# Transcriptional Regulation of the Ovalbumin and Conalbumin Genes by Steroid Hormones in Chick Oviduct\*

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Relative rates of ovalbumin and conalbumin mRNA transcription were measured in isolated oviduct nuclei by allowing endogenous RNA polymerases to synthesize [<sup>32</sup>P]RNA that was then hybridized to immobilized recombinant DNA containing the respective gene sequences. Administration of either estrogen or progesterone to withdrawn birds increased the rate of conalbumin mRNA (mRNA<sub>con</sub>) transcription 2- to 3-fold within 30 min. The rate of ovalbumin mRNA (mRNA<sub>ov</sub>) transcription was undetectable before hormone administration and increased at least 20-fold during the first 12 h. The maximum rates of transcription achieved after 12 h of hormonal stimulation are only 10 to 25% of those observed after several days of hormone treatment; these transcription rates are consistent with the low levels of nuclear estrogen or progesterone receptors measured during the first 12 h of induction.

The induction of mRNA<sub>ov</sub> and mRNA<sub>con</sub> in oviduct explant cultures was quantitatively comparable to that observed in vivo. Relative rates of transcription were also measured in this system by pulse-labeling with [<sup>3</sup>H]uridine. In addition, absolute rates of transcription were determined by measuring the specific activity of the UTP pool during the labeling period. The accumulation of mRNA<sub>ov</sub> sequences was consistent with the absolute rate of transcription measurements, indicating that this mRNA has a long  $t_{1/2}$  (>20 h) in the presence of estrogen or progesterone. Comparable calculations for mRNA<sub>con</sub> indicate that its  $t_{1/2}$  increases from  $\sim$ 3 h in the absence of steroids to  $\sim$ 8 h during the restimulation with hormones. The results indicate that both estrogen and progesterone regulate the rate of transcription of these mRNAs and suggest that there may be significant effects of these hormones on mRNA stability as well.

Estrogen initiates the differentiation and proliferation of tubular gland cells in the chick oviduct magnum, and these specialized cells synthesize and secrete large amounts of the major egg white proteins (ovalbumin, conalbumin, lysozyme, and ovomucoid). When estrogen administration is discontinued (withdrawal), the synthesis of egg white proteins declines rapidly, although some of the differentiated tubular gland cells remain and can be restimulated by estrogen (secondary stimulation). Secondary stimulation is also observed with progesterone (1) and glucocortocoids,  $^{1}$  steroids that are inactive as a primary stimulation.

We, and many others, have been interested in the mechanisms by which steroid hormones regulate the expression of specific genes. Techniques have been perfected during the last 8 years to measure accurately the cellular level of specific mRNAs by either cell-free translation (2) or hybridization to cDNA probes (3), and both of these techniques have been applied extensively to the chick oviduct, demonstrating that steroid hormones regulate the accumulation of egg white mRNAs in tubular gland cells (4, 5). Since the accumulation of a specific mRNA could be regulated by either an increase in its rate of synthesis or a decrease in its rate of degradation, we have been interested in developing techniques to measure these parameters directly during hormonal stimulation.

Measurements of the rates of specific mRNA transcription in the chick oviduct have been difficult because the fraction of the total transcript expected for the single copy egg white genes is low (0.2% or less) and large amounts of endogenous mRNA sequences are present which contaminate nuclei and must be distinguished from the newly transcribed mRNA. Nevertheless, a method has been developed by Dale and Ward (6) and further modified by others to allow the incorporation of HgUTP into the newly transcribed RNA synthesized in isolated nuclei. This mercurated RNA then can be purified by affinity chromatography on sulfhydryl (SH)-Sepharose, eliminating the endogenous contamination. Although this method can be plagued with problems due to aggregation and contamination, several groups have overcome these difficulties and measured the rate of mRNA<sub>ov</sub><sup>2</sup> synthesis in fully stimulated birds and laying hens (7, 8). Recently, Nguyen-Huu et al. (9) have used this technique to demonstrate that the rates of transcription of both the ovalbumin and conalbumin genes increase during secondary stimulation with estrogen. We have confirmed and extended these observations by transcribing isolated nuclei in the presence of [<sup>32</sup>P]UTP and hybridizing the radioactive RNA to filter-bound recombinant DNA containing the coding sequence of either the ovalbumin or conalbumin gene. With this technique, we have defined in more detail the effects of estrogen and progesterone on the transcription of these egg white genes.

We have also used the filter hybridization technique to measure the rates of mRNA<sub>ov</sub> and mRNA<sub>con</sub> synthesis during the hormonal induction of these genes in tissue culture. Using a modification of the culture conditions previously described (10), we can now induce mRNA<sub>ov</sub> and mRNA<sub>con</sub> in cultured tissue fragments at a rate similar to that observed *in vivo* after administration of hormones. At various times during the *in* 

<sup>1</sup>L. Hager, G. S. McKnight, and R. D. Palmiter, unpublished observations.

 $^2$  The abbreviations used are:  $mRNA_{ov}\!,$  ovalbumin mRNA;  $mRNA_{con}\!,$  conalbumin mRNA; SDS, sodium dodecyl sulfate.

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*vitro* induction, tissue fragments were pulse-labeled with [<sup>3</sup>H]uridine and radioactive RNA isolated and hybridized to plasmid DNA immobilized on filters to determine the relative rates of transcription from the ovalbumin and conalbumin genes. The specific activity of the [<sup>3</sup>H]UTP precursor pool was also determined and used to convert relative rates of mRNA synthesis to absolute rates. A comparison of the observed accumulation of these mRNAs with that predicted from the absolute rates of transcription can be used to evaluate the role of mRNA degradation as well as synthesis during hormonal stimulation.

## EXPERIMENTAL PROCEDURES

Animals and Tissue Culture—Four-day-old White Leghorn chicks were implanted with pellets containing 15 mg of diethylstilbestrol (Rhodia Inc.) for 15 days, withdrawn from the hormone for at least 10 days, and then repelleted (secondary stimulation) for 5 to 10 days. Chicks were withdrawn from the secondary stimulation for 2 to 3 days before being used for either tissue culture or *in vivo* experiments. For *in vivo* measurements, chicks were injected (subcutaneously) with either estradiol benzoate (1 mg) or progesterone (1 mg). Chicks were killed and the magnum portion of the oviduct was used to prepare nuclei as previously described (11). A sample of the oviduct magnum was also used to prepare total nucleic acid and determine the cellular concentration of mRNA<sub>ov</sub> and mRNA<sub>con</sub> as described below.

Tissue fragments were induced in culture by mincing oviduct magnum from 2- to 3-day-withdrawn chicks in Ham's nutrient mixture F-10 (Gibco) and incubating the cultures at 41°C as previously described (10) with the addition of 5  $\mu$ g/ml of porcine insulin (Lilly) to the culture medium. A more thorough discussion of the insulin requirement and optimal culture conditions will be published separately. Tissue fragments were incubated in medium for 3 h before the addition of either estradiol or progesterone at the concentrations indicated in the figure legends. Samples of tissue were removed at the indicated times for determination of ovalbumin and conalbumin mRNA concentrations as described below. Tissue fragments were also removed from the incubation, pulse-labeled in medium containing 1 mCi/ml of [3H]uridine (39 Ci/mmol, Schwarz/Mann), washed briefly in cold phosphate-buffered saline, and frozen immediately on dry ice. Total nucleic acid was isolated using SDS and proteinase K as previously described (10). DNA was removed by DNase I treatment as described below followed by proteinase K digestion and removal of protein with phenol/chloroform. The RNA was concentrated by ethanol precipitation.

