Commitment of Chick Oviduct Tubular Gland Cells to Produce Ovalbumin mRNA during Hormonal Withdrawal and Restimulation

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ABSTRACT Acute withdrawal of estrogen from chicks leads to a precipitous decline in egg white protein synthesis and egg white mRNAs in the oviduct. In this paper we explore the biochemical basis of this phenomenon as well as the capacity of the "withdrawn" tubular gland cells to be restimulated with steroid hormones. During withdrawal, the decline in ovalbumin mRNA was closely correlated with the decline in nuclear estrogen receptors. Within 2–3 d of estrogen removal a withdrawn state was established and then maintained, as defined by a 1,000-fold-lower level of ovalbumin mRNA and a 20-fold-lower level of nuclear estrogen receptors, relative to the estrogen-stimulated state. The number of active forms I and II RNA polymerases declined by 50% during this time. Histological examination of oviduct sections and cell suspensions, combined with measurements of DNA content, revealed that tubular gland cells persisted as a constant proportion of the cell population for 3 d after estrogen removal. Despite a 1,000-fold decrease in the content of ovalbumin mRNA, the ovalbumin gene remained preferentially sensitive to digestion by DNase I. When 3-d-withdrawn oviducts were restimulated with either estrogen or progesterone, *in situ* hybridization revealed that ≥98% of the tubular gland cells contained ovalbumin mRNA. Induction by a suboptimal concentration of estrogen was correlated with a lower concentration of ovalbumin mRNA in all cells rather than fewer responsive cells.

Oviduct differentiation is induced and maintained in sexually immature female chicks by administration of steroid hormones. The initial induction requires estrogens; it results in the differentiation and proliferation of tubular gland cells located in the magnum portion of the oviduct and the synthesis of egg white proteins (15, 26, 27, 35). Tubular gland cells eventually comprise the majority of the magnum cell population (10, 29), and they have been identified immunochemically as the producers of the major egg white proteins, including ovalbumin, conalbumin, ovomucoid, and lysozyme (14, 31). When administration of estrogen is halted, the oviduct regresses and production of egg white protein ceases (7, 12, 27, 28, 30, 31); however, production can be reinduced in the remaining tubular gland cells by both estrogen and nonestrogenic steroid hormones and is attributable to the accumulation of specific mRNAs (5, 8, 12, 20, 29). Thus, as steroid hormones are administered or withdrawn, the chick oviduct grows or regresses, much as occurs naturally in laying hens (49).

The advent of techniques for rapid (acute) withdrawal of estrogen affords an opportunity to study the deinduction of specific gene expression, as well as the fate of the tubular gland cells. Previous studies of the withdrawal process have established that the decline in egg white protein synthesis is paralleled by a loss of the respective mRNAs, that the half-life of egg white mRNAs is considerably shorter during withdrawal than in the presence of estrogen, and that egg white mRNAs are selectively degraded (7, 12, 30). In this study the kinetics of degradation of $mRNA_{ov}$[1] are compared with the loss of $E \cdot R_n$. Our observations suggest that within 2–3 d of acute removal of estrogen, a withdrawal state is established, as defined by a

[1] *Abbreviations used in this paper:* $mRNA_{ov}$, ovalbumin mRNA; $E \cdot R_n$ nuclear estrogen receptors; $cDNA_{ov}$, [^{3}H]DNA complementary to $mRNA_{ov}$; $cDNA_{gb}$, [^{3}H]DNA complementary to globin mRNA.

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1,000-fold-lower level of $mRNA_{ov}$ and a 20-fold-lower level of $E \cdot R_n$. Evidence from histological examination and measurement of DNA content is presented that demonstrates that tubular gland cells are maintained during the first 3 d of withdrawal, but that subsequently the majority are lost from the oviduct. During the period of withdrawal when the oviduct cell population is stable we ask whether all tubular gland cells remain committed to produce $mRNA_{ov}$. We examine whether the DNase I–sensitive configuration of the ovalbumin gene, which is characteristic of the stimulated oviduct (1, 9), is retained in the "withdrawn" oviduct. By *in situ* hybridization we quantitate the ability of individual tubular gland cells to produce $mRNA_{ov}$ upon restimulation with estrogen or progesterone.

MATERIALS AND METHODS

Animals, Hormone Regimen, and Terminology

Female White Leghorn chicks were stimulated with estrogen by subcutaneous implantation of one (15 mg) hexestrol pellet per chick for a minimum of 10 d (30). We call this initial exposure to estrogen "primary stimulation." In the majority of experiments reported here, chicks were pelleted at 2 wk of age and then withdrawn from primary stimulation by pellet removal at 3.5–6 wk of age, when they weighed between 200 and 400 g. When primary stimulation was longer than 3 wk, the original pellet was removed and a fresh pellet implanted at least 4 d before withdrawal. In a few experiments an alternative regimen was used: 3-d-old chicks were given primary stimulation for 10–14 d (hexestrol or diethylstilbestrol), withdrawn for several weeks, repelleted (hexestrol) for 4–5 d (termed "secondary stimulation"), and withdrawn again. For studies of the first 10 h of hormone stimulation, steroid hormones were injected subcutaneously in corn oil (24). 17 β-estradiol benzoate and progesterone were administered at a dose of 5 mg/kg body weight, based upon earlier dose response studies (24, 27, 28). Tamoxifen was given at a dose of 10 mg per chick (34).

To simplify the terminology of the hormone regimen, we use the following conventions. All estrogenic compounds (hexestrol, diethylstilbestrol, 17 β-estradiol benzoate) are referred to as estrogen in the text. Removal of an estrogen pellet is termed withdrawal, and may be from primary or secondary estrogen stimulation. We often refer to pellet removal used here as acute withdrawal, to distinguish it from slow withdrawal, which occurs upon cessation of injections in oil (27, 28, 31, 38, 40, 46). All studies of oviduct tissue are restricted to the magnum of the oviduct, which is the central region capable of egg white production.

In retrospect, our use of hexestrol implants probably did not provide maximal stimulation of oviducts. The histological appearance of these oviducts was similar to that reported previously, after daily injections of 17 β-estradiol benzoate or diethylstilbestrol (15, 26, 35). However, tubular gland cells were 40–65% of the population, vs. 80–85% observed previously (10, 29). In this study we measured 3,000–4,000 molecules of $E \cdot R_n$ per cell in hexestrol-stimulated oviducts, which is about half that reported previously (24) but comparable when the proportion of tubular gland cells is taken into account.

Isolation of Nuclei and Quantitation of Nuclear Hormone Receptors

The isolation of oviduct nuclei and quantitation of $E \cdot R_n$ by exchange assay are detailed by Mulvihill and Palmiter (24).

Quantitation of $mRNA_{ov}$ by Hybridization

Total nucleic acid was isolated by SDS-proteinase K digestion of oviduct homogenates, followed by phenol-chloroform extraction and ethanol precipitation as described (18). The concentration of DNA in the total nucleic acid preparation was determined by a micromodification of the diphenylamine assay (4) or by a fluorometric assay (44).

The purification of $mRNA_{ov}$ and synthesis of $cDNA_{ov}$ are described by Lee et al. (16). Preparations of $cDNA_{ov}$ were supplied by G. S. McKnight and R. Moen; the purity, size, and hybridization characteristics of these preparations have been documented (18). Hybridization of $cDNA_{ov}$ with total nucleic acid, quantitation of hybrids by use of S_1 nuclease, and calculation of the number of $mRNA_{ov}$ molecules per cell were performed as described (18).

