DNA fractions were subsequently neutralized with 0.15 volume of 2 M sodium acetate, pH 4.5, and precipitated with alcohol after the addition of 20 μg/mL yeast tRNA as carrier. The pellets were redissolved in 100 μL of water. Various amounts of the DNA fractions were then allowed to hybridize with 60 ng/mL of [^{125}I]mRNA$_{ov}$ (10 cpm/μg) in a final volume of 50 μL. The hybridization reactions were carried out at 68 °C for 40 h in 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, pH 7.0, containing 0.6 M NaCl, 2 mM EDTA, and 0.5 mg/mL yeast tRNA; ^{125}I content of individual reaction mixture was determined using a gamma spectrometer after S1 nuclease treatment which was performed as described in Figure 3: (B, ●-●) partially purified coding ovalbumin DNA after the addition of poly(dC) using terminal transferase; (B, ▲-▲) DNA fraction bound to the sulfhydryl-Sepharose column; (B, ■-■) flow-through DNA fraction on sulfhydryl-Sepharose column; and (C, *-*) full-length cDNA$_{ov}$.

mRNA$_{ov}$ at 55 °C. To ensure that this was the case, [^{3}H]-cDNA$_{ov}$ was allowed to hybridize with either mercurated mRNA$_{ov}$ or intact mRNA$_{ov}$ at 55 °C to an apparent R_0t value of 0.1 and subsequently chromatographed through sulfhydryl-Sepharose columns. It was observed that greater than 80% of the [^{3}H]cDNA$_{ov}$ became bound to the column after hybridization with mercurated mRNA$_{ov}$, whereas only 0.5% of the [^{3}H]cDNA$_{ov}$ became bound when intact mRNA$_{ov}$ was employed. This experiment suggested that the sulfhydryl-Sepharose column chromatographic procedure could indeed be employed for the purification of specific gene sequences.

Further Purification of Coding Ovalbumin DNA by Sulfhydryl-Sepharose Column Chromatography. The tritium-labeled coding strand of ovalbumin DNA was allowed to hybridize with a large excess of mercurated mRNA at 55 °C to an apparent R_0t value of 0.1, and the mixture was chromatographed on the sulfhydryl-Sepharose column. Although the majority of the radioactively labeled DNA was not bound to the column as expected, 4% of the DNA was specifically bound (Figure 3A). The flow-through and the bound nucleic acid fractions were pooled separately and analyzed for the concentrations of ovalbumin gene sequence by hybridization with [^{125}I]mRNA. When the saturation hybridization method of Young et al. (1974) was employed, it was observed that the initial slope of the column-bound DNA fraction had increased by a factor of 18 when compared to that of the starting DNA sample (Figure 3B), indicating that a purification of 18-fold for the ovalbumin gene was effected by this procedure. The initial slope of the column flow-through fraction was much less than that of the starting DNA sample, indicating depletion of the ovalbumin gene sequences as expected. These relative values of initial slopes were then converted to absolute numbers

TABLE I: Purification of the Ovalbumin Gene.

Material	DNA (μg)	Initial slope (% hybridization) / (DNA/[^{125}I]mRNA$_{ov}$)	Purification Fold	Purity (%)	Yield (%)
Total DNA	1 000 000	0.000025[a]	1	0.00025[b]	100
Affinity column purified-DNA	40	0.26	10 000	2.6	40
SH-Sepharose purified DNA	1.1	4.5	180 000	45.0	20

[a] This value was drived from Figure 4C in the following manner. Full length cDNA$_{ov}$ has a complexity of 1800 nucleotides (Monahan et al., 1976) while there are approximately 2×10^9 nucleotides in the haploid chick genome (Mirsky and Ris, 1951; Rosen et al., 1973). Since the ovalbumin gene is represented by a unique DNA sequence (Harris et al., 1973; Sullivan et al., 1973), cDNA$_{ov}$ constitutes approximately 1800 nucleotides/2×10^9 nucleotides = 0.9×10^{-6} of the chick genome. The initial slope of the hybridization reaction between total chick DNA and [^{125}I]mRNA$_{ov}$ should thus be $28 \times 0.9 \times 10^{-6} = 25 \times 10^{-6}$, where 28 is the initial slope of the hybridization reaction between full-length cDNA$_{ov}$ and [^{125}I]mRNA$_{ov}$ obtained from Figure 4C. [b] This value was derived from the following formula: 5000 nucleotides/2×10^9 nucleotides $\times$ 100%, where 5000 nucleotides and 2×10^9 nucleotides are the length of the sheared DNA and the total nucleotide content in haploid chick genome, respectively.