Recombinant DNA—The recombinant plasmid, pCR1-OV, containing 95% of the sequence coding for ovalbumin mRNA, was cloned by Humphries *et al.* (12). The cDNA clone containing a 2500-nucleotide sequence specific for conalbumin mRNA was isolated in the laboratory of P. Chambon (Faculté de Médecine, Strasbourg, France) using the vector pBR322 and appropriate containment as specified by the French National Control Committee. pCR1-OV has been certified for use under P2-EK1 containment by the National Institutes of Health and was grown in our laboratory according to these guidelines. Plasmid DNA from pBR322-CON was generously provided by the laboratory of P. Chambon and used in our laboratory according to the National Institutes of Health *Guidelines.*<sup>3</sup>

Preparation of cDNAs and cRNAs<sup>+</sup>-Complementary DNAs specific for ovalbumin and conalbumin mRNA were synthesized and used to determine the concentration of specific mRNA/cell as previously described (10, 13). The results are reported as molecules/cell without correcting for the per cent of the cell population which represents functional tubular gland cells. Nonradioactive cDNAs were also prepared and used as templates for Escherichia coli DNAdependent RNA polymerase. The radioactive cRNAs<sup>+</sup> synthesized were included as internal standards in the filter hybridizations. The nonradioactive cDNA  $_{\scriptscriptstyle ov}$  was synthesized from pure mRNA  $_{\scriptscriptstyle ov}.$  However, the template used to prepare large amounts of nonradioactive cDNA<sub>con</sub> was purified only by repeated sedimentation on sucrose gradients; hybridization of this cDNA to pure  $mRNA_{con}$  revealed that the product was only  $\sim 50\%$  cDNA<sub>con</sub>. The remaining 50% of the cDNA hybridized to a low abundance, heterogenous class of mRNAs and did not hybridize to mRNA<sub>ov</sub>. The cRNAs<sup>+</sup> were prepared in a 10 µl volume containing a final concentration of 50 mM Tris-Cl (pH 8), 10 mm MgCl<sub>2</sub>, 0.5 mm GTP, CTP, and ATP, 1 mm dithiothreitol. 1 mM K<sub>2</sub>HPO<sub>4</sub>, 10% glycerol, 50 mM KCl, 200 µg/ml of E. coli RNA polymerase (Miles), 5 to 10  $\mu g/ml$  of cDNA, and either 0.07 mM [ $\alpha$ -<sup>2</sup>P]UTP (350 Ci/mmol, Amersham or ICN) or 0.16 mm [<sup>3</sup>H]UTP (30 Ci/mmol, Schwarz/Mann). The reaction mixture was incubated at 37°C for 90 min, diluted with 200 µl of DNase buffer (20 mM 4-(2hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, pH 7.5), 5 mm MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>), and digested at 37°C for 30 min with 10 µg/ml of DNase I from which contaminating RNase was removed according to Brison and Chambon (14). The DNase was removed by the addition of 20  $\mu$ l of 10  $\times$  SET (1  $\times$  SET is 10 mM Tris-Cl (pH 7.5), 5 mm EDTA, 1% SDS) and 10 µg of proteinase K (Merck) followed by a 30-min incubation at 45°C. Unincorporated nucleotides were removed by chromatography on Sephadex G-75 in  $1 \times \text{SET}$ , the void volume was extracted with phenol/chloroform (1: 1), and the cRNA<sup>+</sup> was ethanol-precipitated with carrier E. coli tRNA.

Transcription with Nuclei and Isolation of RNA for Hybridization-Nuclei were isolated from 0.3 to 1.0 g of oviduct magnum as described by Mulvihill and Palmiter (11), suspended in 40% glycerol, 50 mm Tris-Cl (pH 8.3), 5 mm MgCl<sub>2</sub>, 0.1 mm EDTA at a concentration of 0.7 to 1.2 mg of DNA/ml, and stored at  $-70^{\circ}$ C. For transcription, 40 µl of nuclei were incubated in a 100-µl reaction mixture containing: 16% glycerol; 20 mm Tris-Cl, pH 8.0; 5 mm MgCl<sub>2</sub>; 150 mm KCl; 0.4 mM concentrations of ATP, GTP, and CTP; and 100  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]-UTP (about 100 to 350 Ci/mmol, Amersham or ICN) for 45 min at 26°C. The reaction was then treated with DNase I (10  $\mu$ g/ml) for 5 min at 26°C and 25  $\mu$ g of carrier rabbit reticulocyte RNA was added. The reaction was deproteinized by digestion with 100  $\mu$ g/ml of proteinase K in  $1 \times SET$  followed by phenol/chloroform extraction. The aqueous phase was precipitated at  $-20^{\circ}$ C with 0.1 M NaCl and 2 volumes of ethanol. After washing the ethanol precipitate with 0.04 M NaCl, 66% ethanol, the nucleic acid was dissolved in DNase buffer and incubated with 10  $\mu$ g/ml of DNase I for 60 min at 26°C. The reaction was again digested with proteinase K in  $0.2 \times SET$  and extracted with phenol/chloroform. The aqueous phase then was precipitated at 4°C with cold 10% trichloroacetic acid in the presence of 30 mm Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> and 1 mm UTP to remove unincorporated <sup>32</sup>Pnucleotides completely. The precipitate was collected by centrifugation, washed four times with 5% trichloroacetic acid, 10 mM  $Na_4P_2O_7$ , dissolved in 200 µl of 0.1 M sodium acetate (pH 7), and ethanolprecipitated. The ethanol precipitate was dissolved in  $0.1 \times SET$  and aliquots were used to measure specific mRNA transcription by filter hybridization. The total incorporation of UTP in nuclei varied between 0.14 and 1.6 pmol/ug of DNA and was a function of the  $\left[\alpha^{-32}P\right]$ UTP concentration (2 to 10  $\mu$ M) which was always suboptimal and the quality of the  $[\alpha^{-32}P]UTP$  available commercially. We have transcribed the same nuclei under a variety of labeling conditions and found that relative transcription rates for specific genes remained the same, although total incorporation varied.

Hybridization to Immobilized DNA-Plasmid DNAs were attached to nitrocellulose filter discs and hybridized to RNA using a modification of the techniques described by Gillespie (15). DNA was dissolved in 2 M NaCl, 0.2 M NH4OH, heated to 100°C for 1 min, and 4  $\mu$ l (containing 0.2  $\mu$ g of DNA) was spotted on 7-mm diameter Sartorius filter discs (Beckman) and allowed to air dry. The filters then were baked at 80°C for 2 h, washed for 30 min at 45°C in Buffer A (0.3 M NaCl. 2 mm EDTA, 10 mm Tris-Cl, pH 7.5) containing 0.1% SDS, and allowed to air dry. More than 90% of the plasmid DNA binds to filters using this method and remains attached during subsequent hybridization and washing. Hybridizations were performed in 5-ml polypropylene tubes (Falcon) in a final volume of  $30 \,\mu l$  containing 0.5 м NaCl, 0.05 м 1,4-piperazineethansulfonic acid (Pipes, pH 7), 33% for mamide, 0.4% SDS, and 2 mm EDTA in addition to the radioactive RNA being assayed. Appropriate <sup>32</sup>P- or <sup>3</sup>H-labeled cRNAs<sup>+</sup> for ovalbumin and conalbumin were also included in each reaction as standards. Three filters containing immobilized pBR322 wild type DNA, pBR322-CON DNA, and pCR1-OV DNA were added to each tube and overlaid with 0.2 ml of light paraffin oil. The tubes were centrifuged briefly at 200  $\times$  g and incubated at 45°C for 2 to 3 days.