Digestion of Nuclei with DNase I and Quantitation of Ovalbumin and Globin DNA by Hybridization

Nuclei were treated with DNase I (Worthington Biochemical Corp., Freehold, N. J.) by a modification of the procedure described by Weintraub and Groudine (48). Nuclei were suspended in RSB buffer (10 mM Tris-Cl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$) at 0.5 mg of DNA/ml and incubated with DNase I (10 μg/ml) for 2.5 min at 37°C. Reactions were terminated by the addition of EDTA to 10 mM and transfer to 0°C. The percent DNA digested was determined from the absorbance at 260 nm of the acid soluble and acid insoluble fractions.

Nucleic acid was isolated from DNase I–treated nuclei as described above, incubated in 0.3 N NaOH for 10 min at 100°C, transfered to a second tube and incubated for another 10 min at 100°C (to ensure complete hydrolysis of RNA), neutralized, and ethanol precipitated. Total chick DNA (prepared from chick erythrocytes; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) was also base hydrolyzed and ethanol precipitated.

Globin mRNA was prepared essentially as described (17) and copied with reverse transcriptase to produce a complementary [^{3}H]DNA ($cDNA_{gb}$), using conditions established for $cDNA_{ov}$ (16). Hybridization of $cDNA_{ov}$ and $cDNA_{gb}$ with DNA and quantitation of hybrids by use of S_1 nuclease were performed as described (18). For presentation of the data in graph form, the fraction hybridized (H) was expressed as $H/(1 - H)$, as suggested by Garel and Axel (9).

Quantitation of Nucleotide Incorporation in Oviduct Nuclei

Assays of nucleotide incorporation were performed in a final volume of 50–125 μl containing 140 mM KCl, 20 mM Tris-Cl (pH 8.2), 3 mM $Mg(CH_2CO_2)_2$, 0.5 mM dithiothreitol, 10% (vol/vol) glycerol, 0.2–0.5 mg of DNA/ml as nuclei, 0.4 mM (each) ATP, GTP, and UTP, 1.8–2.5 μM [^{3}H]CTP (Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg N. Y.; 18–21 Ci/mmol), and 1 μg/ml α-amanitin where indicated. Alternatively, assays contained 0.4 mM CTP and 1.8–2.8 μM [^{3}H]UTP (New England Nuclear, Boston, Mass.; 28 Ci/mmol). After the addition of nuclei, samples were incubated for 5 min at 4°C, then transferred to 26°C for the times indicated in the figure legends. Reactions were terminated by acid precipitation and the precipitates were collected, washed, and counted. Background radioactivity was determined by the addition of 10 mM EDTA before incubation. The DNA content of nuclei preparations was assayed by a micromodification of the diphenylamine assay (4).

Incorporation of [^{3}H]UTP into internal vs. 3′ termini of RNA chains was quantitated by base hydrolysis of in vitro synthesized RNA and separation of UMP and uridine by thin-layer chromatography as described by Cox (6), with the following modifications. Reaction mixtures were incubated at 26°C under the conditions described above and either terminated by acid precipitation or further incubated for 3 min at 26°C in the presence of at least 0.1 mM unlabeled UTP before termination. Nucleotide incorporation at the salt concentration employed, in the absence of heparin, eliminated the need for charcoal extraction of the hydrolysate. The comparison of parallel reactions plus or minus a chase with excess unlabeled UTP allowed for monitoring of the artificial generation of 3′ termini during in vitro incubation or sample handling, thus obviating the need for separately quantitating and correcting for RNA degradation.

Histology

After rapid dissection of oviduct tissue, central portions of magnum were placed in fixative, consisting of 3% glutaraldehyde in 100 mM sodium cacodylate, pH 6.8, and 5 mM $CaCl_2$. Cross sections (1–2 mm thick) were quickly cut with a razor blade and left in the fixative for 1 h at 20°C, then rinsed with the primary fixative and postfixed with 2% osmium tetroxide in 100 mM sodium cacodylate, pH 6.8, 5 mM $CaCl_2$ for 0.5 h at 20°C. After fixation, tissue fragments were rinsed with distilled water, stained with 1% uranyl acetate for 1 h at 4°C in the dark, dehydrated through a series of alcohols from 70 to 100%, cleared with propylene oxide, and embedded in Spur (Polysciences, Inc., Warrington, Pa.). For light microscopy, 1-μm sections were cut on an Ultratome (LKB Instruments, Inc., Rockville, Md.) and stained with Toluidine Blue.

Preparation of Oviduct Cell Suspension and Detection of $mRNA_{ov}$ by In Situ Hybridization

To prepare cell suspensions from oviduct magnum, finely minced tissue (50 mg) was incubated in a total volume of 5 ml containing 20 mM HEPES (pH 7.5), 130 mM NaCl, 2.5 mg/ml pronase (Sigma Chemical Co., St. Louis, Mo.), 0.5

mg/ml collagenase (Worthington Biochemical Corp.; crude grade) in a shaking water bath at 37°C for 15 min. At 5-min intervals fragments were further dispersed by repeated passage in and out of a 5-ml pipette. Digestion was terminated by dilution (1:1) with ice-cold Ham's Nutrient Mixture F-10 (Grand Island Biological Co., Grand Island, N. Y. [GIBCO]), supplemented with 1.2 mg/ml $NaHCO_3$ and 10% fetal calf serum (GIBCO). Undigested fragments were removed by brief centrifugation. The cells released by protease digestion were washed by centrifugation through a pad of fetal calf serum, resuspended in medium, and dispersed by passage through a 10-gauge steel needle. Cells were viewed by phase-contrast microscopy and scored as: (*a*) tubular gland cells, recognized by secretory granules, (*b*) ciliated cells, which remained mobile for several hours, (*c*) erythrocytes, or (*d*) other cell types, which included fibroblasts, leukocytes, and goblet cells.

In situ hybridization of oviduct cells was carried out by modification of procedures described for mouse liver (11) and thymus (39) cells. A 1-μl drop of an oviduct cell suspension, containing ~5 × 10^4 cells, was placed on an acid-cleaned glass slide, gently spread into a 15-mm^2 area, and air-dried; drops from several cell suspensions were positioned adjacent to one another. Air-dried cells were fixed in absolute methanol for 3 min at 20°C, treated with 0.2 N HCl for 25 min at 20°C, and dehydrated in 50, 70, and 90% ethanol. Cells were hybridized *in situ* with $cDNA_{ov}$, prepared as described above except that [^{3}H]dTTP (New England Nuclear, 100 Ci/mmol) was used for labeling. Cells were overlaid with $cDNA_{ov}$ (~10,000 cpm/μl, with 5 μl per 80 mm^2 area) in 3 × NT (3 × NT = 0.5 M NaCl, 50 mM triethanolamine, pH 7.8) with 2 mM EDTA and 33% formamide; a coverslip was added and slices were incubated in a moist environment for 18 h at 45°C. After hybridization, slides were washed with 2 × NT (=2/3[3 × NT]) at 20°C, incubated with 2 × NT for 1 h at 55°C, treated with S_1 nuclease for 1 h at 37°C (21), and extensively washed with 2×NT at 4°C, to remove free and nonspecifically bound $cDNA_{ov}$. Finally, slides were processed for autoradiography, which included coating with Kodak NTB-2 emulsion, exposure for 1–2 wk at 4°C, and staining of cells with MacNeal's tetrachrome.

