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Use of a Specific Probe for Ovalbumin Messenger RNA to Quantitate Estrogen-Induced Gene Transcripts†

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ABSTRACT: DNA complementary to purified ovalbumin messenger RNA ($cDNA_{ov}$) was synthesized in vitro using RNA-directed DNA polymerase from avian myeloblastosis virus. This $cDNA_{ov}$ was then employed in hybridization assays to determine the effect of estrogen on the number of ovalbumin mRNA ($mRNA_{ov}$) molecules per tubular gland cell of the chick oviduct. The changes in $mRNA_{ov}$ were measured in immature chicks during primary stimulation, after hormone withdrawal and again following secondary stimulation of the chick oviduct with estrogen. The number of $mRNA_{ov}$ per tubular gland cell was also determined for egg-laying hen. Daily estrogen administration to the immature chick resulted in the growth of the oviduct, differentiation of epithelial cells to tubular glands, and a corresponding increase in the concentration of $mRNA_{ov}$ in the tubular gland cell from essentially zero before estrogen administration to 48,000 molecules per cell after 18 days of estrogen treatment. Upon withdrawal of estrogen from the chick, the $mRNA_{ov}$ concentration decreased to a level of 0–10 molecules/tubular gland cell after 12 days. Readministration of a single dose of estrogen to these chicks resulted in a dramatic and rapid increase in the concentration of $mRNA_{ov}$. Within 30 min, the $mRNA_{ov}$ concentration approximately doubled and by 29 hr the tubular gland cell concentration had reached 17,000 molecules. The initial transcription rate for the ovalbumin gene was 12 $mRNA_{ov}$ molecules/min. With these data, we have calculated that the half-life of the ovalbumin messenger RNA should be on the order of 40–60 hr and that the steady-state concentration of $mRNA_{ov}$ per tubular gland cell was 50,000 molecules. Similarly, each messenger RNA molecule was translated approximately 50,000 times during its lifetime in order to effect the necessary quantity of ovalbumin required for egg production. These data substantiate the hypothesis that estrogen exerts its primary action at the level of transcription to effect the synthesis of nascent mRNA molecules which in turn code for synthesis of hormone-induced proteins.

The cytodifferentiation of the chick oviduct under the influence of estrogen has proven to be an excellent system in which to study the induction of the specific messenger RNA molecule for the secretory egg-white protein ovalbumin (O'Malley and Means, 1974; Kohler et al., 1969; O'Malley et al., 1969; Oka and Schimke, 1969). When the immature chicks are given daily injections of either estradiol-17β or diethylstilbestrol, the oviducts begin to grow and differentiate into several new cell types. The predominant cell is the tubular gland cell which actively produces the major egg-white proteins.

No tubular gland cells exist in an immature chick prior to estrogen treatment. However, after approximately 20 days of daily estrogen injections this cell type comprises greater than 80% of the oviduct magnum (Kohler et al., 1969). Upon cessation of estrogen administration, the tubular gland cells become "inactive" and no longer secrete egg-white proteins. Readministration of estrogen results in the renewed synthesis of these proteins as well as their respective messenger RNAs (Rosenfeld et al., 1972; Means et al., 1972; Palmiter and Smith, 1973). Ovalbumin is the major egg-white protein and can reach concentrations as high as 60% of the total intracellular soluble protein of the oviduct in an active egg-laying hen (O'Malley et al., 1969; Harris et al., 1973).

We have previously employed viral RNA-directed DNA polymerase to transcribe a purified ovalbumin mRNA template and synthesize DNA complements of extremely high specific radioactivity (Harris et al., 1973). This radioactive complementary DNA ($[^3H]cDNA_{ov}$) can then be used as a sensitive hybridization probe to determine the gene dosage for ovalbumin in a chick oviduct cell. Our laboratory (Harris et al., 1973) as well as Sullivan et al. (1973) have recently utilized this technique to demonstrate that only one ovalbumin gene copy is present in the haploid chick genome. More importantly, this $[^3H]cDNA_{ov}$ can also be utilized as a sensitive probe in an RNA excess hybridization reaction to determine the concentration of ovalbumin messenger RNA in any given RNA population.

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In the present study the induction of $mRNA_{ov}$ during 18 days of daily estrogen injection to immature chicks (primary stimulation) was examined by hybridization with [^{3}H]$cDNA_{ov}$. In addition the $mRNA_{ov}$ concentration was determined during short-term readministration (0–30 hr) of estrogen to chicks previously stimulated by estrogen and subsequently withdrawn from hormone for 10–12 days (secondary stimulation). Finally, by comparing the messenger RNA activity of oviduct RNA assayed in a wheat germ translation system and the concentration of $mRNA_{ov}$ molecules determined by hybridization with $cDNA_{ov}$, an assessment was made of the relative sensitivities of these two assays for mRNA.

Materials and Methods

Animals. Ten-day-old white-leghorn chicks received daily subcutaneous injections of the synthetic estrogen, diethylstilbestrol (DES,[1] 2.5 mg in oil) and were killed at the indicated times. Oviducts were removed and frozen in liquid nitrogen for subsequent extraction. For experiments involving secondary stimulation with estrogen, the chicks were first treated with DES for 10 days followed by 11 days withdrawal from hormone. On the 12th day of withdrawal, chicks were given one subcutaneous injection of 2.5 mg of DES and oviducts were collected at the indicated time intervals.

Preparation of RNA. Total cell RNA was prepared from unstimulated, 4 day, 9 day, and 18 day estrogen-stimulated chick oviducts as previously described (Liarakos et al., 1973). In general the tissue was homogenized with 5 volumes of 0.01 *M* sodium acetate buffer (pH 5.0) containing 0.5% in SDS, and 5 volumes of phenol–*m*-cresol mixture. Homogenates were extracted 5 min at 65°, rapidly cooled to 0°, and then centrifuged at 12,000 rpm for 10 min. The aqueous phase was removed, adjusted to 0.5 *M* in NaCl, and reextracted as described above. The RNA was precipitated with 2 volumes of ethanol and maintained at −20° overnight. The pellet was dissolved in 0.01 *M* Tris-HCl (pH 7.0), 0.002 *M* $CaCl_2$, 0.01 *M* $Mg(C_2H_3O_2)_2$, 0.025 *M* NaCl, and 200 μg/ml of DNase I and incubated for 1 hr at 0°. Pronase (100 μg/ml) was then added and the mixture was incubated at 37° for an additional 10 min. The sample was then again treated with phenol–SDS, the aqueous phase was removed, and the RNA was precipitated with 2 volumes of ethanol. DNA oligonucleotides were removed by precipitation of the RNA from 2 *M* potassium acetate and 0.001 *M* Na_2EDTA (pH 5.2) in 25% ethanol. The final pellet was resuspended several times in ethanol, recentrifuged, lyophilized, and finally dissolved in distilled water.

In secondary stimulation experiments, oviduct total nucleic acid was prepared as described above with a few modifications. First, RNA was extracted by homogenization in 0.075 *M* NaCl–0.025 *M* Na_2EDTA (pH 8.0) containing 0.5% SDS and an equal volume of phenol. Secondly, the DNA was physically removed by adding carefully two volumes of ethanol to the 0.5 *M* NaCl solution containing the nucleic acid. The DNA was then spooled onto a glass rod. The RNA was precipitated, washed with ethanol to remove residual phenol, and finally dissolved in distilled water.

Preparation of Nitrocellulose-Adsorbable RNA. Each RNA preparation was adsorbed to nitrocellulose filters as previously described (Rosenfeld et al., 1972; Means et al., 1972) except that only 2 mg of RNA was applied to each 25-mm HAWP Millipore filter. After elution of the filter-bound RNA with a buffer containing 0.5% SDS and 0.1 *M* Tris-HCl (pH 9.0), the solution was adjusted to 0.5 *M* KCl and cooled at 4° for 30 min to precipitate SDS, and the SDS was removed by centrifugation. The RNA was then precipitated with two volumes of ethanol, resuspended in ethanol, recentrifuged, dried, and finally dissolved in distilled water.

