Effect of Estrogen on Gene Expression in the Chick Oviduct. Regulation of the Ovomucoid Gene†

Sophia Y. Tsai, Dennis R. Roop, Ming-Jer Tsai, Joseph P. Stein, Anthony R. Means, and Bert W. O'Malley*

ABSTRACT: A restriction endonuclease (*Hha*I) fragment was obtained from a cDNA clone of ovomucoid, pOM100, which contains approximately 650 base pairs of ovomucoid mRNA ($mRNA_{om}$) sequence. This *Hha*I fragment was radioactively labeled to high specific activity by nick translation. A single-stranded ovomucoid specific hybridization probe was prepared from the labeled fragments and then used to study the regulation of transcription of the ovomucoid gene by estrogen. About 1900 copies of the ovomucoid mRNA sequences were found in each tubular gland cell nucleus of the chick oviduct after 14 days of diethylstilbestrol treatment. Only very low levels of ovomucoid mRNA sequences were detected in nuclear RNA isolated from chick liver or chick spleen tissues. After 14 days of withdrawal of diethylstilbestrol from the chick, the concentration of ovomucoid mRNA in the chick oviduct decreased to 3 molecules per tubular gland cell nucleus. Readministration of a single dose of diethylstilbestrol to these chicks resulted in a gradual increase in the concentration of $mRNA_{om}$ within the first 4 h (from 3 to 38 molecules per tubular gland cell nucleus). By 16 h, the nuclear concentration of $mRNA_{om}$ was 120 molecules per tubular gland cell nucleus. However, with a second injection of estrogen at 48 h, the amount of $mRNA_{om}$ sharply increased to a level (620 molecules/tubular gland cell nucleus) approximately one-third of that observed for chicks stimulated chronically with estrogen. In vitro synthesis of ovomucoid mRNA in isolated nuclei was measured by hybridization to nitrocellulose filters containing cloned ovomucoid DNA and compared with the synthesis of ovalbumin mRNA under the same conditions. In oviduct nuclei isolated from chronically stimulated chicks, the in vitro accumulation of the nascent ovomucoid mRNA transcripts on a molar basis was twofold slower than that determined for ovalbumin mRNA sequences.

The chick oviduct has proved to be an excellent system for studying the regulation of gene expression by steroid hormones (O'Malley & Means, 1974, O'Malley et al., 1969). Administration of estrogen to immature chicks leads to cytodifferentiation of the chick oviduct and the appearance of egg-white proteins in tubular gland cells (O'Malley et al., 1969; Oka & Schimke, 1969; Kohler et al., 1969; Palmiter, 1972). Upon withdrawal of hormone, the tubular gland cells become inactive and no longer synthesize or secrete egg-white proteins (Palmiter et al., 1970). Readministration of estrogen stimulates synthesis of these proteins (Rosenfeld et al., 1972; Means et al., 1972; Palmiter & Smith, 1973). All four egg-white proteins, ovalbumin, ovomucoid, conalbumin, and lysozyme seem to be under coordinate control; yet the level of accumulation of these proteins are significantly different (O'Malley et al., 1969; Palmiter, 1972). The regulation of the major egg-white protein, ovalbumin, has been studied extensively. Its synthesis has been shown to correlate well with the accumulation of $mRNA_{ov}$ (Chan et al., 1973; Rhoads et al., 1973; Harris et al., 1975; Palmiter et al., 1976; Cox et al., 1974). In order to further understand how steroid hormones exert their control on differential expression of these genes, it is important to study the regulation of other egg-white proteins. To this aim, ovomucoid was chosen for the present study.

Recently, ovomucoid mRNA was partially purified and used as a template for the synthesis of dsDNA (Stein et al., 1978). The dsDNA was linked to the bacterial plasmid pBR322 and used to transform *Escherichia coli* X 1776 (Stein et al., 1978). A clone, pOM100, was shown to contain approximately 650 of 900 bases in the $mRNA_{om}$ sequence. Plasmid DNA from this clone was digested with the restriction enzyme *Hha*I. The largest *Hha* fragment of pOM100, which contained the entire inserted DNA sequence, was purified by gel electrophoresis. This fragment was labeled by nick translation and a single-stranded hybridization probe specific for ovomucoid was then prepared. With this specific probe, the gene dosage of ovomucoid in the chick genome and the concentration of $mRNA_{om}$ in target and nontarget tissues was determined. Also, the time course for accumulation of nuclear $mRNA_{om}$ was measured during short-term readministration (0–48 h) of estrogen to chicks previously stimulated with estrogen and subsequently withdrawn from hormone for 14 days. Finally, in vitro synthesis of radioactively labeled $mRNA_{om}$ in isolated nuclei was carried out and compared with that of ovalbumin mRNA.

Materials and Methods

Materials

Oviducts were obtained from White Leghorn chicks. In the case of stimulated oviducts, chicks were weekly implanted subcutaneously with 20 mg of diethylstilbestrol (DES) pellet (Sigma Chemical Co.) which provided continuous release of DES for 8–9 days. In the case of withdrawn oviducts, chicks were subcutaneously injected daily with 2.5 mg of diethylstilbestrol for 14 days and then withdrawn from all hormone for 14 days. For experiments involving acute stimulation with estrogen, chicks received an injection of 2.5 mg of DES on the 14th day of withdrawal and then 24 h later. Oviducts were collected at the indicated time intervals. Restriction endonuclease *Hha*I was purchased from Bethesda Research Labs. S_1 nuclease was obtained from Miles Laboratories. DNA polymerase I was a product of Boehringer. [^{3}H]dCTP and [^{3}H]-CTP were purchased from New England Nuclear Corp. [α-^{32}P]ATP was generously supplied by Dr. Lutz Birnbaumer. All chemicals were reagent grade and were purchased from Fisher Scientific Co.

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Methods

Preparation of HhaI Fragment from pOM100. Two hundred and forty micrograms of pOM100 DNA was incubated with 60 units of restriction endonuclease *Hha*I in a buffer containing 6 mM $MgCl_2$, 6 mM mercaptoethanol, 50 mM NaCl, and 6 mM Tris-HCl, pH 7.6 at 37 °C overnight. The largest *Hha*I fragment, which was shown to contain the entire inserted ovomucoid sequence, was then purified by agarose gel electrophoresis as described previously (Lai et al., 1978).