RESULTS

The Content of $mRNA_{ov}$ and $E \cdot R_n$ during Withdrawal

The concentration of $mRNA_{ov}$ and $E \cdot R_n$ during withdrawal of chick oviducts from estrogen stimulation is shown in Fig. 1. In 250-g chicks, we observed a lag of 11 h before the decline of $mRNA_{ov}$, which coincided with a lag of 10 h preceding the onset of $E \cdot R_n$ loss. After the lag, $mRNA_{ov}$ declined with a $t_{1/2}$ of 6 h and $E \cdot R_n$ with a $t_{1/2}$ of 10 h. Similar results were obtained in 350-g chicks, except that after the lag $mRNA_{ov}$ declined with a $t_{1/2}$ of 3.3 h. Upon withdrawal of intermediate size chicks (~300 g) a $t_{1/2}$ of 4–5 h was observed for $mRNA_{ov}$ (data not shown). New steady-state levels of $mRNA_{ov}$ and $E \cdot R_n$ were established within 3 d of pellet removal (Fig. 1). The level of $mRNA_{ov}$ averaged 24,000 molecules/cell in stimulated oviducts and declined to 8–10 molecules/cell by 2 or 3 d of withdrawal. The level of $E \cdot R_n$ averaged 3,100 molecules/cell in stimulated oviducts; between 3 and 25 d of withdrawal, the level ranged from 70 to 200 molecules/cell. When the antiestrogen, tamoxifen, was injected at the time of pellet removal, the decline in $E \cdot R_n$ began immediately and the lag before the decline in $mRNA_{ov}$ was shortened to ~3 h.

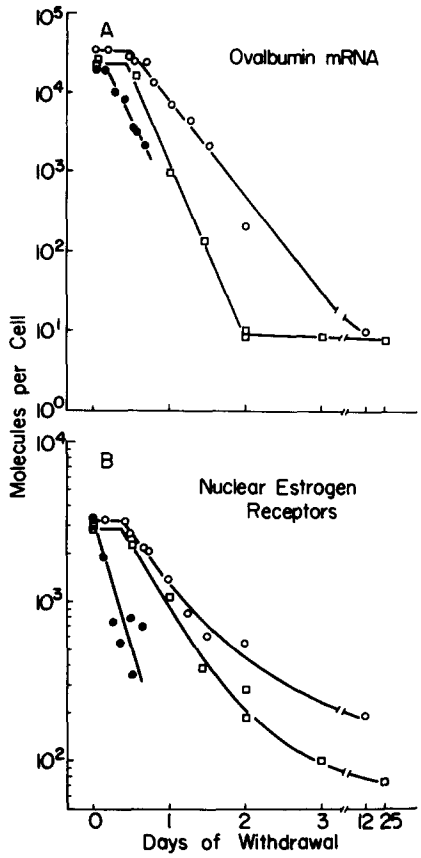

FIGURE 1 Decline of $mRNA_{ov}$ and $E \cdot R_n$ over 25 d of withdrawal and in the presence of tamoxifen. Chicks were given primary estrogen (hexestrol) stimulation and then withdrawn by pellet removal, and were 4 wk old at the time of sacrifice, when they averaged 250 g (○), or were 6 wk old and weighed 350 g (□). Alternatively, chicks were withdrawn by pellet removal plus injection of tamoxifen (●). Portions of oviduct tissue were used to isolate nucleic acid for quantitation of $mRNA_{ov}$ (*A*) and nuclei for assay of $E \cdot R_n$ (*B*). Each point is the mean of three ($mRNA_{ov}$) or five ($E \cdot R_n$) determinations on preparations from three or four chicks.

RNA Synthesis during Withdrawal

We measured the synthesis of total RNA, by following the incorporation of [^{3}H]ribonucleotides into oviduct nuclei in vitro. Under the assay conditions outlined in Materials and Methods, ribonucleotide incorporation: (*a*) was linear for at least 10 min, using nuclei from either hormone-stimulated or withdrawn oviducts; (*b*) was proportional to DNA concentration; (*c*) required all four ribonucleotides, was sensitive to ribonuclease A, and was 90% inhibited by actinomycin D; and (*d*) ~50% of incorporation was sensitive to α-amanitin at 1 μg/ml, whereas the remainder was resistant to concentrations of α-amanitin as high as 200 μg/ml. Incorporation sensitive to α-amanitin was attributed to form II RNA polymerase, whereas resistant incorporation was due predominantly to form I rather than form III RNA polymerase (47). Fig. 2 illustrates the rate of RNA synthesis as a function of the time of withdrawal, relative to the content of $E \cdot R_n$. By 2 or 3 d of withdrawal, CMP incorporation was 40–50% of that observed in stimulated oviducts. This decline in total incorporation reflected similar declines in α-amanitin-sensitive and -resistant incorporation, and was observed in the presence of 1 mg/ml heparin (data not shown).

The data described above demonstrate that the loss of $E \cdot R_n$ during withdrawal was correlated with an ~50% reduction in nuclear RNA synthesis. To determine whether this reduction resulted from a change in the number of active RNA polymerases or the rate of elongation, we quantitated incorporation into 3′ termini vs. internal residues of RNA chains. Nuclei were pulse-labeled with [^{3}H]UTP, and the acid-precipitable material was base-hydrolyzed and chromatographed on polyethylimine cellulose (EM Laboratories, Elmsford, N. Y.) to separate UMP (internal residues) from uridine (3′ termini). To control for the nonspecific conversion of UMP to uridine by ribonucleases or phosphatases, parallel reactions were followed by a chase with excess unlabeled UTP. The kinetics of UTP

incorporation into uridine and UMP in hen oviduct nuclei are illustrated in Fig. 3 to demonstrate the method of analysis. Incorporation into UMP increased linearly for 10 min and was unaltered by a chase with unlabeled UTP. The amount of radioactivity recovered as [^{3}H]uridine increased with time, but the amount that was sensitive to a cold UTP chase remained constant. We interpret the difference between the pulse and chase levels of [^{3}H]uridine to reflect the number of active RNA polymerases, whereas the increase in nonchaseable [^{3}H]uridine is most likely attributable to posttranscriptional degradation of labeled RNA molecules generating new 3′ ends.

An analysis of incorporation into uridine and UMP at three times of withdrawal is presented in Table I. The decline in incorporation into UMP was accompanied by a decline in incorporation into uridine, for total incorporation as well as α-amanitin-sensitive and -resistant fractions. The ratio of UMP to uridine did not change. We interpret these results as indicating that the 50% decline in RNA synthesis observed by 3 d of withdrawal is attributable to a decline in the number of active RNA polymerases, both forms I and II, rather than a decline in the rate of elongation.

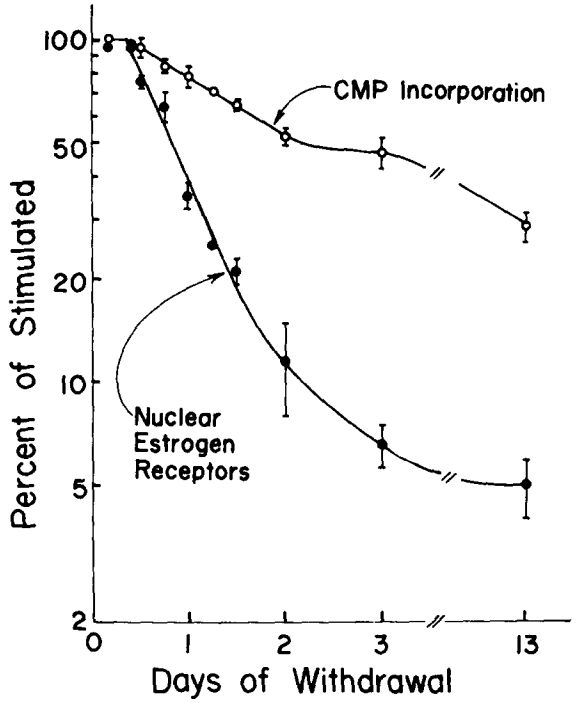

FIGURE 2 CMP incorporation of oviduct nuclei relative to E·R_n content during withdrawal from estrogen stimulation. Data are presented from six experiments: for each experiment oviducts were combined from 3–10 chicks at the same time of withdrawal, nuclei were prepared and assayed five times for E·R_n (●) and three times for CMP incorporation (○). Points with error bars indicate the mean ± the standard error for several experiments. Estrogen-stimulation values averaged 0.15 pmol/μg DNA per 10 min for CMP incorporation and 3,300 molecules/cell for E·R_n.