After hybridization the filters were washed for 2 h in Buffer A containing 0.1% SDS at 45°C. The filters then were washed in the same buffer without SDS for 30 min, digested with 10  $\mu$ g/ml of RNase A and 1.0  $\mu$ g/ml T1 RNase in Buffer A at 37°C for another 30 min, and then washed in Buffer A containing SDS for another 1 to 2 h at 45°C. The filters were treated with 250  $\mu$ l of 0.04 m NaOH to release the RNA and neutralized with 100  $\mu$ l of 0.1 N acetic acid, and the radioactivity was determined after the addition of 4 ml of Triton/

 $<sup>^{\</sup>rm 3}\,Guidelines$  (1976) p. III-19, National Institutes of Health, Bethesda.

xylene scintillation fluid (25% Triton X-100, 75% xylene, and 4 g/liter of Omnifluor (New England Nuclear)). The Packard (model 3003) scintillation counter had a background of 4 cpm for <sup>3</sup>H and 18 cpm for <sup>32</sup>P and, since much of the data relies on samples having 100 cpm or less, each sample was counted for 30 to 50 min to reduce counting error.

Determination of UTP Specific Activity-Oviduct tissue fragments were pulse-labeled with [<sup>3</sup>H]uridine as described above for 3 to 20 min. After a brief wash in cold phosphate-buffered saline, the tissue fragments were extracted with 0.3 N HClO<sub>4</sub> at 0°C for 1 to 2 h. The tissue then was removed, washed in phosphate-buffered saline, and used to prepare total nucleic acid. The HClO4 extract was neutralized with KOH and the KClO<sub>4</sub> precipitate was removed by centrifugation. UTP was partially purified by applying the extract to a 0.3-ml column of polyethyleneimine-cellulose (Sigma). The column was washed successively with H<sub>2</sub>O and 0.2 M LiCl, and the UTP then was eluted with 1 M LiCl. The UTP was absorbed onto activated charcoal, washed with H<sub>2</sub>O, and then eluted with 50% ethanol:5% pyridine:1% ammonia. Residual charcoal fines were removed by filtration through a 0.45-µm Millipore filter; the eluant then was dried and redissolved in 200  $\mu$ l of  $H_2O$ . At this stage the recovery of UTP was ~65%, the UDPG in the original extract was completely removed, and 80% of the total radioactivity migrated with UTP on polyethyleneimine-cellulose sheets (EM Reagents).

Enzymatic conversion of UTP to UDPG was accomplished using the incubation conditions described by Cheung and Suhadolnik (16). For each sample, two 50- $\mu$ l reactions were set up containing: 10  $\mu$ l of [<sup>3</sup>H]UTP (10<sup>5</sup> to 10<sup>6</sup> cpm), 167 pmol of [<sup>14</sup>C]glucose-1-P (300 mCi/ mmol; Amersham), 7 mm MgCl<sub>2</sub>, 128 mm KCl, 80 mm Tris-Cl (pH 8), 1 mM CDP, and 1 unit of inorganic pyrophosphatase (Sigma). One reaction contained 0.2 units of UDPG-pyrophosphorylase (Sigma) and the other served as a control. After incubation for 30 min at 37°C, 10-µl aliquots were spotted on polyethyleneimine-cellulose sheets with carrier unlabeled UDPG. The sheets were developed with methanol to remove salt from the sample and then with 0.1 M LiCl. 1.0 M Ch<sub>3</sub>COOH. In this solvent, glucose-1-P has an  $R_F$  of 0.6, UDPG has an  $R_F$  of 0.21 to 0.31, UDP has an  $R_F$  of 0.1, and UTP remains at the origin. The UDPG spot was scraped off the sheet, extracted with 0.1 ml of 0.5 M Na<sub>2</sub>CO<sub>3</sub>, 0.5 ml of Soluene 350 (Packard), and counted in 5 ml of toluene/Omnifluor (New England Nuclear).

The specific activity of UTP was calculated from the  ${}^{3}H/{}^{14}C$  ratio of the synthesized UDPG (plus enzyme) after subtracting background (minus enzyme). As a control, [ ${}^{3}H$ ]UTP of known specific activity (21 Ci/mmol, Schwarz/Mann) was used as a substrate; this method gave a value of 20.1 Ci/mmol.

*Nuclear Estrogen Receptor Measurements*—Nuclear estrogen receptors were assayed by an exchange assay with isolated nuclei as previously described (11).

## RESULTS

Filter Hybridization Techniques-The method described under "Experimental Procedures" for the hybridization of radioactive RNA to plasmid DNA immobilized on nitrocellulose filters was chosen to maximize the efficiency of hybridization while reducing nonspecific binding to a minimum. The technique for attaching the plasmid DNA to nitrocellulose filters was derived from previously published methods (15, 17, 18) and resulted in filters with a high capacity for binding specific mRNAs as determined by competition hybridization experiments. Problems with nonspecific binding of radioactive RNA to wild type plasmid DNA filters are significantly reduced by the formamide/salt/SDS buffer system employed in these studies and by the complete removal of protein and DNA with proteinase K and DNase, respectively. [<sup>32</sup>P]RNA transcribed by isolated nuclei in vitro gave a nonspecific background of 3 to 5 ppm; [<sup>3</sup>H]RNA synthesized by tissue fragments in culture gave a background of 0 to 1 ppm.

The efficiency of hybridization was monitored in all reactions by including [<sup>3</sup>H]RNA or [<sup>32</sup>P]RNA synthesized from single stranded cDNA<sub>cov</sub> or cDNA<sub>con</sub> using *E. coli* RNA polymerase. We refer to this internal standard, which is the equivalent of labeled mRNA fragments, as cRNA<sup>+</sup>. The use of an internal standard is especially important when measuring rates of mRNA synthesis in tissues which contain high levels