Nick Translation. DNA was labeled by nick translation using a modification of the procedure described by Mackey et al. (1977). The reaction was performed in a final volume of 100 μL containing the following: 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.12 mM dATP, dTTP, and dGTP, 0.02 mM [^{3}H]dCTP (25 Ci/mmol), 5 μg of BSA, and 1.5 μg of DNA. These compounds were assembled on ice and then 0.5 ng of DNase (Worthington, DPFF) was added. The mixture was incubated at room temperature for 60 s and immediately cooled in an ice–water bath. *E. coli* DNA polymerase I (20 μL, 80 units) was added and the mixture incubated at 14 °C. After 6 h, the reaction was stopped by the addition of 100 μL of 0.2 M EDTA, 100 μg of *E. coli* DNA, and heating at 68 °C for 10 min. Unincorporated dNTPs were separated from the labeled DNA by gel filtration on a Sephadex G-50 column using 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10 mM NaCl. The radioactive fractions eluting in the void volume were pooled, made 0.2 M with NaCl, and precipitated by the addition of 2 volumes of ethanol. The specific activity of the final product was 6×10^6 cpm/μg.

Isolation of Chick Oviduct Nuclei. Chick oviduct nuclei were isolated by a modified method of Busch (1967). Chick oviduct tissue was homogenized in 8 volumes of ice-cold 5% citric acid with a tissuemizer at 4 °C for 60 s. The homogenate was filtered through two layers of cheesecloth and one layer of organza, and nuclei were collected by centrifugation at 600*g* for 10 min. The nuclear pellet was suspended in 20 volumes (v/w) of 0.25 M sucrose in 1.5% citric acid. Eighteen milliliters was layered over a 10-mL cushion of 0.88 M sucrose in 1.5% citric acid and centrifuged at 600*g* for 20 min. The purified nuclei were again washed with 20 volumes (v/v) of 0.5% Triton X-100 in 0.25 M sucrose, 1.5% citric acid before isolation of total nuclear RNA. DNA and RNA content was determined by diphenylamine and orcinol assays as described previously (Tsai et al., 1975).

Isolation of Nuclear RNA. RNA was isolated from chick oviduct nuclei by a modified method of Holmes & Bonner (1973). Purified nuclei were lysed at room temperature in 20 volumes of buffer containing 2% $NaDodSO_4$[1], 7 M urea, 0.35 M NaCl, 1 mM EDTA, and 0.01 M Tris-HCl, pH 8.0, by means of a glass–Teflon homogenizer. Protein was extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, v/v). The interface was reextracted with 0.5 volume of lysis buffer and the combined aqueous phase was again extracted. The aqueous phase was adjusted to 0.2 M NaAc, pH 5, and precipitated with 2 volumes of ethanol. The nucleic acid was resuspended in 3 mM $MgCl_2$, 0.1 M NaCl, and 10 mM Tris-HCl, pH 7.5, and treated at room temperature for 1 h with 40 μg/mL of affinity column purified DNase I (Maxwell et al., 1977). $NaDodSO_4$, proteinase, K, and EDTA were added to final concentrations of 0.5%, 20 μg/mL, and 5 mM, respectively. The mixture was incubated at 37 °C for 15 min and extracted with phenol–chloroform–isoamyl alcohol twice before precipitation with ethanol. The resultant RNA was again treated with DNase I at 20 μg/mL for 40 min, extracted with the phenol mixture, and then passed through a Sephadex G-50 column in 0.1 mM EDTA, 10 mM Tris-HCl, pH 7.5. The purified RNA was lyophilized, stored at −20 °C, and used for hybridization. Nuclear RNAs from chick spleen and liver were also prepared as above.

Hybridization of the Ovomucoid [^{3}H]DNA Probe to Chick DNA. Chick oviduct or liver DNA, sheared to ~400 nucleotides in length, was used in DNA excess hybridization experiments. Chick DNA (1.3 mg) and 0.06 ng of the tritiated ovomucoid DNA ([^{3}H]DNA_{om}) probe were mixed in a final volume of 200 μL in tapered reaction vials containing 0.6 M NaCl, 10 mM Hepes (pH 7.0), and 2 mM EDTA. Samples were boiled at 100 °C for 5 min and incubated for various times at 68 °C. Following hybridization, samples were frozen in dry ice/EtOH and stored at −20 °C.

Samples were then treated with S_1 nuclease (4800 units) in 600 μL of 0.4 N NaCl, 0.2 N sodium acetate, 25 mM zinc acetate, pH 4.5, and the S_1 nuclease resistant hybrids were determined as described elsewhere (Harris et al., 1976).

Hybridization of Nuclear RNA to the Ovomucoid Probe. These RNA excess hybridization experiments were performed in a final volume of 30 μL under conditions as described earlier (Harris et al., 1976). RNA, 4–5000 μg/mL, was boiled together with 0.14 ng of [^{3}H]DNA_{om} probe for 5 min. The mixture was incubated at 68 °C for time intervals ranging from 15 min to 72 h. Following hybridization the samples were treated with S_1 nuclease (1600 units) and the S_1 nuclease resistant hybrids were determined as previously described (Harris et al., 1976).

In Vitro RNA Synthesis in Isolated Nuclei. Nuclei were isolated from oviducts of chronically stimulated chicks as previously described (Towle et al., 1977) and then used for in vitro RNA synthesis. [^{3}H]RNA was synthesized in a buffer containing the following: 80 mM Tris-HCl, pH 7.9, 2.5 mM $MnCl_2$, 3 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 0.6 mM each of ATP, GTP, UTP, and 25 μM [^{3}H]CTP (20 Ci/mmol), 30 μM EDTA, 6 mM dithiothreitol, 15% glycerol, and 400 μg/mL DNA. Mixtures were incubated at 37 °C for 30 min. DNase I was then added to a final concentration of 20 μg/mL and incubated at 37 °C for 15 min. Digestion was terminated by addition of 5 mM EDTA and 0.5% $NaDodSO_4$. [^{3}H]RNA was then isolated by proteinase K treatment, phenol extraction, and alcohol precipitation as described earlier (Towle et al., 1977). The resultant RNA was again treated with DNase I (20 μg/mL) and then chromatographed on Sephadex G-50 columns and lyophilized.