Translation Assay. The wheat germ translation system of Roberts and Paterson (1973) was used to test the RNA samples for functional $mRNA_{ov}$. The 30,000*g* supernatant fluid (S-30) was prepared essentially as described except that the wheat germ was ground before adding buffer and the S-30 was not preincubated. The preparation was stored as 50 μl of frozen beads in liquid N_2.

The assay system for protein synthesis contained in a 100-μl final volume: 20 μl of wheat-germ S-30, 24 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes, pH 7.6), 2 m*M* dithiothreitol, 1 m*M* ATP, 20 μ*M* GTP, 8 m*M* creatine phosphate, 40 μg/ml of creatine phosphokinase, 20 μ*M* each of the unlabeled amino acids, 84 m*M* KCl, 2.5 m*M* $Mg(C_2H_3O_2)_2$, and 1 μ*M* [^{35}S]methionine (100 Ci/mmol). Aliquots of RNA (4–10 μg) prepared from oviducts of chicks that received estrogen for various periods of time were added to the assay and the tubes were incubated for 120 min at 25°. A 5-μl aliquot was removed to test for total radioactivity incorporated into protein while the remaining 95 μl was used to determine the amount of ovalbumin synthesis by precipitation with specific antibody to ovalbumin (Means et al., 1972).

AMV RNA-Directed DNA Polymerase. Avian myeloblastosis virus was purified from infected chick plasma as described (Harris et al., 1973). The RNA-directed DNA polymerase was prepared by the method of Kacian et al. (1971) and as described previously (Harris et al., 1973). Recently, purified enzyme has been the generous gift of Neil Burnett and William M. Mitchell of Vanderbilt University Medical School, Nashville, Tenn.

Purification of Ovalbumin mRNA. The purified $mRNA_{ov}$ was prepared from active egg-laying hens (Rosen et al., 1975). Briefly, the poly(A)-containing mRNA present in a total nucleic acid extract of oviduct tissue was adsorbed to Millipore filters by the usual procedure (Rosenfeld et al., 1972), and the RNA eluted from the filter with 0.5% SDS was then passed over a Sepharose 4B column (90 × 1.5 cm), in 0.1 *M* NaOAc (pH 5.0) buffer containing 1 m*M* EDTA. The RNA eluting just prior to the 18S rRNA peak, but following the DNA peak, was collected and assayed in the wheat-germ translation system. The $mRNA_{ov}$ activity at this stage in the purification was increased >35-fold over that of the original total nucleic acid extract as assayed in the wheat germ system. Next, the Millipore filter technique was repeated which resulted in removal of 20–30% of the remaining rRNA in the preparation. The mRNA was then subjected to preparative agarose-urea gel electrophoresis and a highly purified messenger RNA for ovalbumin was obtained (Rosen et al., 1975). This RNA was assayed for ovalbumin mRNA activity in the wheat-germ translation system and the sole detectable product was demonstrated to be ovalbumin (Rosen et al., 1975). Furthermore, when this material was analyzed on either analytical acid–urea agarose gels or polyacrylamide gels in the presence of 99% formamide, only one demonstrable band

[1] Abbreviations used are: DES, diethylstilbestrol; SDS, sodium dodecylsulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

was observed. This single band migrated to 21 S. Finally, when the 21S preparative gel RNA was centrifuged through analytical formamide–sucrose gradients, a single optical density peak was obtained (Rosen et al., 1975).

Synthesis and Purification of [^{3}H]cDNA to mRNA$_{ov}$. The tritiated DNA complementary to ovalbumin mRNA ([^{3}H]cDNA) was synthesized as previously described (Harris et al., 1973). The reaction mixture contained in a final volume of 1 ml: 50 m*M* Tris-HCl (pH 8.1), 10 m*M* dithiothreitol, 6 m*M* $MgCl_2$, 30 m*M* KCl, 30 μg/ml of actinomycin D, 18 μM [^{3}H]dGTP, 25 μM [^{3}H]dATP, 12.5 μM [^{3}H]dCTP, and 1.5 m*M* TTP. Also included in the reactions were: 10–20 μg of mRNA$_{ov}$, 1 μg of oligo-dT$_{12-18}$, and AMV RNA-directed DNA polymerase. Under these conditions this [^{3}H]cDNA$_{ov}$ had a specific radioactivity of approximately 3.5×10^7 cpm/μg.

The reaction was carried out at 20° for 60 min and then adjusted to an EDTA concentration of 2 m*M*. Sheared *Escherichia coli* DNA (400 nucleotides in length) was added as a carrier at a concentration of 100 μg/ml; the solution was adjusted to 0.5% SDS, and the entire sample was placed on a Sephadex G-50 column (80 × 1.5 cm) equilibrated with 0.1 *M* NaCl–2 m*M* EDTA. The excluded fraction was collected and precipitated with 2 volumes of ethanol. The precipitate was then dissolved in 0.1 *N* NaOH–10 m*M* EDTA (3 ml) and incubated at 66° for 2 hr to hydrolyze the RNA. The solution was neutralized with 5 *N* HCl and the [^{3}H]cDNA and *E. coli* DNA were precipitated with 2 volumes of ethanol overnight at −20°. The final precipitate was dried and dissolved in cold distilled water to give a concentration in which 10 μl represented about 2000 cpm or about 60 pg of [^{3}H]cDNA and 4–10 μg of *E. coli* DNA.

Purification of S$_1$ Nuclease. Single strand specific nuclease (S$_1$) was purified from *Aspergillus oryzae* powder (a crude α-amylase, Taka-Diastase, from Parke-Davis) by the method of Hutton and Wetmur (1973). A 30-g sample of Taka-Diastase was suspended in 200 ml of cold distilled water and 105 g of ammonium sulfate (Ultra-pure, Mann) was slowly added. The precipitate was then removed by centrifugation. An additional 30 g of ammonium sulfate was added to the supernatant fluid. The precipitate was collected by centrifugation, dissolved in 10–15 ml of distilled water, and dialyzed overnight against 2 l. of 0.01 *M* sodium phosphate buffer (pH 6.2).

This protein solution was applied to a DEAE-cellulose (DE-52, microgranular, 2.5 × 15 cm) column which had been previously equilibrated with 0.5 *M* sodium phosphate buffer (pH 6.2). A linear salt gradient from 0.1 *M* NaCl to 0.3 *M* NaCl (in 10 m*M* phosphate buffer, pH 6.2) was then applied to the column. The major protein peak eluted at approximately 0.20 *M* NaCl while the S$_1$ nuclease activity eluted at approximately 0.28 *M* NaCl. The active fractions were combined, divided into 2-ml aliquots, and stored in liquid nitrogen.

RNA Excess Hybridization to [^{3}H]cDNA$_{ov}$. RNA excess hybridization experiments with the [^{3}H]cDNA$_{ov}$ were carried out in sealed sterile plastic disposable tubes. During the incubation, the tubes were submerged to prevent condensation. The hybridization reaction was performed in a final 50 μl volume as follows: 0.6 *M* NaCl, 0.01 *M* Tris-HCl (pH 7.0) at 25°, 0.002 *M* EDTA, approximately 1 ng/ml of [^{3}H]cDNA$_{ov}$ (10 μl), and RNA samples were added to the reaction in a concentration range of 0.01–10,000 μg/ml, depending on the RNA preparation being tested. All samples contained 400 μg/ml of rat mammary RNA as a heterologous RNA control. Two to four samples per experiment were assayed with no added oviduct RNA for determination of the total radioactivity that was trichloroacetic acid precipitable prior to treatment with S$_1$ nuclease and the total radioactivity remaining after S$_1$ nuclease treatment. Generally 5–10% of the total radioactivity remained after treatment. Samples were initially placed in a boiling water bath for 30 sec and then incubated as described above at 66° for time intervals ranging from 0.5 to 70 hr, depending on the RNA preparation being tested (e.g., highly purified mRNA$_{ov}$, 0.5 hr; RNA from withdrawn oviduct, 70 hr). Separate controls were utilized when extremely high RNA concentrations (5–10 mg/ml) were used. For instance, after the hybridization incubation was carried out in the absence of oviduct RNA but with 400 μg/ml of mammary gland RNA, oviduct RNA (from withdrawn chicks) was added to the sample to adjust the concentration to 5–10 mg/ml. Generally, the background (i.e., percent of the cDNA not hydrolyzed) would rise to 15–20% due to the fact that high concentrations of RNA inhibited the hydrolysis of cDNA by S$_1$ nuclease.