of endogenous  $mRNA_{ov}$  and  $mRNA_{con}$  since these samples frequently exceed the mRNA binding capacity of the filters. In Table I we show the results obtained when [<sup>32</sup>P]RNA transcribed in secondary stimulated nuclei was hybridized in the presence of known amounts of competitor mRNA<sub>ov</sub>. The hybridization of both the [<sup>32</sup>P]RNA transcript and the [<sup>3</sup>H]cRNA<sup>+</sup><sub>ov</sub> standard was inhibited by the same proportion and when the rate of  $mRNA_{ov}$  synthesis is corrected to 100% hybridization of the standard the values obtained are in good agreement, giving an average rate of 2419 ppm or 0.24% of total for mRNA<sub>ov</sub> synthesis in secondary stimulation. In other experiments with different groups of birds on secondary stimulation the rate of mRNA<sub>uv</sub> synthesis has varied from 1000 to 2400 ppm (average, 1540 ppm) and the rate of mRNA<sub>con</sub> synthesis has varied from 600 to 1280 ppm (average, 800 ppm). This variability in the rates of mRNA<sub>ov</sub> and mRNA<sub>con</sub> synthesis between birds is not due to the transcription assay; we have transcribed and reassayed the same preparation of nuclei and find that the relative rates of specific transcription remain constant. The variability may be related to the rate of hormone release from the diethylstilbestrol implants or the presence of other endogenous hormones which augment the induction. Transcription using hen nuclei gave 2310 ppm for the rate of mRNA<sub>ov</sub> synthesis and 1990 ppm for mRNA<sub>con</sub> synthesis. We have corrected all rates of synthesis measurements to 100% efficiency. Our assumption is that the  $cDNA_{ov}$  template is pure and that all of the  $\ensuremath{\mathsf{cRNA}^{\scriptscriptstyle +}_{ov}}$  transcribed from this template should be capable of hybridizing to pCRI-OV DNA. The cDNA<sub>con</sub> template used was only 50% pure as discussed under "Experimental Procedures" and we, have therefore, assumed that only 50% of the  $cRNA_{con}^+$  standard is potentially able to hybridize to pBR322-CON DNA. Experimentally we find that only 25 to 40% of the  $cRNA_{ov}^+$  and 10 to 20% of the cRNA<sup>+</sup><sub>con</sub> hybridizes to the appropriate plasmid DNA filter; this is due, at least partially, to the stringent hybridization, washing, and RNase conditions employed to reduce nonspecific background.

Messenger RNAs are Transcribed from the Ovalbumin and Conalbumin Genes by RNA Polymerase II—When nuclei are transcribed in the presence of  $\alpha$ -amanitin (1 µg/ml), the incorporation of [<sup>32</sup>P]UTP is inhibited by about 50% and the specific transcription of mRNA<sub>con</sub> is blocked completely in either withdrawn or hormone-stimulated oviduct nuclei as shown in Table II. The specific transcription of mRNA<sub>ov</sub> in withdrawn nuclei is undetectable, as discussed below, but in stimulated nuclei  $\alpha$ -amanitin effectively blocks the transcription from the ovalbumin gene. Since  $\alpha$ -amanitin has been

#### TABLE I

### Hybridization of radioactive RNA, transcribed in isolated nuclei, to pCR1-OV filters in the presence of competitor mRNA<sub>nc</sub>

Isolated oviduct nuclei from 6-day secondary stimulated birds were transcribed in the presence of  $[^{32}P]$ UTP and the  $^{32}P$  transcripts were extensively purified. The radioactive RNA was then hybridized to filters containing 0.2 ug of pCR1-OV DNA as described under "Experimental Procedures." The competitor mRNA<sub>ov</sub> was added to the hybridizations as indicated.

Input of [ <sup>32</sup> P]- RNA	Competitor mRNA <sub>ov</sub>	[ <sup>32</sup> P]RNA hybridized	Hybridiza- tion of stan- dard [ <sup>3</sup> H]- cRNA <sub>ov</sub>	Rate of mRNA <sub>ov</sub> synthesis <sup>a</sup>		
$cpm \times 10^{-3}$	ng	cpm	%			
480		312	26.3	2479		
480	25	135	11.7	2403		
480	50	72	5.7	2640		
480	250	22	2.1	2154		
Average				2419		

 $^{\rm c}$  The results were corrected to an internal standard efficiency of 100%.

#### TABLE II

# The effects of $\alpha$ -amanitin on the transcription of mRNA<sub>ov</sub> and mRNA<sub>con</sub> in isolated nuclei

Nuclei were allowed to incorporate [ $^{32}$ P]UTP in the presence or absence of  $\alpha$  amanitin. The  $^{32}$ P transcripts were hybridized to filters containing immobilized pCR1-OV DNA, pBR322-CON DNA, or pBR322 wild type DNA as in Table I. The nonspecific hybridization to wild type filters was 3 to 5 ppm of the input but has not been subtracted from the counts per min hybridized to pCR1-OV filters.

	Transcribed with $1 \ \mu g/ml \ \alpha$ -amanitin	Incorpora- tion of [ <sup>32</sup> P]UTP	Input of [ <sup>32</sup> P]- RNA in each hy- bridization	Added mRNA <sub>ov</sub> competi- tor	[ <sup>32</sup> P]- RNA hy- bridized to pCR1- OV filter	Hybridiza- tion of standard [ <sup>3</sup> H]- cRNA <sub>ov</sub>	Rate of mRNA Synthe- sis	
Nuclei							Ovalbu- min <sup>a</sup>	Conalbu- min <sup>o</sup>
		pmol/µg DNA	cpm × 10 <sup>-6</sup>	ng	cpm	% of input	ppm	
Oviduct (withdrawn) <sup>c</sup>	_	0.16	2.35		21	39	2.2	56
	_	0.16	2.35	400	19	1.3		
Oviduet (withdrawn) <sup>c</sup>	+	0.065	0.89		8	39	2.9	8
	+	0.065	0.89	400	7	1.4		
Oviduct (estrogen, 3 h) <sup><math>d</math></sup>	-	0.18	1.78		51	38	60	184
o . Iaaot (1999 - 899 - 979 - 979	-	0.18	1.78	400	10	0.8		
Oviduct (estrogen, 10 h) <sup><math>d</math></sup>		0.15	2.46		175	38	163	223
<b>0 / /</b>	_	0.15	2.46	400	23	0.9		
Oviduct (progesterone, 7 h) $^{e}$		0.14	1.6		89	38	131	125
	-	0.14	1.6	400	9	0.94		
Oviduct (progesterone, 7 h) <sup>e</sup>	+	0.07	0.97		10	39	8	2.6
- · · · · · · · · · · · · · · · · · · ·	+	0.07	0.97	400	7	1.2		
Liver (withdrawn) <sup>c</sup>	_	0.29	3.22		28	37	1.7	138
	_	0.29	3.22	400	26	1.4		

<sup>a</sup> The rate of mRNA<sub>ov</sub> synthesis was calculated by subtracting the counts per min bound to pCR1-OV filters in the presence of competitor from the counts per min bound in the absence of competitor. The difference was divided by the input and corrected for the efficiency of hybridization as determined by the standard [ ${}^{3}$ H]cRNA<sub>ov</sub>.

 $^{b}$  The rate of mRNA<sub>con</sub> synthesis was determined by subtracting the nonspecific hybridization to wild type filters (3 to 5 ppm) from the counts per min bound to pBR322-CON DNA filters and dividing by the input. The rate was corrected for the efficiency of hybridization which was 40%.

<sup>c</sup> Withdrawn from diethylstilbestrol pellets for 2 days.

<sup>d</sup> Birds withdrawn for 2 days were injected with 1 mg of estradiol benzoate for either 3 or 10 h as indicated.