In Vitro Synthesis of [^{32}P]RNA from pOM100. RNA was transcribed by *E. coli* RNA polymerase under the following conditions: 50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 2 mM 2-mercaptoethanol, 0.8 mM sodium phosphate, 5 μg/mL pOM100 DNA, 1 mM each of GTP, CTP, and UTP, 1 mM [^{32}P]ATP (0.25 mCi, 700 Ci/mmol), and 300 μg/mL *E. coli* RNA polymerase. RNA synthesis was terminated after 2 h at 37 °C. This [^{32}P]RNA, with a specific activity of 9×10^8 cpm/μg, was used as an internal standard for filtered hybridization experiments.

Preparation of DNA Filters. Two hundred and fifty micrograms of pOM100 DNA was suspended in 4 mL of 10 mM Tris-HCl, pH 7.9 buffer and heat denatured at 100 °C for 10 min. The solution was quick cooled in an ice bath and immediately diluted with 41 mL of ice-cold 4 × SSC. The suspension was then slowly passed through a Millipore filter (3.5 cm in diameter) at 0.4 mL/min at 4 °C and washed with 40 mL of cold 4 × SSC. The filter was air dried and baked in a 60 °C

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; poly(A), poly(T), and poly(U), poly(adenylic acid), poly(thymidylic acid), and poly(uridylic acid), respectively.

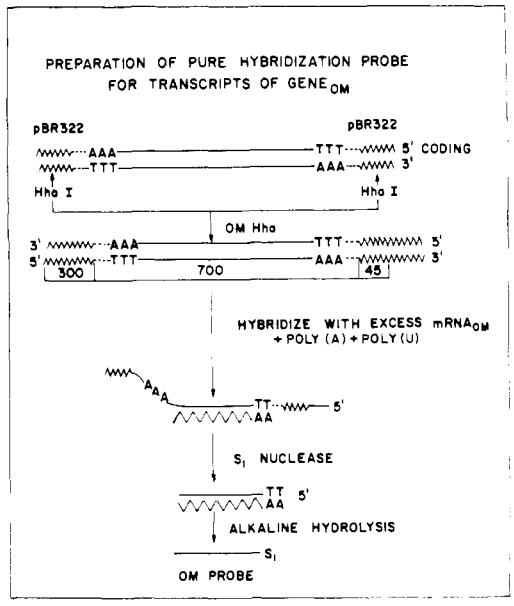

FIGURE 1: Schematic representation of the preparation of [^{3}H]DNA$_{om}$ probe. Fragments corresponding to DNA$_{om}$ were labeled with [^{3}H]dCTP by nick translation to a specific activity of 6×10^6 cpm/μg. Pure single-stranded probes were obtained as described in the text.

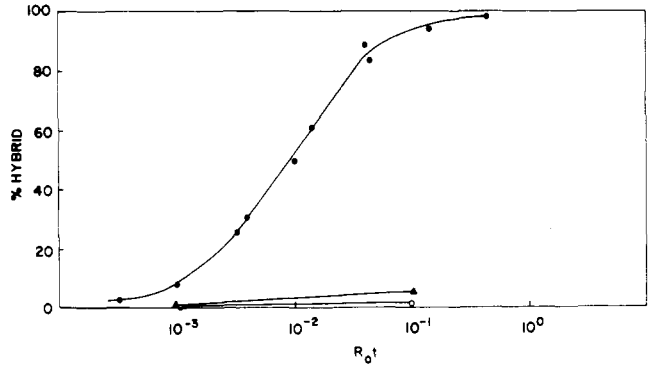

FIGURE 2: Analysis of purity of the [^{3}H]DNA$_{om}$ probe. Hybridization of 0.15 ng of [^{3}H]DNA$_{om}$ to 20–200 ng/mL of partially purified mRNA$_{om}$ (●). Reassociation of [^{3}H]DNA$_{om}$ alone (▲). Hybridization of 0.4 ng of [^{3}H]DNA$_{om}$ to 0.5 μg of pBR322 plasmid DNA (○). The percentage of [^{3}H]DNA$_{om}$ hybridized is plotted against equivalent R_0t or C_0t.

vacuum oven overnight. The filter was then immersed in Denhardt solution (0.02% Ficol, 0.02% BSA, 0.02% polyvinylpyrrolidone in 4 × SSC) for 1 h, dried, and then baked at 70 °C in vacuum oven for 4 h. The amount of DNA on a single filter disc was determined by spectrophotometry after boiling in 0.5 N $HClO_4$.

Hybridization [^{3}H]RNA to DNA Filter. [^{3}H]RNA ($1–5 \times 10^6$ cpm) and [^{32}P]RNA$_{pOM100}$ (2000 cpm) were heated in siliconized glass vials at 80 °C in 40 μL of 50% formamide, 0.21 M NaCl, 0.021 M sodium citrate, pH 7.0, for 5 min to denature any double-stranded sequences. For competition studies, mRNA$_{om}$, prepared according to Stein et al. (1978), was included prior to the heating step. Hybridization reactions were started by addition of DNA filters (with 1.2 μg of bound DNA) to the mixtures and incubated at 42 °C for 18 h. Paraffin oil was added to cover the hybridization mixture in order to prevent evaporation of the solution. At the end of hybridization, filters were removed and rinsed with 1.4 × SSC and then chloroform. Subsequently, filters were washed twice at 30 °C with 5 mL of 1.4 × SSC for 1 h and twice with 5 mL of 0.1 × SSC for 20 min. Finally, filters were treated with pancreatic RNase (20 μg/mL) in 0.5 mL of 2 × SSC for 1 h at room temperature and then washed with 2 × SSC at 30 °C for 15 min. The filters thus obtained were solubilized with 1 mL of Cellusolve and counted in 10 mL of Aquasol scintillation solution.