Assay of Hybrid with S$_1$ Nuclease. After hybridization incubation, the samples were removed, rapidly frozen in Dry Ice–2-propanol, and stored at −20°. All the samples for a given experiment were treated at the same time with the single-stranded S$_1$ nuclease. A final volume of 0.4 ml contained approximately 100–150 μg of S$_1$ nuclease protein (DEAE-cellulose enzyme), 0.2 *M* NaOAc (pH 4.5), 0.4 *M* NaCl, 0.001 *M* $ZnCl_2$, and the hybrid sample or the controls as mentioned above. After incubation at 30° for 2 hr, the samples were placed in ice and 2.5 ml of 10% Cl_3CCOOH was added to each tube. They were allowed to stand in ice for 30 min and then collected on Millipore filters. The filters were dried and counted in a liquid scintillation counter. It was noticed that if S$_1$ nuclease digestion was performed at 45° rather than 30°, the $Cr_0t_{1/2}$ of highly purified mRNA$_{ov}$ increased but all of the rates for the other RNA preparations were also increased proportionately. Thus, it was decided to use the lower temperature for S$_1$ digestion since at the higher temperature it was possible that the potentially short hybrids were less stable and/or other nucleases which might contaminate the S$_1$ preparation were more active.

The data were expressed as the percent of the [^{3}H]cDNA$_{ov}$ which was resistant to the S$_1$ nuclease treatment (hybrid) vs. the product of the RNA sample concentration × time (Cr_0t). All data are reported as the equivalent Cr_0t one would observe if the reactions are carried out in 0.12 *M* phosphate buffer rather than 0.6 *M* NaCl (Britten and Smith, 1969).

Results

Induction of Ovalbumin mRNA during Chronic Administration of Estrogen. The quantitation of mRNA$_{ov}$ in oviduct tissue during chronic estrogen treatment is presented in Figure 1. This figure shows that [^{3}H]cDNA can serve as an effective probe for determining a highly accurate estimate of the concentration of mRNA$_{ov}$ in most RNA preparations. It should be noted that 80% or more reaction was usually achieved and most curves were parallel within a given experiment. However, in the case of RNA preparations with extremely low concentrations of RNA sequences complementary to the cDNA$_{ov}$ (i.e., oviduct RNA from unstimulated or estrogen withdrawn chicks), the hybridization

Table I: Induction of $mRNA_{ov}$ during Chronic Estrogen Administration.

Hormonal State	$Cr_0t_{1/2}$	Fraction $mRNA_{ov} \times 10^3$ [c]	RNA/DNA [a]	Tubular Gland Cells [d]/ Total Cells	$mRNA_{ov}$ (pg)/ Tubular Gland Cell $\times 10^2$	No. Molecules of $mRNA_{ov}$/ Tubular Gland Cell	No. Molecules of Ovalbumin [b]/ No. Molecules of $mRNA_{ov}$
Unstimulated	$\sim 3 \times 10^5$	0.00007	1.1	0			
4 Days × DES	4×10^1	0.5	3.2	0.34	1.2	20,000	6,400
9 Days × DES	1.3×10^1	1.5	3.5	0.50	2.7	44,000	38,000
18 Days × DES	7.2×10^0	2.7	3.6	0.85	3.0	48,000	52,000
Hen-laying	3.1×10^0	6.4	4.9	0.90	9.1	147,000	
30 Days withdrawn	1.2×10^5	0.00016	1.5	0.15	0.00042	7	

[a] From Yu et al. (1971) and Oka and Schimke (1969). [b] The concentration of ovalbumin was calculated from the data of Comstock et al. (1972). [c] The $Cr_0t_{1/2}$ of pure $mRNA_{ov}$ is 2.0×10^{-2} under our assay conditions and the fraction $mRNA_{ov} = Cr_0t_{1/2}$ pure message/$Cr_0t_{1/2}$ given RNA. [d] From Kohler et al. (1969) and Palmiter and Wrenn (1971).

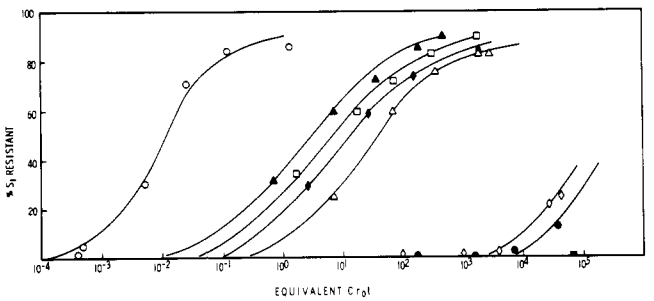

FIGURE 1: Hybridization kinetic curves ("Cr_0t" curves) of total chick oviduct RNA with $[^3H]cDNA_{ov}$. The RNA samples were prepared from oviduct tissue during primary stimulation with estrogen. RNA was extracted from unstimulated (●), 4-day DES treatment (△), 9-day DES treatment (♦), 18-day DES treatment (□), hen oviduct (▲), and 30 days DES withdrawn chick oviducts (◇). The "Cr_0t" curve for pure ovalbumin is also included for comparison (○).

reactions do not go to completion and only an estimate can be made for the $Cr_0t_{1/2}$. Nevertheless, the reaction observed at a very high Cr_0t (estimated $Cr_0t_{1/2} = 3 \times 10^5$) with RNA prepared from oviducts of unstimulated 30-day-old chicks is real since chick liver RNA shows no reaction with $cDNA_{ov}$ up to a Cr_0t of 7×10^4. This amount of reaction with the unstimulated chick oviduct RNA represents much less that one $mRNA_{ov}$ molecule per cell and could be attributed to one oviduct out of a pool of several hundred oviducts having been contaminated with a small amount of estrogen. Furthermore, since it is known that ovalbumin is synthesized in tubular gland cells (O'Malley et al., 1969; Kohler et al., 1969) and these cells are not present in the immature chick oviduct prior to estrogen administration, one would not expect to detect sequences of $mRNA_{ov}$ in unstimulated chicks.

Highly purified $mRNA_{ov}$ reacts, under our conditions, with an equivalent $Cr_0t_{1/2}$ of 2.0×10^{-2} mol of nucleotides/mol/l.-time in seconds) (the actual $Cr_0t_{1/2}$ under 0.6 *M* NaCl was 4×10^{-3}). This is somewhat higher than one would theoretically predict on the basis of the known complexity of the $mRNA_{ov}$ (1161 nucleotides for the coding part). This observation can be explained by several possibilities. The molecular weight, as determined by formamide sucrose gradient, is 500,000 or about 1600 nucleotides. However, the true complexity of the $mRNA_{ov}$ may actually be higher since the molecular weight determinations using acid–urea acrylamide gel or 100% neutral-formamide electrophoresis indicate a theoretical complexity of almost 2600 nucleotides (assuming all of the $mRNA_{ov}$ has no repetitive sequences). It is also possible that the small size of the $[^3H]cDNA$ influences the rate of the hybridization reaction (Hayes et al., 1970) and/or the reaction with S_1 nuclease. Oviduct RNA prepared from chicks pretreated and then withdrawn from estrogen reacted at a rate only slightly faster than RNA from unstimulated chicks. This indicates that removal of estrogen from chicks results in both marked diminution of $mRNA_{ov}$ synthesis as well as nearly complete destruction of existing $mRNA_{ov}$. Also, included in Figure 1 is a curve for RNA prepared from an egg-laying hen which was under constant endogenous stimulation by estrogen; this RNA preparation reacted rapidly with a $Cr_0t_{1/2}$ of 3. Moreover, as the chicks are exposed to estrogen for prolonged periods of time (4, 9, and 18 days), the rate of the reaction of the oviduct RNA with $[^3H]cDNA_{ov}$ increased. This indicated a higher concentration of $mRNA_{ov}$ sequences in those preparations (i.e., kinetic rate constant $\sim 1/Cr_0t_{1/2}$).