<sup>e</sup> Birds withdrawn for 2 days were injected with 1 mg of progesterone for 7 h.

shown to be a specific inhibitor of polymerase II at this concentration (19), we conclude that both of these genes are normally transcribed by RNA polymerase II. Our results with ovalbumin are consistent with those published recently by Bellard *et al.* (8) and Schutz *et al.* (20) showing that mRNA<sub>ov</sub> transcription is inhibited by  $\alpha$ -amanitin.

Rates of mRNA<sub>ov</sub> and mRNA<sub>con</sub> Transcription in Hormone-withdrawn Oviduct Nuclei-The rates of specific mRNA synthesis in nuclei from birds withdrawn from estrogen for 2 days were measured in order to evaluate whether the hormone-induced change in mRNA accumulation could be explained by an increased transcriptional rate of the same magnitude. For example, the concentration of mRNA<sub>ov</sub> in 2day-withdrawn oviduct is approximately 7 molecules/cell. Upon secondary stimulation mRNA<sub>ov</sub> increases to about 30,000 molecules/cell and the rate of mRNA<sub>ov</sub> synthesis is 1540 ppm. If the absolute rate of total RNA synthesis/cell and the  $t_{1/2}$  of mRNA<sub>ov</sub> were the same in fully stimulated and withdrawn tissue, then we would expect a rate of mRNA<sub>ov</sub> synthesis 4,000-fold lower, or 0.4 ppm, in withdrawn nuclei. The results in Table II show that  $\sim 9$  ppm (21 cpm out of 2.35)  $\times 10^6$  cpm) of the [<sup>32</sup>P]RNA transcribed in withdrawn nuclei hybridized to pCRI-OV DNA filters (in this experiment the nonspecific binding to wild type plasmid DNA filters (3 to 5 ppm) has not been subtracted). However, this hybridization of [<sup>32</sup>P]RNA to pCR1-OV filters is presumably nonspecific since it was not significantly reduced by excess competitor mRNA<sub>ov</sub>. In contrast, the rate of mRNA<sub>con</sub> synthesis in withdrawn oviduct nuclei is 56 ppm (Table II) and competition hybridization experiments with mRNA<sub>con</sub> indicate that the transcripts which hybridize are specific (data not shown). Specific transcription of mRNA<sub>ov</sub> was observed in oviduct nuclei at 3 and 10 h after estrogen stimulation as shown in Table II; in these two cases, the hybridization of [<sup>32</sup>P]RNA to pCR1-OV filters was decreased >80% with an excess of  $mRNA_{ov}$  competitor. Table II also shows that the conalbumin gene is actively transcribed in the liver as would be expected since conalbumin mRNA and transferrin mRNA are indistinguishable (13) and transferrin is a major liver protein. The transcriptional activity of the transferrin gene in the liver is regulated by both steroids and iron deficiency.<sup>4</sup>

The Effects of Sarkosyl and Heparin on the Transcription of  $mRNA_{ov}$ —We have used the transcription of isolated oviduct nuclei to examine possible explanations for our inability to detect significant rates of mRNA<sub>ov</sub> synthesis in withdrawn nuclei. One possibility is that mRNA<sub>ov</sub> is degraded in withdrawn nuclei soon after synthesis and is not detected until the administration of hormone which inhibits this degradative pathway. A second possibility is that polymerases are present on the ovalbumin gene in withdrawal but are unable to elongate because of chromosomal proteins; the role of steroid hormones would be to remove these inhibitory proteins from the gene. RNA polymerase can continue to elongate in the presence of high concentrations of either sarkosyl or heparin and these two agents were added to the nuclei incubations to test their effects on mRNA<sub>ov</sub> transcription. Heparin inhibits oviduct RNases (21, 22) and sarkosyl effectively removes histones and most non-histone proteins from the DNA (23). Neither of these compounds caused a significant activation of  $mRNA_{ov}$  transcription (Fig. 1) and, when added to stimulated nuclei, they partially inhibited the specific transcription, indicating that rapid degradation or blocked polymerases are unlikely possibilities.

Time Course of mRNA Transcription in Isolated Nuclei— The relative rates of mRNA<sub>ov</sub> and mRNA<sub>con</sub> synthesis were measured using [<sup>22</sup>P]RNA transcribed in isolated oviduct nuclei for 7, 12, or 45 min. Fig. 2 shows that more than 50% of

 $^4$ G. S. McKnight, D. C. Lee, and R. D. Palmiter, manuscript in preparation.



FIG. 1. Effects of sarkosyl and heparin on the rate of mRNA<sub>ov</sub> synthesis in isolated nuclei. Estradiol benzoate (1 mg) was administered to 2-day-withdrawn chicks and oviduct nuclei were isolated before giving hormone and 1 or 7.5 h after injection. Nuclei were allowed to incorporate [<sup>32</sup>P]UTP as described under "Experimental Procedures" without additions ( $\Box$ ), with the addition of 400  $\mu$ g/ml of sodium heparin ( $\Xi$ ), or with the addition of 0.4% sodium sarkosyl ( $\Xi$ ). The rate of mRNA<sub>ov</sub> synthesis is expressed in parts per million and was calculated by first subtracting the nonspecific hybridization to wild type pBR322 filters (3 to 5 ppm) and then correcting for the per cent hybridization of the internal standard as described under "Results." The values shown are the average of duplicate hybridizations and the input counts per min in each hybridization were 0.5 to 1 × 10<sup>6</sup> cpm.



FIG. 2. The rate of specific mRNA synthesis in isolated nuclei as a function of incubation time. Oviduct nuclei were isolated from chicks restimulated for 1 or 10 h with estradiol benzoate. The nuclei were allowed to transcribe at 26°C for the times indicated using the standard conditions described under "Experimental Procedures." At each time the total incorporation of  $[^{32}P]$ UTP was determined ( $\Box - -\Box$ ). The rates of mRNA synthesis were also measured: mRNA<sub>ov</sub> after 1 h of estrogen ( $\bigcirc - \circ$ ), mRNA<sub>ov</sub> at 10 h ( $\bullet - \bullet$ ), mRNA<sub>con</sub> at 1 h ( $\triangle - - \triangle$ ), mRNA<sub>con</sub> at 10 h ( $\bullet - \bullet$ ). The rate of mRNA synthesis is expressed in parts per million determined as in Fig. 1. The input radioactivity in each hybridization was 1 to 2 × 10<sup>6</sup> cpm.

the total incorporation of  $[^{32}P]UTP$  occurred within the first 10 min and that the relative rate of mRNA<sub>ov</sub> and mRNA<sub>con</sub> synthesis decreased slightly between 7 and 12 min and then remained constant. This indicates that most of the mRNA transcribed remains large enough to form stable hybrids even after 45 min of incubation. We have routinely used an incubation time of 45 min with isolated nuclei to maximize the incorporation of  $^{32}P$ .

