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RETENTION OF ESTROGEN RECEPTORS *IN VITRO* REQUIRES LIMITED ESTRADIOL EXPOSURE *IN VIVO*

MARY E. RISER†, CHARLES E. MANNER* and EUGENE C. LAI
†Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030 and
*Department of Medicine, University of Texas Medical School, 6431 Fannin, Houston, TX 77030, U.S.A.

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Summary—Maintenance of functional estrogen receptors in culture has been accomplished in chick oviduct cells by manipulating the estrogen exposure before tissue dissociation. Tissue from chicks pre-treated with daily 17-β-estradiol injections for 2 weeks or with 2 weekly diethylstilbestrol implants can be established in culture using a variety of enzymes. Tissue from animals with chronic estrogen stimulation must be withdrawn from hormone in culture at least 4 days before the digestion procedure. When tissue is digested using collagenase and pancreatin buffered by bovine serum albumin (Fraction V), large quantities of virtually fibroblast-free cultures can be established. The estrogen and progesterone receptors remain intact at normal levels using this procedure. The receptors have maintained biological function as evidenced by two hormone-dependent measurements. The first was an increase in the amount of ovalbumin mRNA transcribed in response to estrogen supplementation of the cultures compared to cultures with no estrogen. The second function was an increase in ovalbumin protein secreted into the medium upon estrogen stimulation. The protein increment demonstrated that the hormone-induced levels of mRNA were functional and capable of being translated.

INTRODUCTION

Interest in the molecular organization of the avian ovalbumin gene has prompted us to develop a non-transformed cell culture system in which gene transfer experiments can be done [1]. The requirements for these cells are that: they are purified epithelial components; they are capable of producing ovalbumin in culture; they form monolayers; they have retained functional estrogen and progesterone receptors; and there are large enough quantities routinely established to provide material for biochemical analysis. The methods published [2, 3] do not provide enough material for our experiments. We have devised a method that will provide grams of purified epithelial cells using digestion techniques alone. The cells have retained estrogen and progesterone receptors in numbers similar to those measured directly from the animals. The cells respond appropriately to estrogen withdrawal and restimulation using physiological levels of hormone and provide a suitable environment for gene transfer experiments.

EXPERIMENTAL

Animals

The oviducts were derived from chickens that had different estrogen exposures. Some chicks were given 17β-estradiol (E_2) or diethylstilbestrol (DES) on a daily regimen of 1 mg of hormone in sesame oil injected subcutaneously for 10 days beginning at 2 weeks of age. Animals withdrawn *in vivo* were from either of these injected groups, having been withdrawn from daily injections for at least 14 days. Another source of starting material were chicks given weekly subcutaneous injections of 25 mg of DES in Carbowax starting at 2 weeks of age. Due to the residual depot effect of Carbowax, these animals were never used for *in vivo* withdrawal experiments. Comparisons were made with adult laying hens, commercial birds of proven productivity that had no exogenous hormone manipulation.

Dissociation methods and culture conditions

Oviducts were removed by sterile technique from animals that had been sacrificed by cervical dislocation. The tissue was rinsed three times in Dulbecco's medium containing 300 U/ml penicillin and 300 μg/ml streptomycin. The oviducts were then trimmed of excess connective tissue and the shell gland to leave only the magnum portion for culture. The magnum was then split open lengthwise and minced into pieces approx 3 × 3 mm. Minces to be digested were then added to the digestion solution, composed of Collagenase Type III (Worthington), Pancreatin Type VI (Sigma), and Bovine Serum Albumin Fraction V (BSA, GIBCO) in Dulbecco's Medium (GIBCO). The collagenase was 2 mg/ml with 0.5 mg/ml pancreatin and 20 mg/ml BSA dissolved in Dulbecco's medium without $NaHCO_3$ but with 15 mM Hepes and adjusted to pH 7.4 with 5 M NaOH. Tissue was placed in a closed Erlenmeyer

†To whom correspondence should be addressed.