Using these data, the number of $mRNA_{ov}$ molecules per tubular gland cell were calculated. Explicit in this calculation are the following assumptions: first, that the messenger RNA_{ov} standard is highly purified $mRNA_{ov}$; second, that the molecular weight of the coding part of message can be determined; third, that a reasonable estimate of the proportion of the oviduct cells which are tubular gland cells is available (Palmiter and Wrenn, 1971; Palmiter, 1973); and finally, that we have an estimate of the RNA/DNA ratio of the tubular gland cell (Yu et al., 1971; Oka and Schimke, 1969). In the 18-day DES-treated animals, the tubular gland cells represent over 80% of the total cells in the oviduct. Upon withdrawal of estrogen from the chick, the RNA/DNA ratio of the oviduct drops from 3.5 to 1.5 (Table I). This drop in RNA/DNA ratio for the total oviduct primarily represents a drop in the RNA/DNA ratio of the tubular gland cell since there is only a 10–20% drop in the total number of cells as measured by DNA content. Thus, the total RNA/DNA ratio is used as an index of the RNA/DNA ratio of the tubular gland cell in the chick oviduct. As mentioned in Materials and Methods, $mRNA_{ov}$ was purified by preparative agarose gel electrophoresis, and we estimate that the purity of the $mRNA_{ov}$ approaches homogeneity (Rosen et al., 1975; Woo et al., 1975). For calculating the number of mRNA molecules, the value of 1200 nucleotides is used since this amount is sufficient to completely code for the amino acid sequence of ovalbumin plus a short poly(A) addition to the 3′-end and since we are comparing in vitro translation data with data obtained by hybridization of $[^3H]cDNA_{ov}$ to RNA. Recent evidence in our laboratory indicates the actual molecular weight to be 520,000 to 650,000 or 1600 to 1900 nucleotides (Woo et al.,

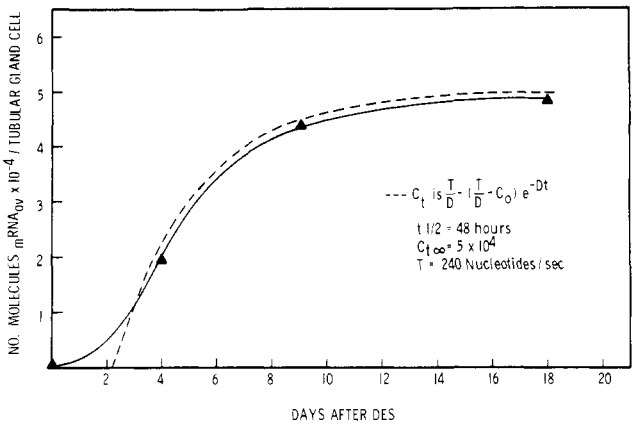

FIGURE 2: Ovalbumin mRNA accumulation in tubular gland cells during primary stimulation with estrogen (▲). (- - -) Theoretical accumulation curve of the number of ovalbumin mRNA molecules per tubular gland cell assuming a constant rate of degradation ($t_{1/2}$ = 48 hr) and a steady-state level of 50,000 molecules.

1975). However, until the amount, if any, of repetitive sequences in the mRNA$_{ov}$ is established, we will use the lower value of 1200 nucleotides for the complexity of mRNA$_{ov}$.[3]

Table I contains the results of the calculations of the number of molecules of mRNA$_{ov}$ per tubular gland cell during chronic estrogen treatment. From the "*Cr*$_0$*t*" analysis, the fraction (or percent) of a given total RNA preparation which is mRNA$_{ov}$ was obtained. Then, from the RNA/DNA ratio, the pg of mRNA$_{ov}$ per cell (2.6 of DNA per cell) was calculated. Next from the mass of mRNA$_{ov}$ per cell, the number of molecules of mRNA$_{ov}$ per cell was determined. Finally, since only the tubular gland cells are producing ovalbumin (Kohler et al., 1969), a correction for the fraction of oviduct cell which are tubular gland cells at various stages of estrogen stimulation was made to arrive at the number of mRNA$_{ov}$ sequences per tubular gland cell.

The concentration of mRNA$_{ov}$ per tubular gland cell increased from barely detectable levels prior to estrogen stimulation to almost 20,000 molecules per cell by 4 days of continuous estrogen adminstration. After 9 days of estrogen administration, the concentration of mRNA$_{ov}$/tubular gland cell more than doubled with respect to the 4-day value and by 18 days a steady-state concentration of about 50,000 mRNA$_{ov}$ molecules/tubular gland cell had been reached. At this stage of oviduct development, the concentration of mRNA$_{ov}$ amounted to 0.3% of the total RNA in a tubular gland cell. In the hen, in which a constant physiologic stimulation by ovarian estrogen occurs and in which other hormones such as testosterone and progesterone play a role in maintenance of egg-white protein synthesis (Palmiter and Haines, 1973), the highest concentration of mRNA$_{ov}$ molecules per tubular cell was found. The 150,000 molecules of mRNA$_{ov}$ accounts for approximately 0.7% of the total RNA found in each tubular gland cell. Finally, upon withdrawal of estrogen, the level of mRNA$_{ov}$ in oviduct decreased from some 50,000 molecules to a level of 0-10 molecules/tubular gland cell within 10-30 days.

The time course for mRNA$_{ov}$ accumulation is a function of both its intrinsic rate of synthesis and its degradation rate, which can be expressed by the following equation (Kafatos, 1972)

$$dc/dt = T - CD \quad (1)$$

where C is the concentration of mRNA in molecules/tubular gland cell; T is the *transcription* rate in molecules of mRNA$_{ov}$/tubular gland cell/sec; and D is the first-order mRNA degradation constant. D is related to the half-life ($t_{1/2}$) in seconds of the message by the equation (Palmiter, 1973; Kafatos, 1972)

$$D = \ln 2/t_{1/2} \quad (2)$$

When the oviduct reaches a steady-state condition with respect to mRNA$_{ov}$ concentration (i.e., no net increase in mRNA$_{ov}$ molecules per tubular gland cell) this state can be expressed as

$$Ct_{ov\infty} = T/D \quad (3)$$

where $Ct_{ov\infty}$ equals the concentration of mRNA$_{ov}$ molecules at steady-state.

The data from Table I is replotted in Figure 2 and a theoretical mRNA$_{ov}$ accumulation (C_t) curve is presented. It was assumed that it required 2-3 days of continuous estrogen stimulation to establish a population of differentiated tubular gland cells stable in the production of mRNA$_{ov}$ (Kohler et al., 1969; Comstock et al., 1972; Palmiter and Wrenn, 1971). The mRNA$_{ov}$ accumulation curve and the equation representing this curve, as indicated on Figure 2, were derived from the integral form of eq 1 (Kafatos, 1972). It could thus be calculated that the theoretical half-life for this ovalbumin mRNA should be about 50 hr (C_0 = initial concentration of mRNA = 0) and that the transcription rate for this single copy gene was on the order of 0.2 molecule of mRNA$_{ov}$ per sec per diploid cell DNA or 240 nucleotides per sec per diploid concentration of ovalbumin gene (12 molecules/min). If both ovalbumin genes are expressed during estrogen stimulation, the transcription rate for the coding part of the message would be 120 nucleotides per sec per ovalbumin gene. The calculated steady-state concentration of mRNA$_{ov}$ in the tubular gland cell of the chick oviduct under the influence of only estrogen would be 50,000 and was quite close to the value observed (48,100) after 18 days of continuous estrogen administration (Figure 2).