Stability of the Oviduct Cell Population during Withdrawal

We examined the stability of the oviduct cell population by measurement of oviduct DNA content, histological examination of oviduct sections, and quantitation of the proportion of tubular gland cells in cell suspensions. By 3 d of withdrawal, oviduct wet weight declined to 50% of the stimulated value; however, DNA content remained relatively constant (Table II). In this experiment, E·R_n declined from 3,100 to 100 molecules/cell by 3 d, $mRNA_{ov}$ dropped from 21,500 to 17 molecules/cell, and nuclear CMP incorporation fell to 47% of the stimulated level. The stability of DNA content suggests no net loss of oviduct cells during a time when the withdrawal state was

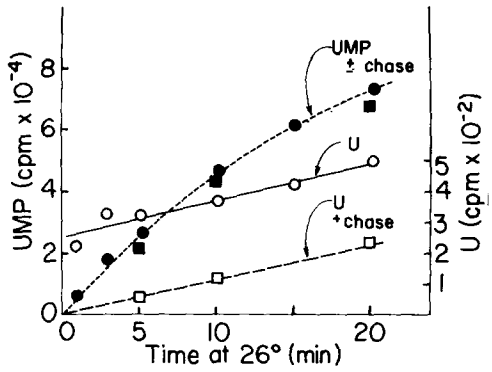

FIGURE 3 Time-course of incorporation of UTP into UMP and uridine (*U*) by oviduct nuclei. Nuclei were isolated from the oviducts of laying hens. Assays were carried out at 26°C with 0.5 mg DNA/ml as nuclei and 1.8 μM [^{3}H]UTP, essentially as described in Materials and Methods. At the indicated times, either samples were terminated by acid precipitation (○, ●), or excess unlabeled UTP was added and the reaction continued for another 3 min at 26°C before termination (□, ■). Radioactivity in UMP (closed symbols) and uridine (open symbols) was separated by thin-layer chromatography; note difference in ordinate scales. Incorporation is per 29 μg DNA.

TABLE I

Incorporation of UTP into Internal Residues vs. 3′ Termini of RNA Chains in Oviduct Nuclei, as a Function of Withdrawal

	Incorporation, *cpm/μg DNA/10 min*‡								
	Total			Form I RNA polymerase			Form II RNA polymerase		
Hours of withdrawal*	UMP	Uridine	UMP/uridine	UMP	Uridine	UMP/uridine	UMP	Uridine	UMP/uridine
0	1,240	9.1	140	530	4.9	110	710	4.2	170
18	960	6.3	150	390	3.6	110	570	2.7	210
71	640	4.0	160	250	2.3	110	390	1.7	230

* Oviducts were combined from three or four chicks. Data on E·R_n content are included in Fig. 2.

‡ Assays were carried out for 10 min at 26°C with 2.8 μM [^{3}H]UTP and 0.3 mg/ml DNA as nuclei, ± 1 μg/ml α-amanitin and ± a 3-min chase with excess unlabeled UTP. Total incorporation was determined in the absence of α-amanitin. Incorporation attributable to form I RNA polymerase was determined in the presence of α-amanitin, and incorporation attributable to form II RNA polymerase was calculated by difference. Incorporation into UMP (internal residues) was determined by averaging pulse and pulse-chase samples. Incorporation into uridine (3′ termini) was determined by difference ([pulse] − [pulse-chase]).

established. In contrast to this initial stability, longer times of withdrawal were accompanied by DNA loss: 50–60% of oviduct DNA was lost by 1–2 wk of withdrawal and 70–80% by 3–4 wk (Table II).

Microscope examination of oviduct sections at 0, 3, and 25 d of withdrawal supported the conclusions drawn from measurements of DNA content. Tubular gland cells of estrogen-stimulated oviducts were easily recognized because of their prominent secretory granules (15–20 granules/cell in the 1-μm sections; Fig. 4*a*) and their organization around lumina. Examination of sections revealed that tubular gland cells predominated in stimulated oviducts, although other cell types were observed: epithelial cells, including goblet and ciliated cells, and interstitial cells, including fibroblasts and blood cells. At 3 d of withdrawal, tubular gland cells could still be identified by their organization around lumina and the presence of secretory granules (Fig. 4*b*). As in stimulated oviducts, tubular gland cells were the predominant cell type, although several morphological changes were apparent. Tubular gland cells contained fewer secretory granules (3–5 granules/cell cross section). This reduction in secretory granules was often accompanied by the appearance of vacuoles that were unstained by periodic acid-Schiff or Toluidine Blue, and were thus presumably devoid of egg white proteins. Tubular gland cells were also decreased in size, by approximately one-half in cross-sectional area, whereas lumina were increased about fourfold in area. By 25 d of withdrawal, secretory granules were not seen in any oviduct cells (Fig. 4*c*). Cells organized around lumina were observed, and presumably these are tubular gland cells capable of responding to hormone restimulation; however, they were no longer the predominant cell type.

We further examined the stability of the oviduct population during 3 d of withdrawal by quantitating the proportion of tubular gland cells in cell suspensions. Cell suspensions were prepared from either estrogen-stimulated or 3-d-withdrawn oviducts and examined by phase-contrast microscopy; tubular gland cells were identified morphologically by their content of secretory granules. A quantitation of the distribution of cell types indicated that tubular gland cells averaged 54% of the cells from stimulated oviducts and 55% of the cells from withdrawn oviducts (Table III). Assuming that the distribution of cell types after isolation was representative of the entire oviduct population, we conclude that the proportion of tubular gland cells remained constant during 3 d of withdrawal.

TABLE II

Stability of the Oviduct Cell Population during Withdrawal

Experiment*	Length of withdrawal	Wet weight/oviduct magnum	DNA/oviduct magnum
	d	*g*	*mg*
A	0	0.91 ± 0.06	1.81 ± 0.21
	1	0.76 ± 0.06	1.68 ± 0.24
	2	0.60 ± 0.07	1.79 ± 0.11
	3	0.46 ± 0.05	1.67 ± 0.18
B	0	0.81	1.57
	6	0.18	0.69
C	0	0.54	0.93
	13	0.071	0.40
	25	0.051	0.30
D	0	0.73	1.40
	24	0.055	0.32

* Within a given experiment, chicks were stimulated with estrogen at the same age and for the same length of time (13–17 d). In experiment *A*, data on wet weight (two determinations) and DNA (three determinations) were collected on single oviducts, then combined from three or four oviducts withdrawn for the same time, and presented as the mean ± the standard error. In experiments *B–D*, oviducts were pooled from 2 to 10 chicks before measurement of wet weight and DNA.