Steroid Hormones Regulate the Relative Rate of  $mRNA_{ov}$ and  $mRNA_{con}$  Synthesis as Measured in Isolated Nuclei— Withdrawn chicks were given a secondary stimulation with estrogen and, at various times during the induction, chicks were killed and oviduct nuclei were isolated as described



FIG. 3. In vivo induction with estrogen. Withdrawn chicks were injected with estradiol benzoate in oil and groups of three or four were killed at various times afterwards. Total nucleic acid was isolated from part of the oviduct tissue and the rest was used to isolated nuclei. Panel A, the rate of mRNA<sub>ov</sub> synthesis (histogram) was measured as in Fig. 1. The concentration of mRNA<sub>ov</sub> ( $\bigcirc$ ) was measured by hybridizing total nucleic acid to cDNA<sub>ov</sub> and is calculated in molecules/cell, assuming 2.5 pg of DNA/cell. Panel B, the rate of mRNA<sub>con</sub> synthesis (histogram) and the concentration of mRNA<sub>con</sub> ( $\bigcirc$ ) was determined as for mRNA<sub>ov</sub> above. The error bars in Panels A and B represent the range of duplicate hybridizations. The input into each filter hybridization reaction was ~1 × 10<sup>6</sup> cpm. Panel C, oviduct nuclei were assayed for the number of estrogen receptors using an exchange assay (11).

under "Experimental Procedures." The nuclei were transcribed in vitro and the relative rates of mRNAov and mRNA<sub>con</sub> synthesis were determined by hybridization to filters containing immobilized plasmid DNA. The nuclei were also assayed for estrogen receptors using an exchange assay with  $[^{3}H]$  estradiol. A portion of the oviduct magnum was used to isolate total nucleic acid which was assayed for mRNA<sub>ov</sub> and mRNA<sub>con</sub> with specific cDNAs. Fig. 3 shows the results of an experiment in which all of the parameters mentioned above were measured during the first 12 h of an estrogeninduced secondary response. The rate of mRNA<sub>ov</sub> synthesis in withdrawn nuclei was undetectable and the earliest increase observed was at 2 h. Between 2 and 12 h the rate of mRNA<sub>ov</sub> synthesis steadily increased and the concentration of  $mRNA_{ov}$ /cell increased exponentially as shown in Fig. 3A. These results are very similar to those previously obtained in our laboratory by measuring the kinetics of accumulation of mRNA<sub>ov</sub> sequences in nuclear ribonucleoprotein particles.<sup>5</sup> The response of the conalbumin gene is shown in Fig. 3B. The rate of mRNA<sub>con</sub> synthesis increased 2- to 3-fold within 30 min after the injection of estrogen and continued to increase

<sup>5</sup> P. Thomas and R. D. Palmiter, manuscript in preparation.

gradually during the next 10 h. The concentration of mRNA<sub>con</sub> sequences increased rapidly with no apparent lag. The increase in nuclear estrogen receptors is shown in Fig. 3*C*; a rapid increase in the first 30 min was followed by a gradual accumulation over the next 10 h. A comparison of the number of nuclear estradiol receptors and the rate of mRNA<sub>con</sub> synthesis suggests a direct correspondence between the receptor concentration and activation of the conalbumin gene. In contrast, transcription of the ovalbumin gene does not begin immediately demonstrating that the lag in mRNA<sub>ov</sub> accumulation previously documented (24–26) is due to a delay before transcription of this gene is initiated. These results are quite similar to those reported by Nguyen-Huu *et al.* (9) using a different assay method.

Progesterone also induces a secondary response; Fig. 4 shows the rate of transcription of  $mRNA_{\rm ov}$  and  $mRNA_{\rm con}$ after administration of progesterone in vivo. The rate of mRNA<sub>ov</sub> synthesis, measured in isolated nuclei, began to increase 1 h after the injection of progesterone and reached a maximal rate of 150 to 200 ppm by 4 h (Fig. 4A). The accumulation of mRNA<sub>ov</sub> was delayed for about 2 h and then increased rapidly. Fig. 4C shows the kinetics of mRNA<sub>con</sub> induction in the same experiment. The rate of mRNA<sub>con</sub> synthesis increased nearly 2-fold in the first 30 min and by 4 h had increased 3-fold over the rate in withdrawn nuclei. The cellular concentration of mRNA<sub>con</sub> increased by about 10 molecules/cell/h for the first 4 h and then abruptly began to accumulate at ~100 molecules/cell/h. This rapid accumulation of mRNA<sub>con</sub> cannot be completely explained by the observed changes in transcription, suggesting that progesterone also causes a stabilization of mRNA<sub>con</sub>. This aspect of hormone action will be discussed later. Fig. 4, B and D, shows data from another experiment in which the amount of progesterone administered was insufficient to maintain the induction over the 12-h time course as shown most clearly for mRNA ov in Fig. 4B. The early kinetics (0 to 4 h) are the same as in Fig. 4A but after 6 h the rate of mRNA<sub>ov</sub> synthesis steadily declined to about 20 ppm at 12 h. This decline in the synthetic rate caused a plateau and then a drop in the mRNA<sub>ov</sub> concentration, suggesting that, as the hormone disappeared, mRNA<sub>ov</sub> became unstable and was rapidly degraded. The effect on mRNA<sub>con</sub> synthesis and accumulation is similar (Fig. 4D), although not as dramatic, undoubtedly due to the fact that lower doses of progesterone are required for induction of conalbumin compared to ovalbumin (27).

The Absolute Rates of Specific mRNA Synthesis Measured in Culture—The oviduct culture system described previously



FIG. 5. Changes in the specific activity of UTP and RNA during the labeling incubation. Withdrawn oviduct tissue fragments were preincubated in culture for 3 h and then induced with estrogen for 4 h. The tissue then was transferred to medium containing  $[^3H]$ uridine (1 mCi/ml, 39 Ci/mmol) and incubated at  $41^{\circ}$ C with shaking for 3 to 20 min. After a 30-s wash in cold phosphate-buffered saline the tissue was extracted with 0.3 N perchloric acid and the specific activity of UTP ( $\bigcirc$ ) was determined as described under "Experimental Procedures." Total RNA was also isolated and its specific activity was determined ( $\bigcirc$ ).

FIG. 4. In vivo induction with progesterone. Groups of chicks were injected with either 1 mg (*Panels A* and *C*) or 0.1 mg (*Panels B* and *D*) of progesterone dissolved in propylene glycol. The rates of mRNA<sub>con</sub> and mRNA<sub>ov</sub> synthesis (histograms) and the accumulation of these specific mRNAs ( $\bigcirc$ ) were determined as in Fig. 3. Filter hybridizations were done in duplicate with ~1 × 10<sup>6</sup> cpm input radioactivity.





FIG. 6. Estrogen induction in culture. Oviduct tissue fragments were incubated for 3 h and then induced at 0 h by the addition of 30 nM estradiol as described under "Experimental Procedures." At various times during the induction tissue was removed to measure mRNA<sub>ov</sub> and mRNA<sub>con</sub> sequences by hybridization to specific cDNAs ( $\odot$ ). Tissue samples were also incubated for 20 min in medium containing [<sup>3</sup>H]uridine (1 mCi/ml) as in Fig. 5 and the rates of mRNA<sub>ov</sub> and mRNA<sub>con</sub> synthesis were determined (histograms). The range of duplicate hybridizations is shown by the *error bars*. The input radioactivity in each hybridization ranged from 2 to 4 × 10<sup>6</sup> cpm. The relative rates of mRNA synthesis were converted to absolute rates (see "Results") and theoretical mRNA accumulation curves were calculated assuming a mRNA  $t_{1/2} > 20$  h (---) or a  $t_{1/2}$  of 8 h (-...). *Panels A* and *B* show the induction of mRNA<sub>ov</sub> and mRNA<sub>con</sub>, respectively.