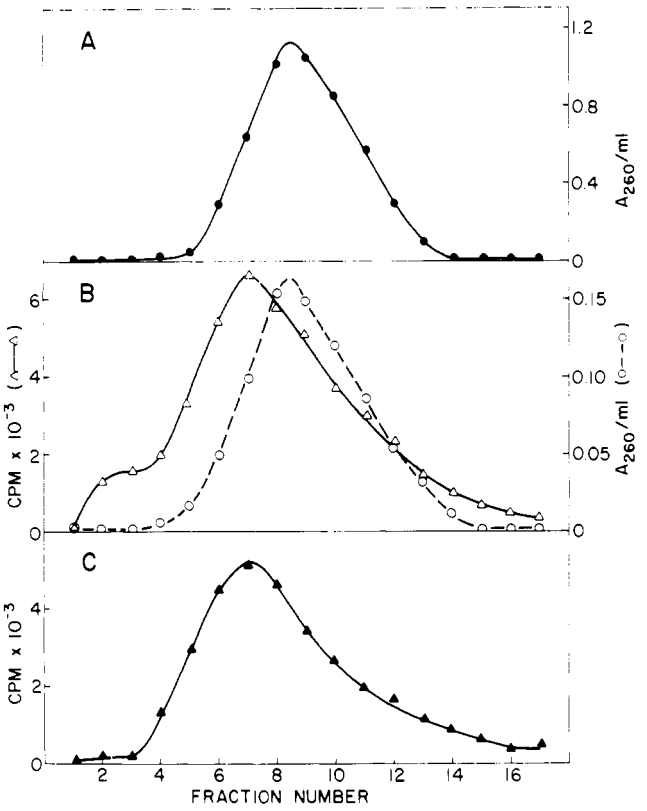

FIGURE 4: Alkaline sucrose gradient centrifugation of total chick DNA that was sheared to a mean weight-average chain length of 5000 nucleotides (A, ●-●); partially purified coding ovalbumin DNA from affinity hybridization using immobilized mRNA$_{ov}$ that was subsequently tailed with ^{3}H-labeled poly(dC) using terminal transferase (B); DNA content was determined by monitoring absorption at 260 nm (○- - -○), and radioactivity content was determined by liquid scintillation counting (△-△); (C, ▲-▲) radioactivity content of final DNA preparation after sulfhydryl-Sepharose column chromatography.

by hybridizing full-length cDNA$_{ov}$ to [^{125}I]mRNA$_{ov}$ under identical conditions (Figure 3C). Since the initial slope of 4.5 for the purified DNA fraction was approximately one-sixth that of the reference curve of 28 and full-length cDNA$_{ov}$ is the coding strand of the structural ovalbumin gene, we have estimated that the purity of the structural ovalbumin gene sequence in our final DNA preparation was approximately 18% of the nucleotide mass present. This represented a total purification of approximately 180 000 (Table I).

Characterization of the Purified Ovalbumin DNA. Although substantial purification of the ovalbumin gene has been achieved with this procedure, the integrity of the purified DNA must be verified before it could be employed for further studies. The size of the DNA was thus analyzed by alkaline sucrose gradient centrifugation. Figure 4A shows the sedimentation profile in an alkaline sucrose gradient of total chick DNA that was sheared to a mean length of 5000 nucleotides. These fragments comigrated with the 16.1S form of ϕx174 DNA. Figure 4B illustrates the sedimentation profile of the mRNA$_{ov}$ affinity column-purified DNA that was subsequently incubated with terminal transferase. Since the mass of DNA has migrated the same distance in the gradient as the starting DNA, no detectable DNA degradation had occurred during the affinity chromatography procedure and the enzymatic treatment. The radioactivity profile in the same gradient, however, was slightly lighter than the mass profile (Figure 4B). This was not unexpected due to the following considerations. Since the DNA was originally sheared mechanically to a mean length of 5000 nucleotides, the DNA lengths actually ranged from 2000 to 10 000 nucleotides and the A_{260} adsorption profile in the alkaline sucrose gradient represents only the weight-average chain length of the DNA preparation. When poly([^{3}H]dC) tracts were added onto the DNA molecules using terminal transferase, all 3′-termini of DNA molecules are substrates for the enzyme regardless of the DNA chain lengths. Thus, the radioactivity profile in the alkaline sucrose gradient represents the number-average chain length of the DNA preparations, which was expected to be smaller than the weight-average chain length of 5000 nucleotides. Figure 4C shows the sedimentation profile of the final DNA preparation. The radioactivity profile was identical to that in Figure 4B, indicating that there was no detectable DNA degradation during hybridization with mercurated mRNA$_{ov}$ and sulfhydryl-Sepharose column chromatography. Thus, the weight-average chain length of the purified ovalbumin DNA should remain at 5000 nucleotides.