DNase I Sensitivity of the Ovalbumin Gene

We examined the DNase I sensitivity of the ovalbumin gene in the chick oviduct during withdrawal from estrogen stimulation. Chick oviduct nuclei were treated with DNase I to solubilize 10–30% of the DNA, and ovalbumin DNA sequences resistant to digestion were quantitated by hybridization with $cDNA_{ov}$. Globin DNA was quantitated in parallel hybridizations with $cDNA_{gb}$, as a control for a gene that is not actively expressed in the oviduct. As can be seen in Fig. 5*a*, $cDNA_{ov}$ and $cDNA_{gb}$ hybridized to an equal extent to total chick DNA. Identical results were obtained with DNA isolated from oviduct nuclei that were undigested with DNase I (data not shown). With DNA isolated from estrogen-stimulated oviduct nuclei, predigested with DNase I, hybridization to $cDNA_{ov}$ was clearly reduced (Fig. 5*b*). The ratio of hybridization to $cDNA_{ov}$ vs. $cDNA_{gb}$ was 0.34; among four preparations from stimulated oviducts, this ratio ranged from 0.30 to 0.55, with a mean of 0.47. Enhanced sensitivity of the ovalbumin gene to DNase I was not observed in nuclei from stimulated livers (Fig. 5*c*). In oviducts withdrawn for 2 d from estrogen stimulation, the ratio of hybridization was 0.39 (Fig. 5*d*), indicating that the ovalbumin gene still was preferentially sensitive to DNase I. At this time, $mRNA_{ov}$ had declined from 22,000 to 10 molecules/cell. In another experiment we measured DNase I sensitivity at 0, 1, 2, and 3 d of withdrawal; the ratio of hybridization to $cDNA_{ov}$ vs. $cDNA_{gb}$ was constant at 0.5. The DNA content of these oviducts also was assayed and found to be stable during this time.

Restimulation of the Withdrawn Oviduct with Estrogen or Progesterone

The following experiments were performed to ascertain whether all of the tubular gland cells remain functionally stable during the first 3 d of withdrawal when the total cell population persists. Functional stability was assessed by restimulating with either the original inducing hormone, estrogen, or another hormone, progesterone. Analysis of the kinetics of $mRNA_{ov}$ accumulation over 10 h of restimulation (data not shown) revealed that $mRNA_{ov}$ began to accumulate after a lag of ~3 h, in response to a single injection of either hormone. With progesterone, the initial rate of $mRNA_{ov}$ accumulation was 17 molecules/min per cell; this rate equals the steady-state rate of $mRNA_{ov}$ production before withdrawal (calculated from the measured concentration of $mRNA_{ov}$ of 35,000 molecules/cell and a $t_{1/2}$ of 24 h, as described in reference 29). With estrogen, the rate of $mRNA_{ov}$ accumulation was ~40% of that observed with progesterone; the number of $E \cdot R_n$ reached only 60% of the prewithdrawal level. (Subsequent experiments have indicated that the dose of estrogen used here was suboptimal.) After 10 h of restimulation, the $mRNA_{ov}$ content of individual cells was assayed by hybridization with $cDNA_{ov}$ *in situ*. The data, summarized in Table III, indicate that ≥98% of tubular gland cells are responsive to restimulation.

The specificity of *in situ* hybridization was evaluated by

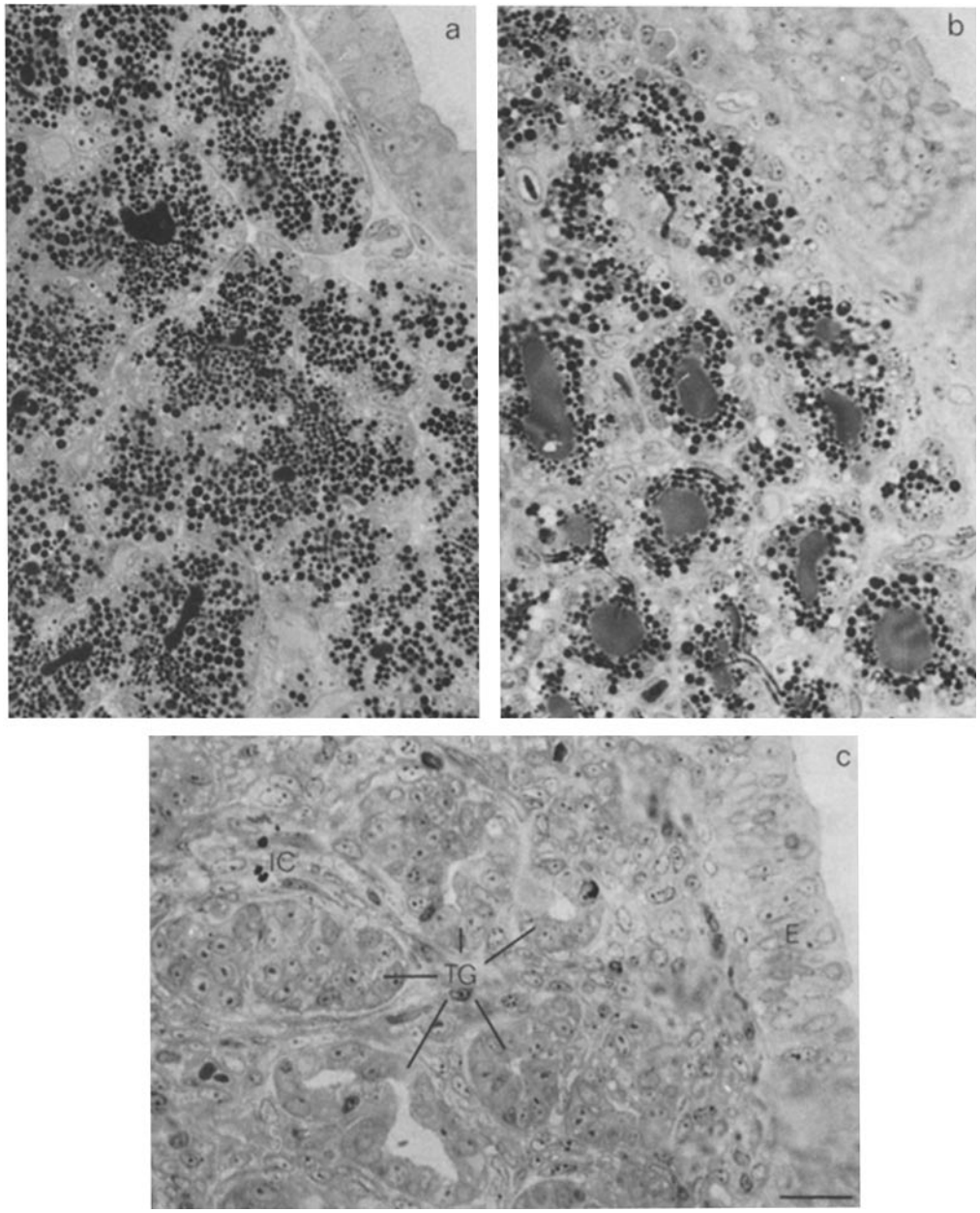

FIGURE 4 Histology of estrogen-stimulated and withdrawn oviducts. Cross sections (1 μm) were stained with Toluidine Blue: (*a*) estrogen-stimulated oviduct, (*b*) 3-d-withdrawn oviduct, (*c*) 25-d-withdrawn oviduct. Data on the content of E·Rn and $mRNA_{ov}$ in these tissues are included in Fig. 1. The oviduct lumen is located in the upper right corner of each micrograph. In *c*, the surface epithelium (*E*), interstitial cells (*IC*), and tubular glands (*TG*) are designated. Bar, 5 μm. × 2,000.