(10) was used to measure the absolute rates of  $m RNA_{\rm ov}$  and mRNA<sub>con</sub> synthesis during the steroid-mediated induction in vitro. Oviduct fragments from 2- to 3-day-withdrawn chicks were incubated in culture medium for 3 h as described under "Experimental Procedures" and then induced with either 30 nM estradiol or 30 nM progesterone. At the indicated times, tissue samples were pulse-labeled with  $[^{3}H]$ uridine (1 mCi/ ml) for 20 min and the rates of  $mRNA_{ov}$  and  $mRNA_{con}$ synthesis were determined by hybridizing the radioactive RNA to filters containing immobilized plasmid DNA. In order to convert relative rates of mRNA transcription to absolute rates, the average specific activity of intracellular UTP was determined during the 20-min labeling incubation using the enzymatic assay outlined under "Experimental Procedures." Fig. 5 shows the specific activity of UTP and of total RNA as a function of time. The average UTP specific activity during the 20-min incubation was determined from the area under the UTP curve and this value was used to calculate the picomoles of UTP incorporated into RNA. The calculation shown below converts a relative rate of 100 ppm for mRNA<sub>av</sub> synthesis to an absolute rate of 0.8 molecule/cell/min:

$$\frac{100 \text{ ppm}}{10^6} \times \frac{1.6 \times 10^5 \text{ dpm}^a}{\mu \text{g RNA}} \times \frac{1.15 \mu \text{g RNA}^b}{\mu \text{g DNA}} \times \frac{1 \text{ pmole/UTP}^a}{3608 \text{ dpm}}$$
$$\times \frac{1.2 \times 10^9 \text{ molecules}^c \text{ mRNA}_{ov}}{\text{pmol UTP}} \times \frac{1 \mu \text{g DNA}^d}{4 \times 10^5 \text{ cells}} \times 1.05^e$$

= 16 molecules/cell/20 min = 0.8 molecule/cell/min

" Data were taken from Fig. 5.

<sup>b</sup> RNA/DNA ratio = 1.15 (determined experimentally).



FIG. 7. Progesterone induction in culture. The culture conditions and methods are the same as in Fig. 6 except that 30 nm progesterone was added instead of estradiol at 0 h. The data from two separate experiments is shown. Experiment 1 is represented by open histograms and closed circles (•) and Experiment 2 is represented by cross-hatched histograms and open circles (O). The input radioactivity in the filter hybridization ranged from 2 to  $4 \times 10^6$  cpm. The error bars indicate the range of duplicate hybridizations. The theoretical accumulation curves for mRNA<sub>ov</sub> and mRNA<sub>con</sub> are calculated as in Fig. 6.

<sup>c</sup> 1 pmol/UTP incorporated =  $1.2 \times 10^9$  molecules of mRNA<sub>ov</sub> determined from the complete sequence of mRNA<sub>ov</sub> (28).

<sup>d</sup> Assuming 2.5 pg of DNA/cell.

 $^{\rm e}$  Correction for the fact that the plasmid pCR1-OV contains only 95% of the mRNA  $_{\rm ov}$  sequence (29).

A similar calculation for mRNA<sub>con</sub> synthesis converts 100 ppm to an absolute rate of 0.57 molecules/cell/min, assuming that mRNA<sub>con</sub> is 2500 nucleotides in length and has a base composition similar to mRNA<sub>ov</sub> and that 100% of the mRNA sequence is contained in the recombinant plasmid pBR322-CON.

The induction of mRNA<sub>ov</sub> and mRNA<sub>con</sub> in cultured oviduct fragments after the addition of estradiol to the medium is shown in Fig. 6. The relative rates of mRNA<sub>ov</sub> synthesis shown in Fig. 6A were converted to absolute rates as described above, assuming that the rate of total RNA synthesis/cell does not change during the first 8 to 10 h of induction in culture. This was verified experimentally by measuring the incorporation of  $[^{3}H]UMP/\mu g$  of DNA at each time. A theoretical accumulation curve for mRNA<sub>ov</sub> was estimated from the absolute rates of mRNA<sub>ov</sub> synthesis, assuming that degradation of newly synthesized mRNA<sub>ov</sub> is negligible during the 10-h induction (*i.e.*  $t_{1/2} > 20$  h) and this is indicated by the dashed line (Fig. 6). The actual concentrations of mRNA<sub>ov</sub> determined by cDNA hybridization are shown as data points and they agree closely with the accumulation of mRNA<sub>ov</sub> predicted from the absolute rates of mRNA<sub>ov</sub> synthesis. The induction of  $mRNA_{con}$  is shown in Fig. 6B; in this case the theoretical accumulation curve calculated by assuming no degradation does not agree with the experimental data. If a  $t_{1/2}$  of 8 h for mRNA<sub>con</sub> is introduced into the theoretical accumulation curve, a much closer approximation to the data points is observed as shown by the stipled curve in Fig. 6B.

A similar experiment is shown in Fig. 7 in which progester-

one was used as the inducing steroid. Again the predicted accumulation for mRNA<sub>ov</sub> was in good agreement with the experimental data. The observed accumulation of mRNA<sub>con</sub> deviated from theoretical curves which assumed either no degradation or a  $t_{1/2}$  of 8 h; the rate of accumulation appeared to undergo a transition at 4 h similar to that seen *in vivo* after progesterone stimulation (see Fig. 4*C*).

The half-life of mRNA<sub>con</sub> in the absence of added steroids can be estimated by assuming that the level of mRNA<sub>con</sub> in withdrawn oviduct (50 molecules/cell) is a steady state level (30). The measured rate of mRNA<sub>con</sub> synthesis before the addition of steroid is about 36 ppm or 12 molecules/cell/h and, therefore, the half-life of mRNA<sub>con</sub> is equal to (50 molecules/cell × ln 2)/(12 molecules/cell/h) or 2.9 h. If this value for the  $t_{1/2}$  of mRNA<sub>con</sub> is correct, it indicates that mRNA<sub>con</sub> is degraded rapidly in the absence of steroid and suggests that both estrogen and progesterone act not only to increase the rate of mRNA<sub>con</sub> transcription but also to stabilize this mRNA<sub>con</sub> observed approximately 4 h after giving progesterone may reflect a lag before the hormone acts to stabilize the induced mRNAs.