Results

Preparation of a Specific Probe for mRNA$_{om}$ Sequences. A specific probe for ovomucoid messenger RNA sequences was prepared from pOM100, a chimeric plasmid previously cloned in our laboratory (Stein et al., 1978). This plasmid contains all but ~150 nucleotides at the 5′ end of the structural sequence. The orientation of the structural ovomucoid sequences in the plasmid and the preparation of a specific probe are diagrammatically illustrated in Figure 1. pOM100 DNA was digested with the restriction endonuclease *Hha*I. The *Hha*I fragment containing the ovomucoid specific sequences and flanking plasmid sequences was then purified from the remaining plasmid fragments by agarose gel electrophoresis (Lai et al., 1978). The purified fragment was labeled with [^{3}H]-dCTP by nick translation to a specific activity of 6×10^6 cpm/μg. A single-stranded ovomucoid probe was then prepared by hybridizing these labeled fragments with a 100× excess of ovomucoid mRNA to a R_0t of 2.5×10^{-2}. Twenty micrograms each of poly(U) and poly(A) was added to avoid self-reannealing of the *Hha*I fragment caused by the poly(A) and poly(T) linker. Under these conditions, the hybridization reaction was complete and essentially no DNA-DNA reassociation occurred (C_0t of 2.5×10^{-4}). At the end of the incubation period, the reaction mixture was treated with S_1 nuclease to destroy the flanking plasmid sequences and anticoding sequences which remained single stranded. The hybridization with excess ovomucoid mRNA and S_1 nuclease digestion were repeated once more to ensure complete removal of the plasmid and anticoding sequences. Finally, the single-stranded DNA probe ([^{3}H]DNA$_{om}$) was obtained by alkaline hydrolysis of the messenger RNA.

Characterization of the Probe. Purity of the probe was ascertained by the following tests. First, the [^{3}H]DNA$_{om}$ probe was reassociated alone to a C_0t of 10^{-1} (Figure 2). Only 5% self-reassociation was detected which indicated the absence of anticoding sequences. Second, pBR322 DNA was hybridized with the probe to a C_0t of 10^{-1} at a ratio of 400:1 (Figure 2) and less than 1% hybridization was observed. This is indicative of the absence of plasmid DNA sequences in the probe. Third, a RNA preparation obtained from Sepharose 4B column enriched for mRNA$_{om}$ (Stein et al., 1978), at concentrations ranging from 20 to 200 ng/mL, was hybridized to the [^{3}H]DNA$_{om}$ probe. A single component hybridization curve was observed (Figure 2), and the probe was protected to the extent of 98%. These results suggest the absence of other RNA sequences in the [^{3}H]DNA$_{om}$ probe. The $R_0t_{1/2}$ of the back hybrid was 9×10^{-3} which is 4.5× slower than the predicted $R_0t_{1/2}$ (2×10^{-3}, Table I) and was due to the fact that the purity of the mRNA$_{om}$ was only approximately 20%.

Hybridization of the Ovomucoid Probe to Chick DNA. The kinetics of hybridization of single-stranded [^{3}H]DNA$_{om}$ probe to excess chick oviduct and liver DNAs are shown in Figure 3. The probe reacted similarly to both chick oviduct and liver DNA. At an equivalent C_0t of 40 000, only 70% of the [^{3}H]-DNA$_{om}$ probe formed stable hybrids with excess DNA. Since the nick translated [^{3}H]DNA$_{om}$ probe was rather small (approximately 5S as determined by alkaline sucrose gradient centrifugation) and the amount of S_1 nuclease used was rather high in order to ensure digestion of [^{3}H]DNA$_{om}$ probe in the

TABLE I: Concentration of $mRNA_{om}$ in Total Nuclear RNA during Secondary Stimulation.

tissue	$R_0t_{1/2}$[a]	fraction of $mRNA_{om}$[b]	RNA/DNA	tubular gland cells/ total cells	molecules of $mRNA_{om}$[c]/ cell nucleus	molecules of $mRNA_{om}$/ tubular gland cell nucleus
$oviduct_{DES}$	1.7	1.18×10^{-3}	0.25	0.8	1550	1940
$oviduct_W$	1.6×10^3	1.25×10^{-6}	0.07	0.15	0.5	3
$oviduct_W + 2h_{DES}$	4.25×10^2	4.70×10^{-6}	0.09	0.15	2	12
$oviduct_W + 4h_{DES}$	2.65×10^2	7.55×10^{-6}	0.15	0.16	6	38
$oviduct_W + 8h_{DES}$	1.65×10^2	1.21×10^{-5}	0.15	0.20	10	50
$oviduct_W + 16h_{DES}$	6.6×10	3.0×10^{-5}	0.15	0.20	24	120
$oviduct_W + 48h_{DES}$	1.2×10	1.67×10^{-4}	0.25	0.35	220	620

[a] The $R_0t_{1/2}$ of pure $mRNA_{om}$ is calculated from the following equation: $(R_0t_{1/2})_{om} = [(N_{om}/\sqrt{N_{om}})/(N_{ov}/\sqrt{N_{ov}})](R_0t_{1/2})_{ov} = 2 \times 10^{-3}$; $N_{om} = 900$; $N_{ov} = 1930$; $(R_0t_{1/2})_{ov} = 3 \times 10^{-3}$. [b] Fraction $mRNA_{om}$ = $R_0t_{1/2}$ of pure ovomucoid message/$R_0t_{1/2}$ of given RNA. [c] Molecules of RNA_{om}/cell nucleus = fraction of $mRNA_{om}$(RNA/DNA) (2.6×10^{-12} g of DNA)[(6.02×10^{23} molecules)/(2.97×10^5 g of $mRNA_{om}$)].

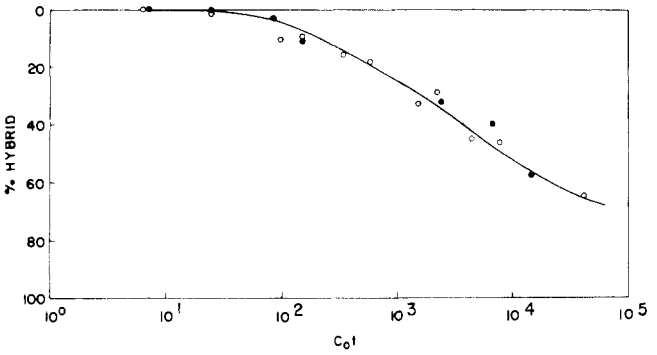

FIGURE 3: Hybridization of ^{3}H-labeled ovomucoid probe to excess chick DNA. Chick DNA (1.3 mg) isolated from oviducts (●) or from liver (○) was hybridized to 0.06 ng of [^{3}H]DNA_{om} in 200 μL as described in Methods.