comparing hybridization to cell suspensions from fully stimulated vs. withdrawn oviducts. Long-term, estrogen-stimulated oviducts (Fig. 6*a*) yielded two populations of cells: heavily labeled (average of 57 grains/cell) or unlabeled above background (2–4 grains/cell). The proportion of labeled cells (54%) equaled the proportion of tubular gland cells in cell suspensions. When cells were quantitated simultaneously for morphology and extent of labeling, all cells that were identified as tubular gland cells were heavily labeled, whereas all cells that were identified as nontubular gland cells were associated with background grain densities. When cell suspensions were prepared from oviducts withdrawn for 3 d (Fig. 6*c*), tubular gland cells were still recognizable; however, ≥99% of the cells were associated with only background levels of hybridization. A few cells were labeled above background, with an average of 7 grains/cell; the number of grains did not overlap that observed over labeled cells of long-term, estrogen-stimulated oviducts. We estimate that 7 grains/cell is equivalent to ~800 molecules of $mRNA_{ov}$/cell. Because 0.25% of the cells were labeled above background, this would account for an average $mRNA_{ov}$ content of ~2 molecules/cell, which is sevenfold less than the level of 13 molecules/cell observed in this experiment. After 24 d of withdrawal (Fig. 6*b*), tubular gland cells were not recognizable in cell suspensions because of a complete loss of secretory granules, and no labeling above background was observed (0 of 847 cells).

When 3-d-withdrawn chicks were restimulated with estrogen or progesterone for 10 h, labeled cells were readily detected (Fig. 6*d*). With progesterone, the average $mRNA_{ov}$ content increased from 13 to 4,010 molecules/cell and the average

TABLE III

Relationship between $mRNA_{ov}$ Content and Tubular Gland Cells during Hormone Withdrawal and Restimulation *

Hormone treatment	$mRNA_{ov}$ content	Number of cells examined	Cells labeled with $cDNA_{ov}$‡	Number of grains over labeled cells‡	Tubular gland cells	
	molecules/cell		*% total*		*% total*	*% labeled with $cDNA_{ov}$*
Stimulated with estrogen for 16–33 d	26,600 ± 1,600	3,076	54 ± 2	57 ± 14	54 ± 5	100§
Withdrawn for 3 d	13 ± 4	3,301	0.25 ± 0.13	7 ± 2¶	55 ± 6‖	0.5**
Restimulated with estrogen for 10 h	1,840 ± 180	1,448	56 ± 1	19 ± 2		100**
Restimulated with progesterone for 10 h	4,010 ± 320	2,773	51 ± 3	28 ± 4		98§

* Data are presented as the mean ± standard error from three or four experiments. Total nucleic acids were isolated and hybridized with $cDNA_{ov}$ in solution to determine the average $mRNA_{ov}$ content per cell (*$mRNA_{ov}$ content*). Cell suspensions were prepared for determination of percent tubular gland cells (*tubular gland cells*); other cell suspensions were prepared for hybridization with $cDNA_{ov}$ *in situ* and determination of percent labeled cells (*cells labeled with DNA_{ov}*). When cell morphology was sufficiently good after *in situ* hybridization, the percent of tubular gland cells labeled with $cDNA_{ov}$ was determined directly (§ under *tubular gland cells*).

‡ A background level of grains (ranging from one to four grains/cell in individual experiments) has been subtracted; only those cells with more than twice background were scored as labeled.

¶ After hybridization with $cDNA_{ov}$ *in situ*, tubular gland cells and labeled cells were quantitated simultaneously. Of nontubular gland cells from estrogen-stimulated oviducts, 0% were labeled.

‖ Data are combined from oviducts withdrawn for 3 d, as well as from oviducts withdrawn for 3 d and restimulated with estrogen or progesterone for 10 h, during which time cell proliferation does not occur. For example, in one experiment cell suspensions from such oviducts contained 65, 55, and 45% tubular gland cells, respectively.

§ ≥99% of cells from 3-d-withdrawn oviducts were associated with background levels of hybridization (one to four grains/cell). A few cells (eight out of 3,301) were observed to be labeled, with an average of seven grains/cell. By comparing the $mRNA_{ov}$ content after restimulation with the number of grains over labeled cells, we estimate that the amount of seven grains is equivalent to ~800 molecules of $mRNA_{ov}$/cell or ~1,500 molecules/tubular gland cell.

** Calculated from the percent labeled cells (*cells labeled with $cDNA_{ov}$*), assuming that 55% of the cells are tubular gland cells‖ and that only tubular gland cells are labeled§.

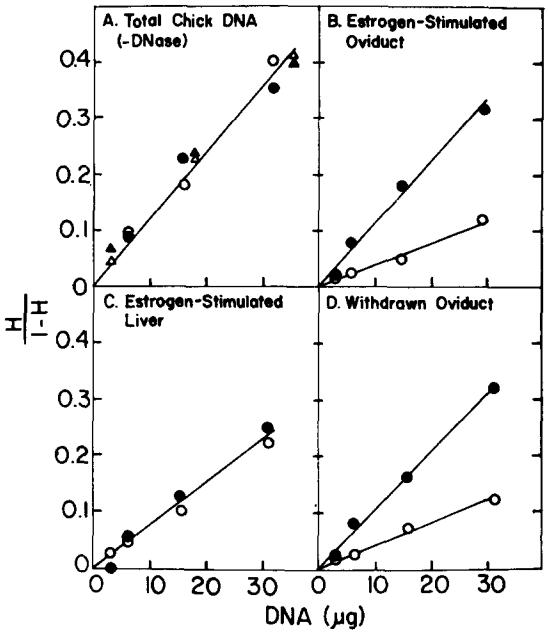

FIGURE 5 DNase I sensitivity of the ovalbumin and globin genes. Oviduct or liver tissue was combined from five or six chicks. Nuclei were isolated and digested with DNase I until 29% of the DNA was acid soluble. The remaining DNA was isolated, alkali digested, and hybridized with ovalbumin (○, △) and globin (●, ▲) cDNAs. The fraction hybridized (*H*) is plotted as H/(1 − H) (see Materials and Methods). (*A*) Total chick DNA (prepared from chick erythrocyte), undigested with DNase I. All other DNA samples were prepared from DNase I-treated nuclei, isolated from: (*B*) estrogen-stimulated oviduct, (*C*) estrogen-stimulated liver, (*D*) oviduct, withdrawn 2 d.

number of grains per labeled cell was >10-fold over background. The lower $mRNA_{ov}$ content observed after restimulation with estrogen compared with progesterone was reflected in fewer grains per cell, although the relationship between grain number and $mRNA_{ov}$ content was not linear; this nonlinearity is most likely because of insufficient $cDNA_{ov}$ excess. Simultaneous quantitation of tubular gland cells and labeled cells indicated that with progesterone 98% of the recognizable tubular gland cells were labeled. In all preparations from chicks restimulated with estrogen or progesterone, approximately half of the oviduct cells were labeled, corresponding to the proportion of tubular gland cells and to the proportion of labeled cells from long-term, estrogen-stimulated oviducts. Taken together, these data indicate that ≥98% of tubular gland cells are responsive to either estrogen or progesterone.