In the hen, the presumed steady-state level is some three times higher than that observed in the estrogen-stimulated chick and the transcription rate (T) was about 300-400 nucleotides per sec per ovalbumin gene or 0.6 molecule per sec per tubular gland cell (36 molecules/min). Thus

$$T = Ct_{ov}D = (147{,}000)\frac{\ln 2}{1.73 \times 10^5 \text{ sec}}$$
$$= (147{,}000)(0.4 \times 10^{-5} \text{ 1/sec})$$
$$= 0.59 \text{ mRNA}_{ov} \text{ molecules/sec}$$

Induction of Ovalbumin mRNA during Acute Estrogen Stimulation. Since removal of estrogen from a fully stimulated chick resulted in a dramatic decrease in the concentration of mRNA$_{ov}$ molecules (from 50,000 to 0-10 molecules per cell), it was of interest to study the temporal aspects of secondary stimulation with estrogen relative to mRNA$_{ov}$ accumulation. Chicks withdrawn from hormone

[3] If one uses the molecular weight of 650,000 and a complexity of 1900 nucleotides for the ovalbumin messenger RNA, then the calculated number of molecules of ovalbumin messenger RNA per tubular gland cell would be about 35% lower. Thus, the steady-state level found in the estrogen-stimulated chick would be 32,500 rather than 50,000. Taking this into consideration, the upper and lower limits are established for the calculated number of mRNA$_{ov}$ molecules. However, this variance does not appreciably alter the interpretation given to these estimates.

Table II: Recovery of $mRNA_{OV}$ Sequences upon Millipore Filtration of Total Oviduct RNA.

RNA	$Cr_0t_{1/2}$	% of Total RNA	% Recovery of $mRNA_{OV}$ Sequences[a]
12 day withdrawn plus 1 hr estrogen			
(a) Total	3.5×10^4	100	
(b) Millipore filter bound	6.0×10^2	0.9	52.5
12 day withdrawn plus 18 hr estrogen			
(a) Total	5.0×10^1	100	
(b) Millipore filter bound	1.3×10^0	1.5	57.7

[a] The % recovery of $mRNA_{OV}$ sequences is calculated as follows: % of total RNA Millipore filter bound $\times$ $Cr_0t_{1/2}$ of total RNA/$Cr_0t_{1/2}$ of the Millipore filtered RNA.

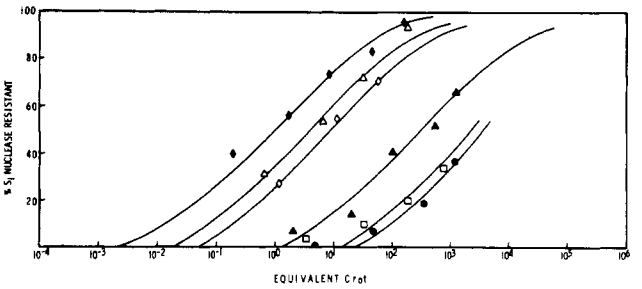

FIGURE 3: Hybridization kinetic curves ("Cr_0t" curves) of Millipore filtered RNA with [^{3}H]$cDNA_{ov}$. The RNA samples were prepared from oviduct tissue during secondary stimulation with estrogen (single injection). The RNA was extracted and then Millipore filtered from 10 day estrogen withdrawn (●), 30-min DES treated (□), 1-hr DES treated (▲), 4-hr DES treated (◇), 8-hr DES treated (△), and 29-hr DES treated chick oviducts (♦).

for 11 days were given a single injection of diethylstilbestrol, oviducts were removed at subsequent intervals and total RNA was extracted and purified as detailed in Materials and Methods.

Figure 3 shows the "Cr_0t" analysis, using [^{3}H]$cDNA_{ov}$ and RNA excess hybridization, of Millipore-filtered oviduct RNA isolated from chicks which received a single injection of estrogen for 30 min, 1 hr, 4 hr, 8 hr, 18 hr, and 29 hr. An increase in the rate of hybridization of [^{3}H]$cDNA_{ov}$ to RNA prepared from oviducts which were exposed to estrogen for only 30 min could be detected by this procedure. By 1 hr of estrogen stimulation, the $Cr_0t_{1/2}$ of the RNA preparation had almost decreased by an order of magnitude (i.e., a five- to tenfold *increase* in the concentration of $mRNA_{ov}$ sequences after 1 hr of exposure to estrogen). At 4 hr, the concentration of $mRNA_{ov}$ sequences had increased 30-fold over that found at 1 hr of estrogen. Therefore, within 4 hr after estrogen administration, a 600-fold increase in $mRNA_{ov}$ sequences was observed. Finally, the RNA from oviducts stimulated with estrogen for 29 hr reacted with a $Cr_0t_{1/2}$ = 1.0, indicating another fourfold increase in $mRNA_{ov}$ sequences over that noted at 4 hr. Thus, estrogen caused almost a 4000-fold increase in a specific oviduct transcript within 29 hr following a single injection.

Since Millipore-filtered RNA was used in these acute induction experiments in order to permit greater sensitivity in detection of $mRNA_{ov}$ sequences during the early time points, there was some question whether the hormonal state of the animal could effect the recovery of $mRNA_{ov}$ sequences on the filter. Normally, the recovery of $mRNA_{ov}$ is estimated by the in vitro translation activity described under Materials and Methods. With hen RNA, a 30 to 50% recovery of $mRNA_{ov}$ *activity* is obtained when overloading is avoided. Alternatively, the $cDNA_{ov}$ can be used to estimate the recovery of $mRNA_{ov}$ sequences. In Table II, the $Cr_0t_{1/2}$ of the total RNA and the Millipore-filtered RNA are presented, along with the actual mass of RNA recovered for RNA preparations from 1-hr and 18-hr estrogen-stimulated chick oviducts. From both translational assay and hybridization assay, the recovery of $mRNA_{ov}$ is estimated to be about 50%. Thus the recovery of $mRNA_{ov}$ sequences upon Millipore filtration is not a function of the hormonal state of the animal.

Table III contains the information needed to calculate the approximate number of $mRNA_{ov}$ molecules per tubular gland cell during these acute induction experiments. A correction for the amount of RNA collected on the Millipore filters and the recovery of $mRNA_{ov}$ is included in this calculation. As before, the RNA/DNA ratio was used to calculate the pg of $mRNA_{ov}$/tubular gland cell. From this value, the number of molecules of $mRNA_{ov}$/cell was determined. An increase of >15,000 molecules/cell was noted at ~24 hr following a single estrogen injection.

Using the data of Table III for 4–29-hr stimulation, a

Table III: Induction of $mRNA_{OV}$ during Acute Estrogen Administration.