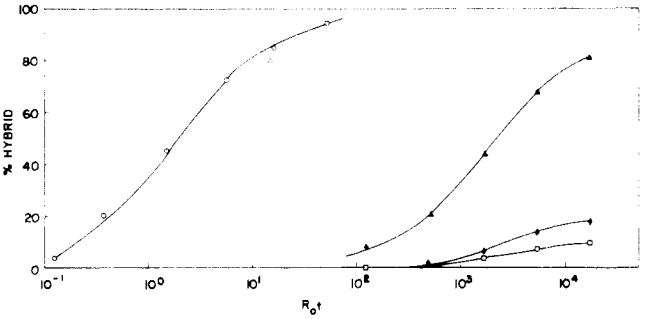

FIGURE 4: Hybridization of nuclear RNA to [^{3}H]DNA_{om}. [^{3}H]DNA_{om} (0.12 ng) was hybridized to 0.6 μg and 0.15 μg of DES-stimulated chick oviduct RNA (○), 150 μg of chick liver RNA (□), 150 μg of chick spleen RNA (♦), or 150 μg of withdrawn chick oviduct RNA (▲).

presence of such vast excess of unlabeled DNA, short hybrids might be unstable under our experimental conditions. Furthermore, the amount of ovomucoid sequences in the DNA used was only in eightfold excess to the [^{3}H]DNA_{om} probe (unlabeled DNA/[^{3}H]DNA_{om} probe = 2×10^7). Thus, the hybridization reaction might not go to completion even at a higher C_0t value. The apparent $C_0t_{1/2}$ value for these reactions was 1.5×10^3. This agrees well with the value predicted for a single copy gene in the haploid chick genome (Harris et al., 1973; Bishop & Rosbach, 1973; Harrison et al., 1974). Also the hybridization curve is quite similar to that of the ovalbumin gene which was determined to be a unique sequence (Harris et al., 1973).

Quantitation of Ovomucoid RNA Sequences in Target and Nontarget Tissues. As shown in Figure 4, nuclear RNA isolated from chick oviducts stimulated chronically with estrogen, reacted rapidly with the probe. At an equivalent R_0t value of 50, the hybridization reaction was essentially complete. The apparent $R_0t_{1/2}$ for the reaction is 1.7. This indicates that 0.1% of the RNA sequences in stimulated oviduct nuclei are ovomucoid sequences (Table I). Nuclear RNA prepared from chicks first stimulated and then withdrawn from estrogen reacted 1000 times more slowly ($R_0t_{1/2}$ of 1.6×10^3) than nuclear RNA isolated from stimulated chicks. Thus, removal of estrogen resulted in a marked decrease in the concentration of ovomucoid sequences. However, the hybridization reactions were almost complete at a R_0t value of 10^4. As for nuclear RNA prepared from liver and spleen, even at a R_0t value of 2×10^4, only 10–18% hybridization was obtained.

The number of molecules of ovomucoid RNA sequences in a given RNA preparation can be estimated by comparing the $R_0t_{1/2}$ value of the hybridization curve to that obtained for purified $mRNA_{om}$. Since the $mRNA_{om}$ is not pure, the $R_0t_{1/2}$ of pure $mRNA_{om}$ to [^{3}H]DNA_{om} probe can only be estimated using $mRNA_{ov}$ as a standard. Ovomucoid mRNA is estimated to be 900 nucleotides in length with a molecular weight of 297 000 (Stein et al., 1978; Buell et al., 1978). Thus, pure $mRNA_{om}$ is calculated to react with the probe at an equivalent $R_0t_{1/2}$ of 2×10^{-3} under our present experimental conditions (Table I). Since the RNA/DNA ratio in the isolated nuclei is 0.25, about 1600 molecules of $mRNA_{om}$ are calculated to be present in the cell nucleus of estrogen-stimulated oviducts (Table I). Withdrawal of estrogen from chicks results in a marked decrease of $mRNA_{om}$ to one molecule per cell nucleus. In the nontarget tissues of spleen and liver, less than one molecule of $mRNA_{om}$ can be detected.

Induction of Ovomucoid mRNA during Secondary Estrogen Stimulation. The marked diminution of ovomucoid sequences in the RNA preparation upon withdrawal of estrogen made it interesting to study the temporal effect of hormone on accumulation of $mRNA_{om}$ during acute stimulation. Chicks withdrawn from hormone for 14 days were subsequently given a single injection of estrogen and sacrificed at the time intervals indicated. For the 48-h time point, one additional injection was given 24 h prior to sacrifice. Nuclear RNA was then isolated and hybridized to the [^{3}H]DNA_{om} probe. Figure 5 shows the analyses of various RNA preparations. Within 2 h after readministration of estrogen to withdrawn chicks, the rate of hybridization increased 4-fold ($R_0t_{1/2} = 4.3 \times 10^2$; Table I). It gradually increased to 10-fold after readministration of estrogen for 8 h ($R_0t_{1/2} = 1.7 \times 10^2$). By 16 h, the rate of hy-

TABLE II: In Vitro Transcription of $mRNA_{om}$ and $mRNA_{ov}$ from Isolated Nuclei.

filter	[^{3}H]RNA[a]	competitor	[^{3}H]RNA hybridized (cpm)	% recovery of ^{32}P internal standard	hybridizable gene sequences (cpm)	% mRNA in total RNA	av % of total RNA[b]
pOM100	$oviduct_{DES}$	—	232	14.3	1077	0.018	0.017 ± 0.001
		$mRNA_{om}$	78				
		yeast RNA	248	13.2	1287	0.021	
E. coli DNA	$oviduct_{DES}$	—	96				
		$mRNA_{om}$	46				
rat liver DNA	$oviduct_{DES}$	—	84				
		$mRNA_{om}$	98				
pHb1001	$oviduct_{DES}$	—	86	7.2			
		$mRNA_{om}$	68				
pOM100	$oviduct_{W}$	—	74	16.1			
		$mRNA_{om}$	86				
pOM100	liver	—	106	13.6			
		$mRNA_{om}$	90				
pOM100	spleen	—	76	10.6			
		$mRNA_{om}$	80				
pOM100	$oviduct_{DES}$ + α-amanitin	—	36	19.0			
		$mRNA_{om}$	38				
pOV230	$oviduct_{DES}$	—	1130	15.3	6300	0.106	0.100
		$mRNA_{ov}$	166				