#### DISCUSSION

Although other methods have been devised for measuring transcription rates from specific genes, the use of recombinant DNA immobilized on nitrocellulose filters gives an assay which is highly specific and more convenient than previous methods. One advantage of this technique is that it does not require the transcription of isolated nuclei or chromatin and can be applied equally well to RNA pulse-labeled in tissue culture. The rates of mRNA<sub>ov</sub> and mRNA<sub>con</sub> transcription were measured during hormonal induction either by pulselabeling the oviduct explant cultures or by allowing isolated oviduct nuclei to continue transcription in vitro. We assume that the assays of mRNA synthesis in isolated nuclei reflect rates of transcription in intact cells. This is borne out by the close correspondence in the kinetics and relative rates of mRNA synthesis measured in nuclei and in cultured oviduct explants. Since the half-life of mRNA  $_{\rm ov}$  changes from 24 h in the presence of steroid (31) to 2 to 4 h in the absence of steroid (32), we were concerned that changes in mRNA accumulation might be entirely due to changes in the rate of mRNA degradation. While this possibility cannot be eliminated it is very unlikely since mRNA<sub>ov</sub> synthesis was not detected in withdrawn nuclei either after short incubation times (Fig. 2) or in the presence of sarkosyl or heparin (Fig. 1). We conclude that the hormone-induced accumulation of  $mRNA_{ov}$  and  $mRNA_{con}$ is at least partially due to an increase in their rates of transcription. The hormone-induced increase in specific  $mRNA_{ov}$ synthesis in the oviduct has now been observed in several laboratories using a variety of techniques (9, 33). Recently Swanek et al. (34) have confirmed this induction using a filter hybridization method similar to that used in this report. They have also determined the kinetics of induction of mRNA<sub>ov</sub> synthesis in vivo and find a rapid return to fully induced rates of synthesis in 8 h. Our results differ in that after 2 to 3 days of withdrawal we find a much slower reinduction; this may be due to the decline in functional estrogen receptors during withdrawal (see below). We have recently observed that birds withdrawn from estrogen for less than 2 days reinduce much more rapidly.<sup>6</sup>

The relative rates of mRNA synthesis were measured by *in* vitro transcription of oviduct nuclei after *in vivo* administration of estrogen to 2-day-withdrawn chicks. The results (Fig.

3) show that estrogen induced mRNA<sub>con</sub> synthesis within 30 min in parallel with the appearance of estrogen receptors in the nucleus. In contrast, mRNA<sub>ov</sub> synthesis was not activated immediately, suggesting that there must be intermediate events between localization of the receptor in the nucleus and activation of mRNA<sub>ov</sub> transcription.

The rates of mRNA<sub>con</sub> and mRNA<sub>ov</sub> transcription achieved during the first 12 h of restimulation range from 10 to 25% of the fully stimulated values which are 800 ppm for mRNA<sub>con</sub> and 1540 ppm for mRNA<sub>ov</sub> synthesis. This result was unexpected since the tubular gland cells which synthesize both ovalbumin and conalbumin (35) are maintained during the first 3 days of withdrawal from estrogen.<sup>7</sup> However, it is clear that the number of functional estrogen receptors declines from a fully stimulated level of about 7000 to 8000 molecules/cell (11) to approximately 1300 molecules/cell after 2 days of withdrawal (Fig. 3*C*), suggesting that receptors are rate-limiting for gene induction during restimulation.

A simple relationship between the number of nuclear estrogen receptors and the specific transcription rates can be derived which approximates the experimental data obtained during restimulation (Fig. 3), although it does not predict the ~2-h lag before mRNA<sub>ov</sub> transcription begins. We assume that the 8000 receptor binding sites present in fully stimulated oviduct nuclei are still available in withdrawn oviduct nuclei and that, if they were completely filled with receptor, the rates of mRNA<sub>con</sub> and mRNA<sub>ov</sub> transcription would equal the fully stimulated values given above. The rate of mRNA<sub>con</sub> transcription during restimulation, when receptors are limiting, appears to be related to the probability that one specific site is filled with receptor:

mRNA<sub>con</sub> synthesis (ppm)

$$= \frac{[\text{nuclear estrogen receptors}]}{8000 \text{ sites}} \times 800 \text{ ppm}$$

The rate of  $mRNA_{ov}$  synthesis is predicted empirically if we assume that two sites must be occupied by estrogen receptors to allow gene activation:

mRNA<sub>ov</sub> synthesis (ppm)

$$= \left[\frac{[\text{nuclear estrogen receptors}]}{8000 \text{ sites}}\right]^{2} \times 1540 \text{ ppm}$$

These relationships are also consistent with the dose-response data previously obtained (11) in which 50% receptor saturation gave half-maximal conalbumin induction but only 15 to 25% of maximal ovalbumin induction. Whether these empirical relationships have any biological significance in terms of receptor sites remains an interesting question for future work. Other theoretical relationships between nuclear receptor levels and the number and affinity of specific regulatory sites have been described by Yamamoto and Alberts (36).

The early time course of hormonal activation of mRNA<sub>ov</sub> and mRNA<sub>con</sub> transcription was also examined in oviduct explants cultures *in vitro*. Transcription from both the ovalbumin and conalbumin genes was increased by estrogen at 1 h and reached a maximal rate by 2 h (Fig. 6). This somewhat more rapid induction of mRNA<sub>ov</sub> synthesis and mRNA<sub>ov</sub> accumulation in culture compared to the *in vivo* response (Fig. 3A) only occurred under optimal culture conditions in the presence of insulin. From this and other data it appears that the lag in mRNA<sub>ov</sub> induction following estrogen administration reflects the physiological state of the tissue. We are currently interested in whether insulin affects the events

<sup>7</sup> J. Shepherd, E. Mulvihill, P. Thomas, and R. Palmiter, manuscript in preparation.

<sup>&</sup>lt;sup>6</sup> G. S. McKnight and R. D. Palmiter, unpublished data.

which determine the lag. The induction of mRNA<sub>ov</sub> transcription by progesterone in culture (Fig. 7A) gave nearly identical results to those observed *in vivo* (Fig. 4A). There was a 1.5-h lag and the rate of mRNA<sub>ov</sub> transcription reached a maximal level by 3 h. The response of the conalbumin gene to progesterone in culture (Fig. 7B) was also quite similar to the *in vivo* response (Fig. 4C). However, the kinetics of mRNA<sub>con</sub> accumulation after progesterone cannot be explained entirely by the changes in mRNA<sub>con</sub> transcription, suggesting that effects on mRNA<sub>con</sub> stability are also occurring.

Several other observations indicate that estrogen and progesterone have significant effects on mRNA stability as well as on transcription. We have converted the relative rates of mRNA transcription measured in culture to absolute transcription rates by correcting for the specific activity of the UTP precursor pool during the pulse-label incubation. This has allowed us to calculate the maximum rate of specific mRNA accumulation, assuming no degradation of newly synthesized mRNA. A comparison of these theoretical accumulation curves with the actual data demonstrates that in the presence of either estrogen or progesterone, mRNAov accumulates as predicted and, therefore, must have a half-life greater than 20 h. The half-life of mRNA<sub>con</sub> appears to be changing during the inductions but is at least 8 h at later times. In the absence of either hormone, mRNA<sub>con</sub> has a calculated  $t_{1/2}$  of 2.9 h using steady state equations. Similar calculations are not possible for mRNA<sub>ov</sub> since in the withdrawn chick the basal level of mRNA<sub>ov</sub> transcription is below the level of sensitivity of our assay. However, the in vivo experiment with a suboptimal dose of progesterone (Fig. 4B) clearly indicates that mRNA<sub>ov</sub> is degraded when its rate of transcription falls. It should now be possible to make direct measurements of the  $t_{1/2}$  of mRNA<sub>con</sub> and mRNA<sub>ov</sub> in the culture system by using pulse-chase techniques similar to those recently described by Lowanhaupt and Lingrel (37) for measuring the  $t_{1/2}$  of globin mRNA. Nevertheless, it is already clear that steroid hormones regulate the accumulation of egg white mRNAs by coordinately increasing transcription and decreasing degradation of these specific mRNAs.

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