Hormonal State	$Cr_0t_{1/2}$	[1] $mRNA_{ov}$[a]/ Total Millipore Filtered RNA $\times 10^3$	[2] Total Millipore Filtered RNA/Total Oviduct RNA	[3] Recovery of $mRNA_{ov}$ Activity upon Millipore Filtration[e]	[4] RNA/ DNA[b]	[5] Tubular Gland Cells[c]/ Total Oviduct Cells	$mRNA_{ov}$ (pg)/ Tubular Gland Cell $\times 10^2$	No. Molecules $mRNA_{ov}$[d]/ Tubular Gland Cell
Withdrawn	3.7×10^3	0.0054	0.009	0.5	1.5	0.15	0.00025	0–4
0.5 hr × DES	2.0×10^3	0.010	0.011	0.5	1.5	0.15	0.00056	9
1.0 hr × DES	3.0×10^2	0.066	0.009	0.5	1.5	0.15	0.003	50
4.0 hr × DES	9.0×10^0	2.2	0.012	0.5	1.6	0.15	0.14	2,300
8.0 hr × DES	$4\ 0 \times 10^0$	5.0	0.014	0.5	1.75	0.20	0.31	5,100
29.0 hr × DES	9.5×10^{-1}	20	0.014	0.5	1.85	0.25	1.0	17,000

[a] $Cr_0t_{1/2}$ for pure $mRNA_{OV}$ = 2.0×10^{-2} under our assay conditions and fraction of $mRNA_{OV}$ = $Cr_0t_{1/2}$ pure message/$Cr_0t_{1/2}$ given RNA. [b] From Yu et al. (1971) and Oka and Schimke (1969). [c] From Palmiter (1973). [d] No. molecules = 2.6 pg of DNA/cell × ([1] [2] [4]/[3] [5]) × K; where K converts pg of $mRNA_{OV}$ to molecules (1.62×10^6) $mRNA_{OV}$. [e] Assayed by translation activity in the in vitro wheat germ translation system (see text).

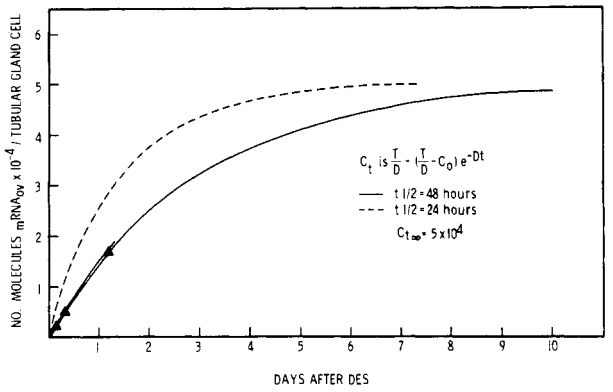

FIGURE 4: Ovalbumin mRNA accumulation in tubular gland cells during the first 29 hr of secondary stimulation with a single injection of estrogen (▲). (—) *Theoretical* accumulation curve of the number of ovalbumin mRNA molecules per tubular gland cell assuming a constant rate of degradation ($t_{1/2}$ = 48 hr) and a steady-state level of 50,000 molecules. Also included in this figure is the theoretical accumulation curve for ovalbumin mRNA assuming the same steady-state level of 50,000 molecules per tubular gland cell but a constant degradation rate where $t_{1/2}$ = 24 hr (- - -).

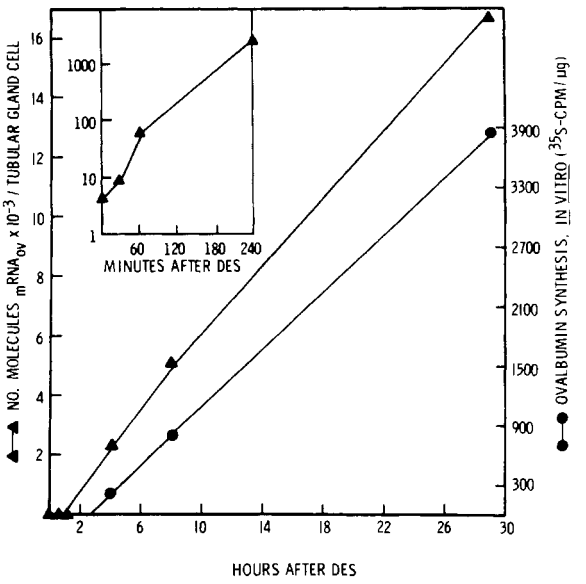

FIGURE 5: Comparison of the amount of mRNA$_{ov}$ sequences as measured by [^{3}H]cDNA$_{ov}$ hybridization and the actual biological activity, as measured by the in vitro wheat germ translation assay system during secondary stimulation with estrogen. (▲—▲) Number of mRNA sequences as measured by [^{3}H]cDNA$_{ov}$ hybridization; (●—●) ovalbumin synthesis as measured by [^{35}S]methionine incorporation into specific immunoprecipitable ovalbumin in the in vitro wheat germ system. Insert: (▲—▲) The early rise after a single injection of estrogen to withdrawn chicks in the hybridizable sequences to the [^{3}H]cDNA$_{ov}$—before translation activity can be detected.

theoretical accumulation curve for secondary stimulation by estrogen was generated as shown in Figure 4. If it is assumed that there was very little degradation of mRNA$_{ov}$ between 4 and 8 hr, an estimate of the initial rate of accumulation can be obtained. The number of mRNA$_{ov}$ molecules/tubular gland cell increased from 2300 to 5100 during this 4-hr period. This indicates that the initial transcription rate (T) was roughly 0.195 molecule/sec. (234 nucleotides/sec; 12 molecules/min). Between 1 and 4 hr, the initial rate was calculated to be 12.6 molecules/min while the rate was 8 molecules/min between 4 and 29 hr of estrogen stimulation (uncorrected for $t_{1/2}$ of mRNA$_{ov}$).

The same theoretical accumulation curve used for analysis of primary induction also is plotted in Figure 4 assuming a $t_{1/2}$ = 48 hr. Here no lag time was included since there was no time required for cytodifferentiation during secondary stimulation. The theoretical accumulation curve, assuming a $t_{1/2}$ for mRNA$_{ov}$ molecule of 24 hr is also included (dashed line). It is obvious that the data best fit the projected $t_{1/2}$ for mRNA$_{ov}$ of 48 hr.

Comparison of the Number of mRNA$_{ov}$ Molecules Quantitated with [^{3}H]cDNA$_{ov}$ vs. mRNA$_{ov}$ Activity Assayed in the Wheat Germ Translation System. As shown in Figure 5, the first mRNA$_{ov}$ activity observed in the wheat germ assay system (see Materials and Methods) was detected in the RNA isolated from oviducts of chicks treated with estrogen for 4 hr. Between 4 and 29 hr of hormone stimulation there was an almost linear increase in the concentration of biologically active (translatable) mRNA$_{ov}$ assayed in the protein synthesis system. These data are then plotted together with the concentration of mRNA$_{ov}$ sequences present in the same RNA preparations but quantitated as determined by RNA-excess hybridization with [^{3}H]cDNA$_{ov}$. It can be seen that there was an excellent correlation between the rate of increase of sequence concentration and biological mRNA$_{ov}$ activity. This indicated that the major rate-limiting step in the induction of ovalbumin synthesis in the chick oviduct by estrogen was the production of new biologically active mRNA$_{ov}$ transcripts, a long held hypothesis which now seems to be unequivocally true for this system (O'Malley et al., 1969; O'Malley and Means, 1974).

In Figure 5, a log plot of the accumulation of mRNA$_{ov}$ sequences during the first 4 hr of hormone treatment is presented as an insert. It is apparent that new sequences of mRNA$_{ov}$ could be detected by [^{3}H]cDNA$_{ov}$ well in advance of the mRNA activity detected by the wheat germ translation system. The concentration of mRNA$_{ov}$ must reach a level of about 1000 molecules per tubular gland cell to detect translation of mRNA$_{ov}$ in the wheat germ assay even using [^{35}S]methionine, which is the most sensitive in vitro isotope for protein synthesis. On the other hand, the [^{3}H]cDNA$_{ov}$ was at least 1000 times more sensitive as a probe for estimating the relative concentration of mRNA$_{ov}$ when compared to this in vitro translation assay.

Discussion

A ^{3}H-complementary DNA to pure ovalbumin mRNA has been employed as a specific probe to determine the concentration of mRNA$_{ov}$ sequences in the chick oviduct during primary stimulation with estrogen, withdrawal from hormone, and secondary stimulation with estrogen. The intracellular concentration of mRNA$_{ov}$ has also been determined for the egg-laying hen. Finally, the mRNA$_{ov}$ translation activity of RNA preparations extracted from oviducts after a single dose of estrogen (secondary stimulation) and the concentration of mRNA$_{ov}$ sequences as determined by [^{3}H]cDNA$_{ov}$-RNA hybridization have been compared. An excellent correlation between biologically active mRNA and presence of mRNA$_{ov}$ sequences has been established.