[a] Input [^{3}H]RNA: $oviduct_{DES}$, 5.9×10^6 cpm; $oviduct_W$, 4.4×10^6 cpm; liver, 4.9×10^6 cpm; spleen, 4.6×10^6 cpm; $oviduct_{DES}$ + α-amanitin, 1.6×10^6 cpm. [b] Average and standard error of mean determined from results of ten separate experiments.

bridization still appeared to increase at a similar rate ($R_0t_{1/2} = 6.6 \times 10$). However, after a second injection of hormone, the $R_0t_{1/2}$ at 48 h further decreased to 1.2×10^{-1}. This rate of hybridization was only 7-fold slower than that calculated for nuclear RNA from oviducts of chronically stimulated chicks. Thus, within 48 h, the concentration of ovomucoid RNA sequences per cell nucleus increased almost 200-fold.

Since the proportion of tubular gland cells in the chick oviduct during secondary stimulation has been determined (Oka & Schimke, 1969; Yu et al., 1971), one can estimate the number of molecules of ovomucoid RNA per tubular gland cell nucleus. As shown in Table I, the number of ovomucoid RNA molecules in chronically stimulated chick oviducts is about 1900. Upon withdrawal of hormone for 14 days, the number of ovomucoid RNA molecules decreased to 3 per tubular gland cell nucleus. Four hours after readministration of hormone, 38 molecules per cell was found. The number of molecules increased further to 600 per cell with a second injection of hormone, which is 200 times higher than that observed for withdrawn chicks.

Transcription of Ovomucoid Sequences in Isolated Nuclei. [^{3}H]RNA was synthesized in nuclei isolated from hormone-stimulated chick oviducts and assayed for the presence of $mRNA_{om}$ sequences by hybridization to filters containing pOM100 DNA. As shown in Table II, approximately 250 cpm was recovered as hybrids to pOM100 DNA. In the presence of excess nonradioactively labeled competitor, $mRNA_{om}$, the level of bound [^{3}H]RNA decreased to 80 cpm. However, no competition resulted when yeast RNA was used as competitor. The net 170 cpm was considered to be from ovomucoid specific RNA sequences. The absolute amount of ovomucoid specific RNA is calculated by correcting this value for the hybridization efficiency of [^{32}P]RNA synthesized from pOM100 DNA which was used as an internal standard to correct for both recovery and hybridization efficiency of the [^{3}H]$mRNA_{om}$. As shown in Table II, only 17% of the input [^{32}P]RNA was recovered as hybrids. The low hybridization efficiencies observed might be due to loss of DNA or unstable hybrids from the filter during the extensive washing employed to reduce the hybridization background. After correction for the hybridization efficiency, the hybridizable and competable ovomucoid sequences were calculated to be 950 cpm which represented 0.018% of the input [^{3}H]RNA. An average value for ovomucoid specific sequences, 0.017%, was obtained from ten sets of such experiments. The standard of the mean of this number between experiments is only 0.001%.

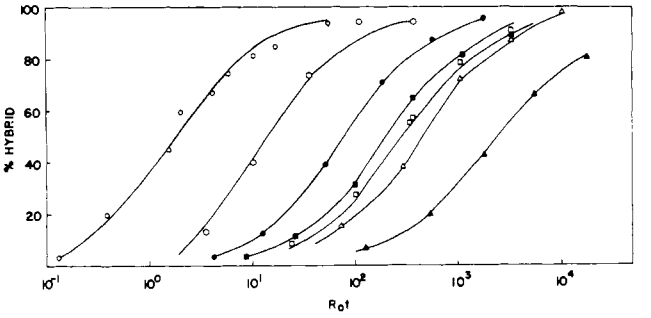

FIGURE 5: Analysis of ovomucoid sequences in nuclear RNA during secondary stimulation. Nuclear RNA was prepared from 14-day DES-withdrawn (▲, 150 μg), 2-h DES-treated (△, 90 μg), 4-h DES-treated (□, 30 μg), 5-h DES-treated (■, 30 μg), 16-h DES treated (●, 15 μg), and 48-h DES-treated chick oviducts (○, 6 μg). Hybridization was carried out in a final volume of 30 μL. The kinetic curve for nuclear RNA isolated from 14-day DES-stimulated chick oviduct is included for comparison (○, 0.15 μg and 0.6 μg).

Since [^{32}P]cRNA internal standard was transcribed from pOM100 DNA which contains only 15% ovomucoid DNA sequences, the hybridization efficiency of ovomucoid RNA sequences might not be represented by this [^{32}P]cRNA standard. In order to rule out this possibility, we have prepared [^{32}P]cRNA from *Hha*I fragment of pOM100 DNA. The

TABLE III: In Vitro Synthesis of mRNA and Anti-mRNA Sequences from Nuclei.

	competitor	[^{3}H]RNA[a] hybridized (cpm)	% recovery of ^{32}P internal control	total hybridizable gene sequences (cpm)	% mRNA
pOM100	—	243	18.0	950	0.017
	$mRNA_{om}$	72			
pOM100 presaturated with $mRNA_{om}$	—	110		161	0.003
	$mRNA_{om}$	81			
pOV230	—	663	18.0	2961	0.085
	$mRNA_{ov}$	100			
pOV230 presaturated with $mRNA_{ov}$	—	102		67	0.002
	$mRNA_{ov}$	90			

[a] [^{3}H]RNA input for pOM100 = 5.52×10^6 cpm; for pOV230 = 3.48×10^6 cpm.

majority of the *Hha*I DNA fragment is ovomucoid DNA sequences; thus, the hybridization efficiency of this [^{32}P]cRNA will better represent the recovery of ovomucoid RNA when [^{32}P]cRNA transcribed from *Hha*I fragment was used as internal standard. A similar hybridization efficiency was observed (18%); 562 cpm which represented 18% of the input was retained by the pOM100 filter. In addition, in the presence of excess $mRNA_{om}$, the hybridizable sequences were reduced to 162 cpm, indicating that 70% of the hybridizable sequences was competed by mRNAom. Since 67% of the *Hha*I fragment was homologous to $mRNA_{om}$ and 33% was the flanking plasmid sequences, this suggested that the transcription of cRNA from the template is a random process. Thus, cRNAs synthesized either from *Hha*I of pOM100 or total pOM100 DNA can serve as an internal standard.