Discussion

In order to study the effects of hormone–receptor complexes and nuclear proteins on the transcription of the ovalbumin gene at the molecular level in vitro, we attempted to prepare substantial quantities of the purified ovalbumin gene. Since ovalbumin is encoded by a unique DNA sequence in the chick genome (Harris et al., 1973; Sullivan et al., 1973) and the base composition of ovalbumin mRNA does not differ substantially from that of total chick DNA (Woo et al., 1975), classical methods successfully employed for gene isolation using sizing techniques and repeated density gradient centrifugation are not adequate. Molecular hybridization was thus employed to

effect a substantial purification of the ovalbumin gene. $mRNA_{ov}$ was covalently linked to phosphocellulose and the coding ovalbumin DNA strand was fractionated from total chick DNA by affinity chromatography. Following repeated affinity chromatography, the coding ovalbumin DNA strand was estimated to be purified about 10 000-fold from total chick DNA (Woo et al., 1976).

In the present study, further purification of the coding ovalbumin DNA preparation partially purified by the mRNA affinity column has been achieved by sulfhydryl-Sepharose column chromatography after hybridization with mercurated ovalbumin mRNA. Since a large excess of mercurated $mRNA_{ov}$ could be used, the hybridization reaction was driven by the mercurated $mRNA_{ov}$ and required only short incubation times. Thus, reannealing of homologous chick DNA molecules present in the preparation was minimized and the DNA fraction that became bound to the column should be further enriched for the coding ovalbumin DNA sequences. Using this method, the ovalbumin gene sequence was substantially purified to yield an overall purification of 180 000-fold from total chick DNA (Table I). From these data it could be calculated that the ovalbumin structural gene sequence was 18% pure. This estimation, however, has not taken into consideration the fact that the DNA was originally sheared to a mean length of 5000 nucleotides. Since the structural ovalbumin gene has a complexity of approximately 1800 nucleotides (Woo et al., 1975; Monahan et al., 1976), the purity of 18% for the final DNA preparation should be corrected by a factor of 5000 nucleotides/1800 nucleotides = 45%. Thus, two out of five DNA molecules in the final DNA preparation contain at least part of the ovalbumin gene.

Using a similar approach, the anticoding strand of the ovalbumin gene has been purified 150 000-fold from total chick DNA and is also approximately 40% pure (Woo et al., 1977). A highly purified ovalbumin gene duplex could thus be obtained by allowing the two DNA preparations to reanneal. Since no DNA degradation occurred during the isolation procedure, the resulting DNA preparation should contain DNA sequences that are adjacent to the structural ovalbumin gene which may play a regulatory role in the expression of this structural gene.

Isolation of a unique sequence eucaryotic gene is not only a formidable task, but also with little value unless the isolated gene can be amplified. Such is the case because only minute quantities of the purified product can be obtained from gram quantities of total DNA. With such low yields, the purified gene cannot be properly characterized and studied with respect to regulation of eucaryotic gene expression. Insertion of the highly purified natural ovalbumin gene into bacterial plasmids for amplification in bacteria is thus our immediate plan. Since there is already a tract of poly(dC) on the 3′-terminus of the highly purified ovalbumin gene, it could be hybridized to-poly(dG)-containing plasmid DNA for bacterial transformation (Rabbit, 1976; Maniatis et al., 1976; Higuchi et al., 1976; Rougeon et al., 1975). Recently, we have successfully inserted into plasmid DNA the full-length structural ovalbumin gene synthesized from $mRNA_{ov}$)mcReynolds et al., 1977a,b). Successful insertion of the highly purified natural ovalbumin gene into plasmid DNA and its amplification in bacteria should yield unlimited quantities of this gene, which could be used to study the mechanism by which steroid hormones regulate gene expression. In addition, this gene could be used to form a "minichromosome" for experiments aimed at elucidation of the basic mechanisms involved in eucaryotic gene transcription at the molecular level.