The use of [^{3}H]cDNA$_{ov}$ is an extremely sensitive method for detecting the presence of mRNA$_{ov}$ sequences in any population of RNA molecules. This method has allowed a lower limit to be set on the number of mRNA$_{ov}$ molecules found in the estrogen-deprived immature chick. The sensitivity of the RNA excess hybridization technique will accurately detect as few as one mRNA$_{ov}$ sequence in 20 cells. It has previously been demonstrated that only tubular gland

cells produce ovalbumin. Since cytological evidence indicates the immature chick oviduct has no tubular gland cells, the low concentration of $mRNA_{ov}$ sequences in the immature chick (0–4 $mRNA_{ov}$/cell) may be attributed either to spontaneous induction of $mRNA_{ov}$ in a few chicks or to a possible accidental exposure of even one chick per 1000 to exogenous estrogen.

In our experiments, the number of $mRNA_{ov}$ molecules per cell increased from essentially zero in the unstimulated oviduct to a steady-state concentration of 50,000/tubular gland cell after approximately 20 days of continuous estrogen administration. Within 9 days the level of $mRNA_{ov}$ per cell was almost 90% of the steady-state level, and between 9 and 18 days of stimulation, estrogen acts to both increase the total number of tubular gland cells as well as maintain the secretory function of preexisting cells. When estrogen was withdrawn from pretreated chicks, the oviduct decreased in weight from about 1.5 to 0.4 g, the RNA/DNA ratio dropped by a factor of 2–3 as active ovalbumin-synthesizing polysomes were disassembled, and the number of tubular gland cells appeared to decrease[2] (Kohler et al., 1969; Oka and Schimke, 1969). During this period of hormone withdrawal, the concentration of $mRNA_{ov}$ decreased from 50,000 molecules to 0–7 molecules/tubular gland cell. These data suggested that the cell must also have an efficient mechanism to destroy what was once a relatively stable mRNA since the [^{3}H]$cDNA_{ov}$ would detect even short fragments of mRNA. In fact, when investigators have attempted to measure $mRNA_{ov}$ half-life following removal of estrogen, data were obtained which indicated a half-life on the order of 12 hr (Chan et al., 1973; Palmiter, 1973). This observation was in marked contrast to the calculated $mRNA_{ov}$ $t_{1/2}$ during continuous estrogen administration (see below) and raised the question as to whether estrogen also acted on the tubular gland cell to effect a stabilization of $mRNA_{ov}$.

Upon secondary estrogen-stimulation of withdrawn chicks, a marked increase in the number of $mRNA_{ov}$ molecules occurred by 30 min. By 29 hr the number of "active" tubular gland cells had doubled, while the concentration of $mRNA_{ov}$ per tubular gland cells had increased over 4000-fold. This impressive change occurred prior to a significant amount of cell division (Socher and O'Malley, 1973).

The theoretically derived $t_{1/2}$ of 45–60 hr for ovalbumin mRNA in chick oviduct can be checked by an independent, theoretical calculation. If the message is translated 40,000–50,000 times (arrived at by dividing the number of ovalbumin molecules by the number of $mRNA_{ov}$ under steady-state conditions) and the ribosomal transit time for translocation of the $mRNA_{ov}$ is known, the kinetics for $mRNA_{ov}$-directed synthesis of 40,000–50,000 ovalbumin molecules can be calculated. Palmiter estimated that the transit time for $mRNA_{ov}$ translation was about 2 min at 35°. With a Q_{10} of 2.2 for the rate of elongation, the transit time at 41° (the body temperature of the chicken) would be about 1.3 min (Palmiter, 1972). Thus, if each $mRNA_{ov}$ accommodates 13–15 ribosomes (Palmiter and Haines, 1973), then 13–15 ovalbumin molecules can be produced in 1.3 min. Therefore, it would take one $mRNA_{ov}$ molecule from 60 to 80 hr to produce 40,000–50,000 ovalbumin molecules. This value is identical with the theoretically predicted mean lifetime of a $mRNA_{ov}$ molecule (i.e., 70 hr) (Kafatos, 1972).

When the initial rate of $mRNA_{ov}$ synthesis was estimated between two short-time intervals (where degradation should have little effect on the estimated "true" transcription rate) and then compared for a greater time interval, it became apparent that the half-life under steady-state conditions was between 40 and 60 hr. The initial rate of transcription was 12 molecules/min, similar to the value reported by Palmiter using data obtained from a translation assay system (Palmiter, 1973). Consider

$$\int_{1\text{ hr}}^{4\text{ hr}} dc/dt = \int T = 12 \text{ molecules/min} \qquad (4)$$

and

$$\int_{1\text{ hr}}^{29\text{ hr}} dc/dt = \int T = 8 \text{ molecules/min} \qquad (5)$$

however

$$\int_{1\text{ hr}}^{29\text{ hr}} \frac{dc}{dt} = \frac{T}{D} - \left(\frac{T}{D} - C_0\right) e^{-Dt} = 12 \text{ molecules/min} \qquad (6)$$

where $t_{1/2}$ = 48 hr for $mRNA_{ov}$ half-life. Furthermore, the initial rate of transcription of the ovalbumin gene was exactly the same as the steady-state rate only if a $t_{1/2}$ of 48 hr was used.

Palmiter (1973) calculated a theoretical steady-state rate on the basis of $C_{t\infty}$ = 70,000 and $t_{1/2}$ = 24 hr. However, if it is considered that the transit time for $mRNA_{ov}$ decreased after 3–4 days of estrogen administration (from 1.3 to 1.0 min) (Palmiter, 1972), then the biphasic nature of the observed accumulation curve for $mRNA_{ov}$ molecules which is derived from his *translation* data can be explained. If this correction for transit time is made, the steady-state level of $mRNA_{ov}$ (54,000 molecules/tubular gland cell) derived by Palmiter was very close to the predicted steady-state level of 50,000 molecules/tubular gland cell described in this communication. The reason for the experimentally derived $t_{1/2}$ of 24 hr for $mRNA_{ov}$ remains obscure. Possibly there are differing pools of UTP within the tubular gland cell or, possibly, the UMP concentration does not properly reflect the UTP pool. These matters cannot be solved until new, more sophisticated methods for measuring half-life of mRNA are developed.

If all the translatable $mRNA_{ov}$ in the oviduct is associated with polysomes (Rhoads et al., 1971; Means et al., 1972) the transcription rate (T) for $mRNA_{ov}$ should approximately equal the translation rate (E) for this mRNA. Otherwise, if the transcription rate exceeded the translation rate, there would be an accumulation of free, nonpolysomal bound $mRNA_{ov}$ with time. When such a calculation is made, it becomes apparent that the transcription rate is very close to the translation rate for this message. The transcription rate (T) is estimated to be 240 nucleotides per sec per 2 ovalbumin genes (diploid). The transit time for the $mRNA_{ov}$, determined by Palmiter (1972), was 1.3 min (78 sec) at 41°. This indicates that the process of polymerizing at least 387 amino acids to form ovalbumin is repeated 14 times every 78 sec/$mRNA_{ov}$–polysome complex. Thus, the rate at which $mRNA_{ov}$ is traversing the ribosomes will be about 15 nucleotides per sec per ribosome. The total polysome complex (14 ribosomes) then translates a $mRNA_{ov}$

[2] Upon examination of tissue sections prepared from a hormone-withdrawn chick oviduct, it becomes apparent that the tubular gland cells have either "disappeared" or are present but no longer are morphologically recognizable as tubular gland cells. We favor a combination of these two alternatives, since only a 10–20% decrease in total DNA content of the oviduct occurs.

molecule at a rate of about 210 nucleotides/sec. This value is very close to the transcription rate (T) of 240 nucleotides.