In order to validate this type of filter hybridization assay, several control experiments were carried out. First, when similar amounts of [^{3}H]RNA were hybridized to filters containing *E. coli* DNA, rat liver DNA or plasmid DNA containing chick β-globin gene (pHb 1001), very low levels of hybridizable sequences were detected (Table II). Second, [^{3}H]RNA synthesized from withdrawn chick oviduct nuclei hybridized only at background levels to pOM100 filters and the low levels of hybridizable sequences were not competed out by excess $mRNA_{om}$. Similar results were also obtained when [^{3}H]RNAs synthesized from nontarget tissues, liver, or spleen were hybridized to pOM100 filters. These results suggested that tissue specificity was maintained in this in vitro transcription system and that the filter hybridization technique could distinguish this difference. However, it is possible that the liver, spleen, or withdrawn oviduct nuclei support small amounts of $mRNA_{om}$ synthesis that are below the level of sensitivity of this assay. Third, addition of α-amanitin at 5 μg/mL during in vitro RNA synthesis resulted in the complete inhibition of $mRNA_{om}$ sequences, when only 50% inhibition of total RNA synthesis was observed. Thus, the level of hybridization detected indeed represents the amount of ovomucoid RNA synthesized in vitro by RNA polymerase II. Fourth, when pOM100 filters were presaturated with excess $mRNA_{om}$ and then hybridized with in vitro synthesized [^{3}H]RNA, only background levels of hybridizable counts could be detected. Control filters that were treated under the same conditions but without $mRNA_{om}$ were still able to hybridize with 0.02% of the input [^{3}H]RNA (Table III). These results indicate that sequences which hybridize to pOM100 filters represent ovomucoid coding sequences.

To further clarify the filter hybridization assay, [^{3}H]RNA was synthesized from oviducts chronically stimulated with estrogen and hybridized with filters containing pOV230 DNA, a cDNA clone containing a full-length ovalbumin DNA insert. [^{32}P]cRNA synthesized from $cDNA_{ov}$ was used as internal standard. As shown in Tables II and III, approximately 0.1% of the input [^{3}H]RNA (650–1000 cpm) represented ovalbumin sequences which were competed out by excess nonradioactive ovalbumin mRNA. When pOV230 filters were presaturated with $mRNA_{ov}$ prior to hybridization, only background levels of hybridization occurred (Table III). The level of in vitro synthesis of ovalbumin mRNA sequences was about 5.5-fold higher than ovomucoid. Taking into consideration the difference in DNA lengths of pOM100 and OV230 on the filters (650 bp vs. 1859 bp), the level of in vitro synthesis of ovalbumin mRNA sequences on a molar basis was only 2-fold higher than ovomucoid.

Discussion

Highly labeled DNA specific for ovomucoid mRNA was prepared from a *Hha*I fragment of cloned pOM100 by nick translation. A single-stranded probe was further purified by hybridization with excess $mRNA_{om}$ and then used for DNA excess hybridization. It was demonstrated that DNA from both chick oviduct and liver cells contain only a single copy of the ovomucoid gene. Therefore, the synthesis of ovomucoid mRNA resembles that observed for other proteins which are synthesized in large amounts (ovalbumin, globin, silk fibroin, etc.) and is accomplished by preferential transcription rather than by gene amplification (Harris et al., 1973; Bishop & Rosbach, 1973; Harrison et al., 1974; Suzuki et al., 1972).

RNA excess hybridization is a sensitive technique which permits an accurate assessment of the number of molecules of a particular messenger RNA in the cell if the $R_0t_{1/2}$ value and the length of the messenger RNA are known. A length of 900 nucleotides was used for ovomucoid mRNA. This value was estimated from the s value determined by sucrose gradient centrifugation. Approximately 1900 molecules of $mRNA_{om}$ was found in each tubular gland cell nucleus of chicks stimulated chronically with estrogen (Table I). After withdrawal of all hormone for 14 days, only 3 molecules of $mRNA_{om}$ per cell nucleus was found. The hybridization observed with the [^{3}H]DNA_{om} probe to nuclear RNA isolated from hormone-withdrawn oviduct was indeed due to DNA–RNA hybrids since no hybridization was detected if the RNA preparation was hydrolyzed with alkali prior to hybridization with the [^{3}H]DNA_{om} probe.

Since the concentration of $mRNA_{om}$ sequence in the cytoplasm is extremely high, approximately 6000 molecules/tubular gland cell (unpublished results), it is important to demonstrate that the $mRNA_{om}$ sequences measured in nuclei are not due to cytoplasmic contamination. It is difficult to perform

experiments that would completely rule out this possibility. Nevertheless, we feel that the contribution of cytoplasmic sequences to those measured in nuclear extracts is minimal for the following reasons: First, similar amounts of $mRNA_{om}$ were detected in RNA isolated from nuclei not treated with detergent as compared with nuclei which were repeatedly washed with 0.5% Triton X-100. Second, the ratio of ovomucoid mRNA to ovalbumin mRNA ($mRNA_{ov}$) sequences in isolated nuclei (0.12% $mRNA_{om}$ vs. 0.4% $mRNA_{ov}$, Table I and Roop et al., 1978) is different from that observed in polysomal poly(A) or in total cellular RNA (6% $mRNA_{om}$ vs. 50% $mRNA_{ov}$ in polysomal poly(A) RNA and 0.04% $mRNA_{om}$ vs. 0.5% $mRNA_{ov}$ in total cell RNA; Hynes et al., 1977; unpublished results). If the majority of the $mRNA_{om}$ sequence in nuclei was due to contamination by cytoplasmic RNA, we would expect the nuclear ratio to be very similar to that observed in the cytoplasm. Most importantly, during secondary stimulation, the kinetics of accumulation of $mRNA_{ov}$ in the nucleus and the total cell are dramatically different. After 48 h of estrogen stimulation, the concentration of $mRNA_{ov}$/tubular gland cell in nuclear RNA has plateaued (3000 molecules), while that in total RNA is still accumulating (13 000 molecules; Harris et al., 1975; Roop et al., 1978). In the chronically stimulated oviduct, the concentration of $mRNA_{ov}$/tubular gland cell in nuclear RNA is 3000 molecules while 50 000 molecules are found in total RNA. These observations suggest that the $mRNA_{om}$ sequence observed in nuclear RNA are not due to contamination by $mRNA_{om}$ in the cytoplasm.