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References

Bishop, J. O. (1972), *Biochem. J. 126,* 171-185.
Britten, R. J., Graham, D. E., and Neufeld, B. R. (1974), *Methods Enzymol. 29,* 363-418.
Chan, L., Means, A. R., and O'Malley, B. W. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1870-1874.
Chang, L. M. S., and Bollum, F. J. (1971), *J. Biol. Chem. 246,* 909-916.
Dale, R. M. K., Martin, E., Livingston, D. C., and Ward, D. C. (1975), *Biochemistry 14,* 2447-2457.
Dale, R. M. K., and Ward, D. C. (1975), *Biochemistry 14,* 2458-2469.
Harris, S. E., Means, A. R., Mitchell, W. M., and O'Malley, B. W. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3776-3780.
Harris, S. E., Rosen, J. M., Means, A. R., and O'Malley, B. W. (1975), *Biochemistry 14,* 2072-2080.
Harris, S. E., Schwartz, R. J., Tsai, M.-J., Roy, A. K., and O'Malley, B. W. (1976), *J. Biol. Chem. 251,* 524-529.
Higuchi, R., Paddock, G., Wall, R., and Salser, W. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3146-3150.
Maniatis, T., Kee, S. G., Efstratiadis, A., and Kafatos, F. C. (1976), *Cell 8,* 163-182.
McReynolds, L. A., Catterall, J., and O'Malley, B. W. (1977b), *Gene* (in press).
McReynolds, L. A., Monahan, J. J., Bendure, D., Woo, S. L. C., Paddock, G. V., Salser, W., Dorson, J., Moses, R. E., and O'Malley, B. W. (1977a), *J. Biol. Chem. 252,* 1840-1843.
Mirsky, A. E., and Ris, H. (1951), *J. Gen. Physiol. 34,* 451-458.
Monahan, J. J., Harris, S. E., Woo, S. L. C., Robberson, D. L., and O'Malley, B. W. (1976), *Biochemistry 15,* 223-233.
O'Malley, B. W., McGuire, W. L., and Korenman, S. G. (1967), *Biochim. Biophys. Acta 145,* 204-207.
O'Malley, B. W., and Means, A. R. (1974), *Science 183,* 610-620.
Palmiter, R. D. (1973), *J. Biol. Chem. 248,* 8260-8270.
Palmiter, R. D., Oka, T., and Schimke, R. T. (1971), *J. Biol. Chem. 246,* 724-737.
Rabbit, T. H. (1976), *Nature (London) 260,* 221-225.
Rosen, J. M., Liarakos, C. D., and O'Malley, B. W. (1973), *Biochemistry 12,* 2803-2809.
Rosen, J. M., Woo, S. L. C., Holder, J. W., Means, A. R., and O'Malley, B. W. (1975), *Biochemistry 14,* 69-78.
Rougeon, F., Kourilsky, P., and Mach, B. (1975), *Nucleic Acids Res. 2,* 2365-2378.
Schwartz, R. J., Tsai, M.-J., Tsai, S. Y., and O'Malley, B. W. (1975), *J. Biol. Chem. 250,* 5175-5182.
Sullivan, D., Palacios, R., Stavnezer, J., Taylor, J. M., Faras, A. J., Kiely, M. L., Summers, N. M., Bishop, J. M., and

Schimke, R. T. (1973), *J. Biol. Chem. 248,* 7530-7539.
Tsai, M.-J., Schwartz, R. J., Tsai, S. Y., and O'Malley, B. W. (1975a), *J. Biol. Chem. 250,* 5165-5174.
Tsai, S. Y., Tsai, M.-J., Schwartz, R. J., Kalimi, M., Clark, J. H., and O'Malley, B. W. (1975b), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4228-4232.
Wetmur, J. G., and Davidson, N. (1968), *J. Mol. Biol. 31,* 349-370.
Woo, S. L. C., Monahan, J. J., and O'Malley, B. W. (1977), *J. Biol. Chem. 252,* 5789-5797.
Woo, S. L. C., and O'Malley, B. W. (1975), *Life Sci. 7,* 1039-1048.
Woo, S. L. C., Rosen, J. M., Liarakos, C. D., Robberson, D. L., Choi, Y. C., Busch, H., Means, A. R., and O'Malley, B. W. (1975), *J. Biol. Chem. 250,* 7027-7039.
Woo, S. L. C., Smith, R. G., Means, A. R., and O'Malley, B. W. (1976), *J. Biol. Chem. 251,* 3868-3874.
Young, B. D., Harrison, P. R., Gilmour, R. S., Birnie, G. D., Hill, A., Humphries, S., and Paul, J. (1974), *J. Mol. Biol. 84,* 555-568.