The hen oviduct is under the influence of optimal concentrations of all hormonal factors necessary to maximize ovalbumin output (i.e., constant endogenous estrogen, testosterone, progesterone, etc.). When the total oviduct cell RNA from egg-laying hen was reacted with the [^{3}H]cDNA$_{ov}$, it was found that the apparent steady-state rate of mRNA$_{ov}$ transcription was about 35 molecules/min or 0.59 molecule/sec or 700 nucleotides per sec per 2 ovalbumin genes. Since the hen oviduct has about 3.5×10^8 tubular gland cells/g of tissue and the average oviduct magnum weighs about 30 g, the daily output of ovalbumin per hen can be calculated. If 148,000 molecules of mRNA$_{ov}$/tubular gland cell have a mean lifetime of 70 hr and are translated some 50,000 times, then they can produce approximately 7×10^9 ovalbumin molecules in that period. This calculates to be a rate of 2.8×10^4 ovalbumin molecules per sec per tubular gland cell. Thus the total output for the hen magnum would be 2.9×10^{14} ovalbumin molecules per sec per hen magnum ($2.8 \times 10^4 \times 3.5 \times 10^8 \times 30$ g). Since there are 14×10^{15} ovalbumin molecules per mg of ovalbumin, this amounts to 20 μg of ovalbumin/sec or 1.8 g/day. This is approximately the amount of ovalbumin in one egg, and an average hen produces only one egg per day.

One possible way to regulate the *amount* of a given mRNA produced per unit time would be to regulate the number of RNA polymerase molecules which may bind and initiate transcription at a specific gene site. The transcription rate for the ovalbumin gene was estimated to be about 120 nucleotides/sec in the chick. Since the fastest rate of transcription per RNA polymerase molecule has been estimated to be on the order of 20 nucloetides/sec (Bremar, and Yuan, 1968), there must be at least 6 RNA polymerase molecules simultaneously transcribing the ovalbumin gene in the chick. In the hen, this value would be about three times larger or 18 RNA polymerases per ovalbumin gene. This number could increase to 36 polymerase molecules if the diploid state were expressed.

Our results confirm that estrogenic hormones have a marked effect on the intracellular concentration of specific, biologically active mRNAs in target cells. A single injection of estrogen resulted in a marked increase in the concentration of ovalbumin mRNA molecules and this increase in mRNA sequences correlated directly with an increase in biologically active mRNA which was assayed in a cell-free translation system. The mechanism of this induction appears to be primarily transcriptional in nature. This view is supported by the following data: (1) essentially no mRNA$_{ov}$ molecules were present in a cell which has not previously been stimulated with estrogen; (2) following induction, withdrawal of the steroid hormone again leads to a reduction in the concentration of mRNA molecules to essentially a zero level; (3) an observable increase in cellular mRNA$_{ov}$ sequences is easily detected as early as 0.5 hr after hormone stimulation; (4) the time required for apparent mRNA sequence processing and transport to the cytoplasm is consistent with the initial appearance of cytoplasmic ovalbumin synthesis following hormone stimulation; and (5) no translational block or biologically inactive mRNA$_{ov}$ sequences were detected in the oviduct cell.

The posttranscriptional theory of hormonal control (Tompkins et al., 1969) states that an inducible gene is continuously transcribed but the mRNA sequences are then quickly inactivated by some regulatory protein and degraded. It would seem that if this theory were operative in our system, we would see a higher base line of oviduct mRNA$_{ov}$ molecules in the uninduced state, especially since the [^{3}H]cDNA$_{ov}$ is capable of detecting even small fragments (100 nucleotides) of the mRNA$_{ov}$ molecule. Our conclusion is further supported by previously published data (Rhoads et al., 1973) which reveal that no detectable increase in translatable mRNA activity for ovalbumin occurs during superinduction of ovalbumin synthesis with actinomycin D. Finally, we have recently transcribed oviduct chromatin from estrogen-induced and uninduced chicks in vitro in the presence of excess bacterial RNA polymerase. The RNA synthesized from these chromatin templates was reacted with [^{3}H]cDNA$_{ov}$ and mRNA sequences for ovalbumin were only detected in the chromatin prepared from hormone-stimulated target cell chromatin. These results imply that the ovalbumin gene in chromatin is accessible to RNA polymerase during hormone stimulation but is inaccessible or "closed" both prior to hormone stimulation or during hormone withdrawal. These results (to be published elsewhere) again strongly support transcriptional control as the major mechanism for the estrogen-mediated induction of ovalbumin in chick oviduct tubular gland cells.

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Structure–Function Relationship in the Binding of Snake Neurotoxins to the Torpedo Membrane Receptor†

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ABSTRACT: The Cys_{30}–Cys_{34} bridge present in all long neurotoxins (71–74 amino acids, 5 disulfide bridges), but not in short toxins (60–63 amino acids, 4 disulfide bridges), is exposed at the surface since it can be reduced rapidly and selectively by sodium borohydride. Reduction and alkylation of the Cys_{30}–Cys_{34} bridge of *Naja haje* neurotoxin III hardly alter the conformational properties of this model long toxin. Although alkylation by iodoacetic acid of the -SH groups liberated by reduction abolishes the toxicity, alkylation by iodoacetamide or ethylenimine does not affect the curarizing efficacy of the toxin. The Cys_{30}–Cys_{34} bridge is not very important for the toxic activity of long neurotoxins. Reduction of the Cys_{30}–Cys_{34} bridge followed by alkylation with radioactive iodoacetamide gave a labeled and active toxin which is a convenient derivative for binding experiments to the toxin receptor in membranes of the Torpedo electric organ. The binding capacity of these membranes is 1200 pmol of toxin/mg of membrane protein. The dissociation constant of the modified toxin–receptor complex at pH 7.4, 20° is 10^{-8} *M*. Reduction with carboxamidomethylation of the Cys_{30}–Cys_{34} bridge decreases the affinity of the native *Naja haje* toxin only by a factor of 15. Carboxymethylation after reduction prevents binding to the membrane receptor. The binding properties of the derivative obtained by reduction and aminoethylation of Cys_{30}–Cys_{34} are very similar to those of native neurotoxin III; the affinity is decreased only by a factor of 5. Binding properties to Torpedo membranes of long neurotoxins (*Naja haje* neurotoxin III) and short neurotoxins (*Naja haje* toxin I and *Naja mossambica* toxin I) have been compared. Dissociation constants of receptor–long neurotoxin and receptor–short neurotoxin complexes are very similar ($5.7–8.2 \times 10^{-10}$ *M* at pH 7.4, 20°). However, the kinetics of complex formation and complex dissociation are quite different. Short neurotoxins associate 6–7 times faster with the toxin receptor and dissociate about 5–9 times faster than long neurotoxins. Acetylation and dansylation of Lys_{53} and Lys_{27} decrease the affinity of long and short toxins for their receptor by a factor of about 200 at pH 7.4, 20°, mainly because of the slower rate of association with the receptor.

During the last decade considerable work has been carried out on snake venoms, and more than 50 toxins with neurotoxic activity have been isolated in a pure state from elapid venoms (cobra, krait, tiger snakes, mambas) as well as from hydrophid venoms (sea-snakes).

These neurotoxins form two different groups—the 60–63 amino acids group and the 71–74 amino acids group. Neurotoxins with a chain length of 60–63 amino acid residues in a single polypeptide chain are cross-linked by four disulfide bridges; they are called "short neurotoxins." Neurotoxins with a chain length of 71–74 amino acid residues are cross-linked by five disulfide bridges; they are called "long neurotoxins." Both short and long neurotoxins are postsynaptic or curariform toxins which combine firmly with the acetylcholine receptor on the motor end plate and produce a nondepolarizing block of neuromuscular transmission, just like *d*-tubocurarine (Lee, 1970).

Both short and long neurotoxins have the same overall arrangement of four disulfide bridges (Figure 1). The two

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