Hybridization of [^{3}H]DNA_{om} probe to nuclear RNA from chick liver or spleen revealed that ovomucoid is expressed only at an extremely low level in nontarget tissues. This conclusion is further supported by the data presented in Table II on the in vitro transcription of ovomucoid mRNA in isolated nuclei. Radiolabeled ovomucoid mRNA sequences were found in the in vitro transcripts from chronically stimulated chick oviducts but very little if any were observed in withdrawn oviduct, liver, or spleen. Thus, transcription of the ovomucoid gene sequences is relatively tissue specific.

The validity of transcription of the ovomucoid gene in isolated nuclei is substantiated by the following criteria. First, the hybridizable [^{3}H]$mRNA_{om}$ sequences can be competed out by excess unlabeled ovomucoid mRNA but not by other RNA such as yeast RNA. Second, in vitro transcripts of stimulated chick oviduct do not hybridize to *E. coli* or rat liver DNA filters. These results indicate the specificity of the hybridization. Third, at a low concentration of α-amanitin, synthesis of ovomucoid sequences is completely abolished indicating that ovomucoid sequences detected are RNA polymerase II specific. Fourth, in vitro synthesized RNA did not hybridize to pOM100 filters that were presaturated with $mRNA_{om}$. This implies that in vitro transcription of the ovomucoid gene is asymmetrical. Thus, based on the above criteria, we conclude that sequences detected by filter hybridization were indeed synthesized by the nuclei in vitro. In these experiments, no attempt was made to distinguish chain completion from chain initiation. The assay was used simply to assess the amount of nuclear $mRNA_{om}$ synthesis in various tissues under varying physiological conditions.

Of the total RNA synthesized in nuclei isolated from stimulated oviducts, 0.018% corresponds to $mRNA_{om}$ sequences. This value is approximately 200-fold greater than would be expected if random transcription of the haploid chick genome occurred. Since only 10% of the DNA is expressed in oviduct cells (Liarakos et al., 1973), ovomucoid sequences are actually transcribed at a frequency 20-fold greater than random transcription of the available sequences. It is thus clear that the ovomucoid gene is transcribed preferentially in stimulated oviduct nuclei. Of interest, also, is the observation that 0.1% of the RNA synthesized corresponds to $mRNA_{ov}$ sequences. This suggests that the ovomucoid gene is transcribed at a rate 5.5× slower than the ovalbumin gene on a mass basis. The amount of $mRNA_{om}$ (nucleotides) present in the nuclear RNA extracted from oviducts of chronically stimulated chicks is 0.11%, while that for ovalbumin sequences is 0.4% (Roop et al., 1978). The fourfold difference in the relative amount of these two nuclear mRNA sequences in vivo is very similar to the relative rate of transcription of the two genes in isolated nuclei. Therefore, transcription rate might contribute significantly to differential expression of the two genes. This by no means rules out potential contributions of posttranscriptional factors as the differences in concentration of these two proteins in the cytoplasm is greater than the difference in concentration with respect to their mRNAs in nuclei.

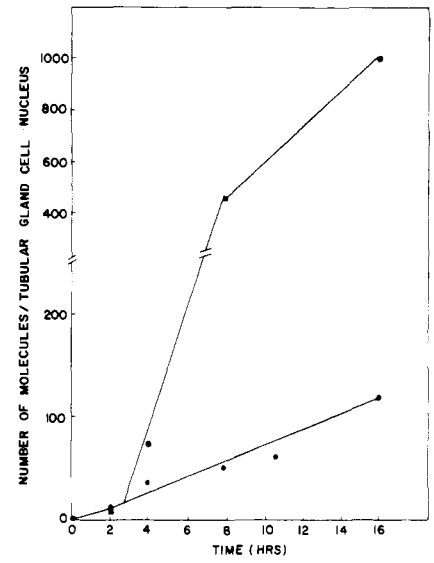

FIGURE 6: Induction of ovomucoid and ovalbumin messenger RNA sequences during secondary stimulation. Molecules of ovomucoid mRNA per tubular gland cell nucleus were calculated as described in Table I. (●) Data for accumulation of ovalbumin mRNA sequences were taken from a manuscript of Roop et al. (1978) (■).

During acute estrogen stimulation, a marked increase in the number of $mRNA_{om}$ molecules in the nucleus occurred. Within a 16-h period after secondary stimulation, it increased from 3 to 120 molecules. However, this increase is gradual and linear for a long period of time and is significantly different from that observed for the induction and accumulation of ovalbumin mRNA. As shown in Figure 6, ovalbumin and ovomucoid mRNA sequences increase at similar rates during the first phase of induction (0–4 h). Four hours after readministration of hormone, the accumulation of ovalbumin mRNA sequences increases at a rate tenfold greater than that of the earlier phase. In contrast, the concentration of ovomucoid mRNA sequences continues to increase at a rate similar to that observed during the first few hours. After 16 h, 1000 molecules of ovalbumin mRNA as compared with 120 molecules of ovomucoid mRNA are present per tubular gland cell nucleus. At steady-state conditions, ovalbumin mRNA accounts for about half of the total cellular mRNA, while ovomucoid mRNA is approximately 6–9% of the total. The difference in the rate and level of accumulation of the two gene products may be due to the following reasons. First, the rate of transcription of ovalbumin gene appears to be greater than the

ovomucoid gene after the initial phase of induction. Second, ovalbumin mRNA sequences may be stabilized by an unknown mechanism and thus degraded at a slower rate than ovomucoid RNA. In order to ascertain the relative roles of processing and transcription in controlling the expression of these two specific genes during secondary stimulation, it is necessary to examine both the transcription rates and processing rates (by pulse-chase) of the two mRNAs in isolated nuclei during different phases of acute induction. These experiments are currently underway in our laboratory.

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