Dependence on Potassium Concentration of the Inhibition of the Translation of Messenger Ribonucleic Acid by 7-Methylguanosine 5′-Phosphate†

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ABSTRACT: The effects of potassium on the inhibition by 7-methylguanosine 5′-phosphate of the translation in the wheat germ cell-free system of globin mRNA and satellite tobacco necrosis virus (STVN) RNA are examined. The concentration of potassium ion and the anion of the potassium salt both influence the effects of 7-methylguanosine 5′-phosphate on the translation of globin mRNA. If potassium concentrations are less than the optimum for protein synthesis, the inhibition of the translation of globin mRNA by 7-methylguanosine 5′-phosphate is greatly diminished. The inhibition increases as the potassium concentration is increased even after the optimal concentration for protein synthesis is exceeded. At equivalent potassium concentrations, the inhibitory effect of 7-methylguanosine 5′-phosphate on translation of globin mRNA is substantially decreased if potassium acetate is substituted for KCl. However, the optimal concentration for protein synthesis is considerably greater for potassium acetate than for KCl and, if examined at the respective optimal concentrations of these salts for protein synthesis, the inhibiting effects of 7-methylguanosine 5′-phosphate are equivalent. The relative importance of the 7-methylguanosine cap for translation of globin mRNA is apparently not decreased at low potassium concentrations since the inhibition of translational activity of globin mRNA resulting from chemical removal of the cap is similar at all potassium concentrations. As expected, inhibition by 7-methylguanosine 5′-phosphate of the translation of STNV RNA, which does not contain a 7-methylguanosine cap, is considerably less than that observed with globin mRNA. However, the inhibition that is observed with STNV RNA exhibits qualitatively the same dependence on the concentration of potassium as that observed with globin mRNA and no inhibition by guanosine 5′-monophosphate is observed. These results illustrate the necessity of optimizing reaction conditions for individual mRNAs if the inhibition of translation of the mRNA by 7-methylguanosine 5′-phosphate is used as a criterion for the presence of a 7-methylguanosine cap.

The 5′ termini of most eukaryotic mRNAs have a unique structure of 7-methylguanosine (m^7G)[1] linked through its 5′-hydroxyl via a triphosphate to the penultimate nucleoside (Shatkin, 1976). The m^7G group is required for maximal translational activity in the wheat germ cell-free system of mRNAs for vesicular stomatitis virus, reovirus, hemoglobin, and parathyroid hormone (Both et al., 1975; Muthukrishnan et al., 1975; Kemper, 1976). Hickey et al. (1976a) observed that, in the wheat germ cell-free system, 7-methylguanosine 5′-phosphate (pm^7G) inhibited the translation of mRNAs containing m^7G but did not inhibit the translation of satellite tobacco necorsis virus (STNV) RNA which does not contain m^7G. Canaani et al. (1976) similarly observed that translation in the wheat germ system of the capped mRNAs, globin mRNA, and SV40 mRNA was inhibited to a much greater extent than that of uncapped mRNAs. However, translation of total poly(A)-containing mRNA from either HeLa cells or sea urchin eggs was inhibited only 75% to 80% by pm^7G compared with greater than 90% for globin mRNA (Weber et al., 1976; Hickey et al., 1976b), suggesting that the translation of some or all of these mRNAs is less sensitive to pm^7G than globin mRNA.

Since different mRNAs have different optimal ionic conditions for translation in the cell-free systems (Mathews, 1972; Tse and Taylor, 1977), it is possible that the sensitivities of presumed capped mRNAs to pm^7G are also a function of ionic conditions. In this report we show that the inhibition of the translation of globin mRNA by pm^7G is enhanced by increasing concentrations of potassium in the wheat germ cell-free system.

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; m^7G, 7-methylguanosine; pm^7G, 7-methylguanosine 5′-phosphate; STNV, satellite tobacco necrosis virus; ATA, aurintricarboxylic acid; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); EDTA, ethylenediaminetetraacetic acid.