DISCUSSION

Injection of chicks with estrogens leads to differentiation and proliferation of oviduct tubular gland cells and the synthesis of egg white proteins and their mRNAs (5, 8, 12, 15, 20, 27, 33, 35). Withdrawal of chicks from estrogen, by cessation of injections, leads to gradual involution of the oviduct over a period of weeks, characterized by a decline in egg white proteins, ribosomal RNA and mRNA synthesis, total RNA, ribosomes, wet weight, $E \cdot R_n$, and eventually total DNA and the proportion of tubular gland cells (8, 12, 24, 27, 30, 31, 38, 40, 43). To facilitate studies of estrogen stimulation and withdrawal, it was of obvious interest to obtain a withdrawal state in the absence of cell population changes. In an earlier study, chicks were stimulated with pellets of the synthetic estrogen, hexestrol, and then withdrawn by pellet removal, which led to an acceleration of the time-course of withdrawal, termed acute withdrawal (30). Acute withdrawal also has been achieved by removal of implants of diethylstilbestrol in silicone tubing (12). This study was carried out to assess the kinetics of $mRNA_{ov}$ loss in relation to estrogen receptors during acute withdrawal, and to determine whether a withdrawal state was achieved before cell population changes.

The hormone withdrawal process can be divided into at least two stages. We show here that the first stage commences when the circulating estrogen concentration falls below a critical threshold, evidenced by a decline in estrogen receptors from

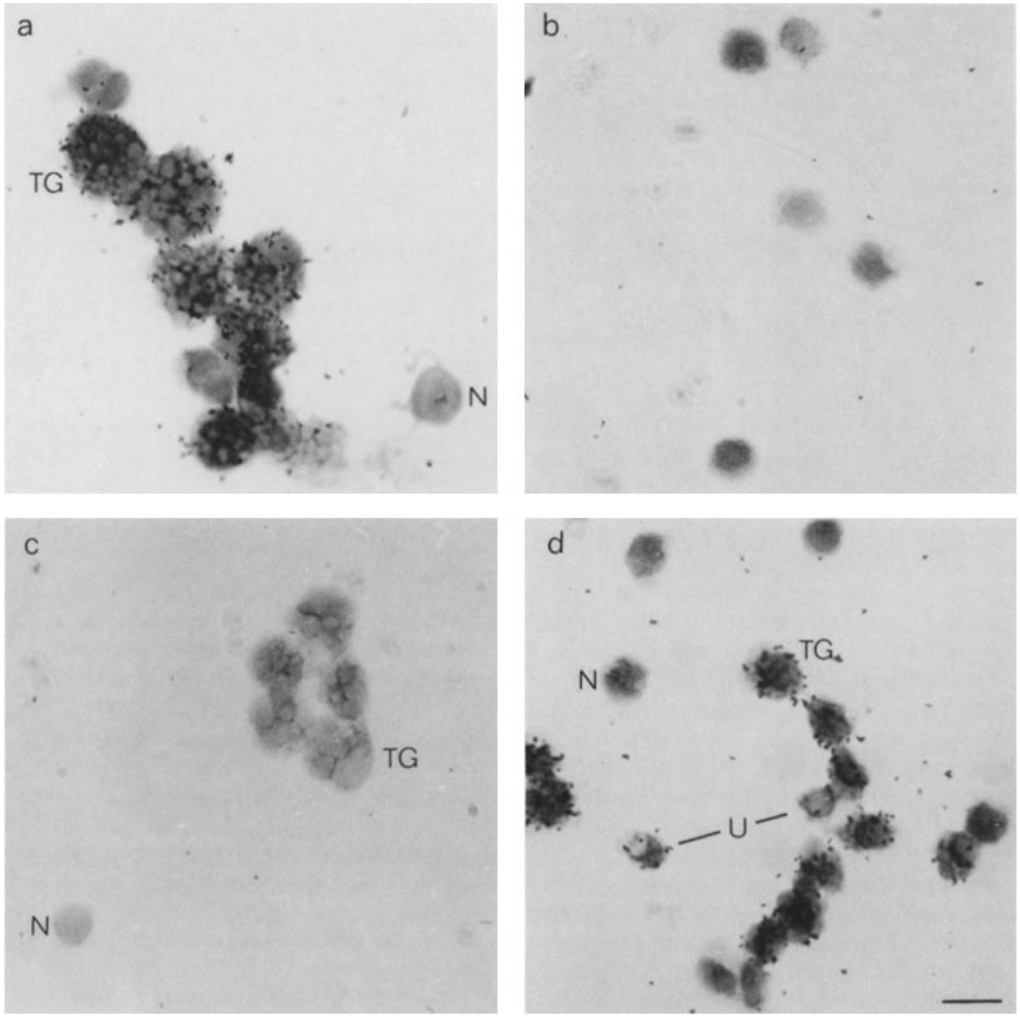

FIGURE 6 Hybridization of $cDNA_{ov}$ to oviduct cell suspension *in situ*. Cell suspensions were prepared from portions of oviduct tissue combined from two chicks that were (*a*) estrogen stimulated, (*b*) withdrawn 24 d, (*c*) withdrawn 3 d, or (*d*) withdrawn 3 d and restimulated with progesterone for 10 h. Examples of tubular gland cells (*TG*) and nontubular gland cells (*N*) are indicated. By 24 d of withdrawal, tubular gland cells could not be distinguished, because of the absence of secretory granules (see Fig. 4 *c*). Two unclassified cells (*U*) in Fig. 6 *d* also are shown. Bar, 2 μm. × 4,000.

the nucleus. The earliest effects of $E \cdot R_n$ decline are manifested in reduced RNA synthesis. Tsai et al. (46) observed that initiation sites on chromatin for *E. coli* RNA polymerase decline in concert with $E \cdot R_n$. Endogenous forms I and II RNA polymerase activities decline upon acute withdrawal (22); we show that this coordinated decline occurs simultaneously with $E \cdot R_n$ and is attributable to a reduction in the number of active RNA polymerases. The rate of egg white mRNA transcription also is tightly coupled with $E \cdot R_n$ concentration (19, 25, 43; and unpublished observations). During this stage, a decrease in egg white mRNA stability is triggered (7, 12, 30), and for $mRNA_{ov}$ we show that this decrease occurs within 3 h of the onset of $E \cdot R_n$ decline. These events lead to a rapid loss of egg white mRNAs followed by a more gradual decline in egg white proteins and secretory granules.

Our observations suggest that the onset of $E \cdot R_n$ and $mRNA_{ov}$ decline are determined by the time necessary for the clearance of residual hormone. Studies with tamoxifen support this conclusion. Injection of this antiestrogen (34, 41) at the time of pellet removal shortened the lag before onset of $E \cdot R_n$ decline from 10 to ≤1 h; $mRNA_{ov}$ declined in parallel but a few hours later (Fig. 1). The $t_{1/2}$ of $mRNA_{ov}$ ranged between 3 and 6 h, with the shortest $t_{1/2}$ being associated with use of tamoxifen or withdrawal of large chicks (which presumably have lower levels of residual hormone). These observations imply that, in the absence of estrogen, $mRNA_{ov}$ has a $t_{1/2}$ of 3 h, which confirms previous estimates (7, 12, 30) and is eightfold less than the $t_{1/2}$ of 24 h measured for $mRNA_{ov}$ in the presence of estrogen (29). Hynes et al. (12) demonstrated that egg white mRNAs are preferentially degraded relative to non–egg white mRNAs during hormonal withdrawal. Because these mRNAs outnumber receptors by ~10-fold in fully stimulated oviducts, they probably are not stabilized by combining directly with receptors; it seems more likely that estrogen regulates the synthesis of stabilization factors or nucleases that recognize this class of mRNAs. The combination of an eightfold decline in $t_{1/2}$ of $mRNA_{ov}$ and a several hundred–fold decrease in the rate of $mRNA_{ov}$ synthesis (19, 25, 43) accounts for the 2,000- to 3,000-fold change in steady-state concentration of $mRNA_{ov}$ upon hormone withdrawal (see Fig. 1).