flask using a ratio of 5 ml/g of tissue, and shaken in a 37°C rotary shaker bath 30–40 min. All dissociated tissue was centrifuged from the dissociation solution and rinsed three times before being seeded into plastic culture dishes. Differential plating of fibroblasts and epithelial cells was possible using the CPBSA procedure although fibroblast contamination was only 10–15% with a single plating. The fibroblasts attached to the dishes in 12–16 h permitting removal of the floating epithelial cells as a purified population into new culture dishes. Alternate methods used to establish cultures were trypsinization using trypsin–EDTA (GIBCO) at a ratio of 5 ml/g tissue, other published methods for endocrine tissues [2, 7–9], and addition of 20 mM molybdate during digestion. All cultures were maintained at 37°C in humidified incubators gassed with 5% CO_2 95% air. Cells were best maintained in Dulbecco's High Glucose medium (GIBCO) supplemented with 5% horse serum (GIBCO) and 10^{-7} M 17β-estradiol (Sigma). Charcoal-stripping of serum was accomplished by adding 2% (w/v) activated charcoal to serum and heating 1 h at 55°C, followed by centrifugation and filtration to clarify and sterilize.

Receptor assays

Estrogen receptors were assayed using exchange conditions and 10 point saturation analysis. Cells were scraped from the plates using TED buffer (10 mM Tris, 1.5 mM EDTA, 1.0 mM DTT pH 7.4, at 30°C) and broken apart using a Dounce homogenizer. The homogenate was filtered through Nitex cloth and 1 ml was layered on top of a 1 ml 1.2 M sucrose pad. The tube was centrifuged at 6,000 rpm for 45 min, at which time the upper cytosol was removed, the lipid and sucrose pad were aspirated away, and the nuclear pellet was remaining. Cytoplasmic and nuclear estrogen receptors were assayed [4] using [^{3}H]estradiol ([2,4,6,7,16,17-^{3}H]estradiol, 140 Ci/mmol, New England Nuclear) with or without 100-fold excess DES to determine total and non-specific binding. Exchange was at 37°C for 30 min. Protamine sulfate (1 mg/ml) was used to precipitate the receptors. Replicate tubes were used for DNA determination [5] and cell counts using a standard of 2.3 pg of DNA per avian cell. Data were analyzed via computer using a modification of the Rosenthal procedure (Dr Ernest Peck, unpublished, copies available on request). Progesterone receptors were determined in the cytosol using R5020 binding assays in buffers that included 10% glycerol [6]. The receptors with bound R5020 were separated from unbound R5020 using hydroxylapatite. Receptors per cell were calculated from the DNA values and knowing the volume of cell homogenate and cytosol from that nuclear pellet.

Ovalbumin solid phase radioimmunoassay

Medium tested for ovalbumin secretion was collected, centrifuged at 1000 *g* to remove cellular contaminants, and frozen at −70°C until assayed by solid phase radioimmunoassay as previously described [10].

Synthesis of [^{3}H]RNA in vitro

Finely minced oviducts removed directly from DES-stimulated chicks, *in vitro* maintained oviduct tissue minces, and monolayer cultures of oviduct epithelial cells were used as starting materials for preparation of [^{3}H]RNA. Minced oviduct tissues or oviduct epithelial cells harvested by a sterile rubber policeman (approx 600–800 mg tissue) were washed twice with tissue culture medium, then incubated at 37°C for 1 h in plastic flasks with 200 ml of tissue culture medium containing 0.5 mCi/ml of [^{3}H]cytidine (30 Ci/mmol) and 0.5 mCi/ml of [^{3}H]uridine (55 Ci/mmol). Synthesis was stopped by the addition of cold 5% citric acid, and [^{3}H]RNA was isolated from purified nuclei using described methods [11]. The amount of ovalbumin mRNA transcribed was quantitated by hybridization to nitrocellulose filters containing either pOV_{230} or pMB9 DNA prepared as described [11]. The pOV_{230} is a chimeric plasmic cloned into pMB9 and contains a full-length ovalbumin cDNA insert lacking only 12 nucleotides at the 5′ end. The pMB9 filters were used a background control for hybridization to pBR322 plasmid sequences and [^{32}P]RNAov_{230} synthesized *in vitro* was used as an internal standard.

RESULTS

In vivo *receptor quantitation*

The overall results from using various digestion protocols demonstrated that many enzymes will digest the oviduct tissue and tubular gland cells will attach. Some methods generated more fibroblast contamination than others. The problem encountered with these protocols was loss of estrogen receptor beginning immediately after digestion. Through various manipulations it was determined that the estrogen state of the material prior to digestion was the main determinanat in the *in vitro* retention of the receptors. When oviducts derived directly from animals given multiple DES-Carbowax or E_2 injections were used as a source of tissue to establish the parameters of the estrogen receptor (ER) assay it was determined that a normal animal variation of 4000 to 13,000 receptors per cell existed. Therefore, pooled oviducts from several animals were used for most experiments, initial estimate of receptors was done when possible, and a ratio of 4000 receptors per cell was used as a minimal estimate of normal levels.