By the end of the first stage, which lasts 2–3 d with the hormonal regimen described here, a 20-fold-lower level of $E \cdot R_n$ is established as well as a 1,000-fold-lower level of $mRNA_{ov}$, but there is no significant loss of cells. Stability of the tubular

gland cell population during this stage was inferred from two observations: (*a*) the DNA content of oviducts remain constant for 3 d, and (*b*) tubular gland cells comprise approximately half of the oviduct population in both estrogen-stimulated and 3-d-withdrawn oviducts. Hynes and co-workers (12) reported the attainment of a withdrawn state without cell population changes within 3 d of removing diethylstilbestrol implants. Oviducts in the first stage of withdrawal are fully responsive to hormone restimulation, as discussed below.

A second stage of withdrawal is characterized by the loss of atrophied tubular gland cells. This loss begins after 3 d of withdrawal and continues for about another week. By the end of the second stage nearly 80% of the oviduct cells have been destroyed, based on the decline in total DNA content of the oviduct, and only 15% of the remaining cells are recognizable as tubular gland cells (29).

Analysis of the $mRNA_{ov}$ content of individual cells in fully stimulated oviducts revealed that measurable levels of $mRNA_{ov}$ were restricted to tubular gland cells. This result was expected from earlier observations that tubular gland cells are responsible for producing ovalbumin (14, 32). By 3 d of withdrawal, $mRNA_{ov}$ had declined to ~10 molecules/cell, and *in situ* hybridization revealed that no tubular gland cells remained completely resistant to withdrawal: of >3,000 cells examined from withdrawn oviducts, we did not see a single cell that contained the fully stimulated level of $mRNA_{ov}$. However, ~0.2% of withdrawn cells were slightly labeled. The $mRNA_{ov}$ content of these few cells did not account totally for the average withdrawal level of $mRNA_{ov}$ per cell. Finding a few $mRNA_{ov}$-containing cells in withdrawn oviducts raises the question as to whether these are normal cells. In a recent study, Tsai et al. (45) observed that the low level of $mRNA_{ov}$ ($\leq$1 molecule/cell) in estrogen-stimulated livers is correlated with the presence of ovalbumin in a few hepatocytes ($\leq$0.1%), which they speculate may be undifferentiated stem cells.

Because tubular gland cells were maintained for 3 d of withdrawal, we asked whether they remain functionally stable, in terms of producing $mRNA_{ov}$ in response to another hormonal stimulus. Restimulation with either estrogen or progesterone led to accumulation of $mRNA_{ov}$ after a lag of ~3 h, in agreement with previous results (8, 12, 20, 33, 36). In the presence of progesterone, $mRNA_{ov}$ was produced initially at the rate observed in prewithdrawal oviducts, suggesting that all tubular gland cells were producing $mRNA_{ov}$. *In situ* hybridization confirmed this point. At 10 h after injection of progesterone, 98% of the tubular gland cells contained $mRNA_{ov}$. Furthermore, the proportion of cells containing $mRNA_{ov}$ was approximately half the population, as in fully stimulated oviducts.

When 3-d-withdrawn chicks were restimulated with estrogen, using a dose that was optimal in long-term-withdrawn chicks (24), $mRNA_{ov}$ was produced at a submaximal rate. This reduced response was correlated with a low level of $E \cdot R_n$ that could reflect a temporary deficiency of functional receptors, or could be the consequence of suboptimal levels of estrogen reaching oviduct cells. The latter possibility is supported by our recent observations that optimal restimulation after acute withdrawal requires a higher dose of estrogen than after long-term withdrawal. Furthermore, Swaneck et al. (42) reported that injection of estrogen directly into the oviduct lumen after acute withdrawal resulted in the synthesis of $mRNA_{ov}$ at the prewithdrawal rate without an appreciable lag. *In situ* hybridization revealed that the submaximal response to estrogen correlates with low $mRNA_{ov}$ production by all tubular gland cells rather than a lower percentage of responsive cells (Table III).

A variety of studies demonstrates that DNA sequences in actively transcribed chromatin are preferentially sensitive to digestion with DNases (1–3, 48). This correlation has been extended to specific gene sequences, including globin and endogenous viral genes in chicken erythrocytes (48) and the ovalbumin gene of the laying hen oviduct (1, 9). We also found that the ovalbumin gene was preferentially sensitive to DNase I in the estrogen-stimulated chick oviduct, relative to the transcriptionally inactive globin gene. However, approximately half of the ovalbumin DNA sequences were insensitive. This fraction correlated with the observations that, of the cells isolated from oviduct magnum, approximately half were cell types other than tubular gland cells by morphology and approximately half did not contain $mRNA_{ov}$ by *in situ* hybridization. We interpret these results to indicate that the ovalbumin gene is preferentially sensitive to DNase I only in tubular gland cells. Preferential sensitivity was not observed in stimulated liver cells, confirming previous observations (9, 34). Because hepatocytes respond to estrogen by synthesizing specific mRNAs for the egg yolk proteins (13, 23), but do not synthesize appreciable levels of $mRNA_{ov}$ (45), the comparison of oviduct and liver suggests that preferential sensitivity of the ovalbumin gene to DNase I is correlated with gene expression. However, preferential sensitivity of the ovalbumin gene to DNase I was maintained in the oviduct withdrawn for 2 or 3 d. This result is consistent with our earlier observation that the ovalbumin gene remains sensitive to DNase I after administration of the antiestrogen, tamoxifen (34). Here we extend this observation by showing that sensitivity is maintained, despite a 1,000-fold decline in $mRNA_{ov}$ content and a depletion of nuclear estrogen receptors.

There are at least two possible explanations for continued DNase I sensitivity of the ovalbumin gene in the withdrawn oviduct. If DNase I sensitivity of a gene is correlated with transcriptional activity, then the level of synthesis in withdrawn oviducts may be sufficient for continued DNase I sensitivity. We calculate a rate of $mRNA_{ov}$ production of 0.03 molecules/min per cell, based upon 10 molecules/cell of $mRNA_{ov}$ at 3 d of withdrawal and a $t_{1/2}$ of 3 h. Endogenous chicken viral sequences, expressed at the even lower level of 0.01–0.2 RNA molecules per viral DNA copy, were found to be preferentially sensitive to DNase I (48). Thus, a low level of transcriptional activity may preserve the DNase I sensitivity of the ovalbumin gene. An alternative explanation is that the biochemical events leading to DNase I sensitivity may not be reversible in terminally differentiated cells. In this regard, the tubular gland cell may be similar to the mature, nondividing erythrocyte, in which the globin gene remains sensitive to DNase I in the absence of globin RNA synthesis (48). The same mechanism could underlie the maintenance of sensitivity in both of these cell types if a change in sensitivity requires cell division. Division of tubular gland cells slows markedly after hormone withdrawal (37).

Attainment of a withdrawal state without cell population changes allows more meaningful comparisons of hormone-induced and -uninduced oviduct. For example, the DNase I studies would have been uninterpretable in a comparison of fully induced and long-term-withdrawn oviduct nuclei because of the cell population changes. The use of oviduct tissue derived from birds withdrawn for only 2 or 3 d has facilitated recent

studies of egg white mRNA synthesis and accumulation during hormone restimulation as well as attempts to achieve physiological hormone responses in cultured oviduct tissue (12, 19, 25, 36, 42). These observations on hormone withdrawal provide a framework for further investigation into the events triggering deinduction of specific gene expression and the catabolic mechanisms involved. They may also help to elucidate the biology of hormone action during the normal egg-laying cycle of seasonal birds.

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