In vitro *tissue mince receptor levels*

Mince material from pooled oviducts was maintained in culture for several days to use for comparable estrogen receptor values for the digested material. The tissue minces maintained receptor levels

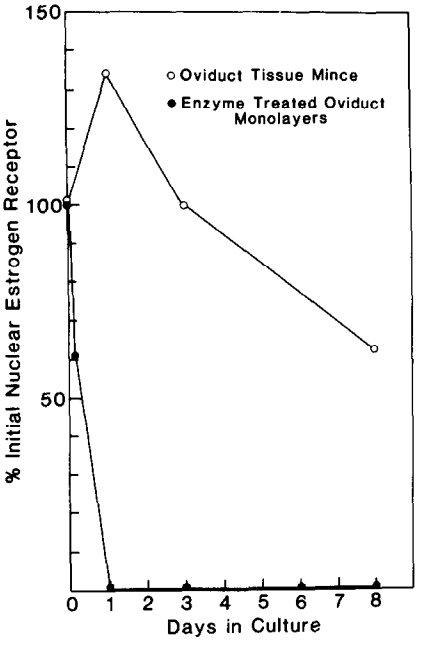

Fig. 1. Time course of nuclear estrogen receptors in mince and companion digested tissue. Each point was determined using a 7 point exchange assay with and without 100-fold excess DES to determine total and non-specific binding. See text for details.

for several days then they gradually declined to approx 60–75% of the starting level by day 14 (Fig. 1). This is in contrast to tissue dissociated with collagenase and pancreatin (CPBSA), or any enzyme, which rapidly lost receptors and never recovered them when the cells were cultured up to 3 weeks. Additions to the dissociation medium of 20 mM molybdate or 50 μM 2-β-mercaptoethanol or digestion at 4°C or room temperature did not preserve receptors.

The tissue used for these experiments was from animals that had received four or more DES-Carbowax implants to develop large stimulated oviducts. It was decided to withdraw this tissue from estrogen exposure before digestion. Because of the depot nature of these implants, the withdrawal was done *in vitro*.

In vitro *withdrawal—effects on receptor levels*

Oviducts pooled from chicks given DES-Carbowax were minced and placed into a withdrawal culture medium for 5 days. Withdrawal medium was Dulbecco's medium supplemented only with 5% charcoal-stripped horse serum and thus was free of steroid hormones. On the sixth day the minces were divided into five aliquots: one aliquot was tested then for estrogen receptors; one remained in the withdrawal medium for a future receptor assay; one was supplemented with 10^{-7} M E_2; and two groups were digested using 2 mg/ml collagenase, 0.5 mg/ml pancreatin and 20 mg/ml BSA (CPBSA). Half of these latter digested cells were culture into medium supplemented with 10^{-7} M 17-β-estradiol (E_2) and the other half in 10^{-8} M E_2. On the eleventh day the groups were assayed for estrogen receptors (see Fig. 2). On day 6, there were 3215 receptors per cell in the withdrawn minces and 4020 per cell on day eleven. Minces supplemented with E_2 for days 6 to 11 had 6577 receptors per cell. The monolayers of cells digested then maintained in 10^{-7} M E_2 had 8214 receptors per cell while those in 10^{-8} M E_2 had 9108 receptors per cell. Thus, the cells withdrawn from estrogen before digestion maintained normal levels of receptors. To test more thoroughly for the influence of time on *in vitro* withdrawal, oviducts were removed from animals on sequential days for 5 days, minced, and placed into withdrawal medium. On day 5 all minces were digested in separate flasks and transferred into medium with 10^{-7} M E_2 for assay 3 days later. Cells withdrawn 4 days before digestion had 7046 receptors; those withdrawn 3 days had 8495; those withdrawn 2 days had 4105; those withdrawn one day in advance had 3714; and those with no advance withdrawal had 4006 receptors. Table 1 gives the details of these assays which also demonstrate that the 3 and 4 day withdrawal minces had classical estrogen receptor binding affinities whereas the other oviducts had lower affinities for estradiol. Due to animal variation, these numbers cannot be held as absolutes, but it appeared that removing the tissue from high levels of estrogen at least 72 h before digestion will provide cells that have receptor levels normally found *in vivo*, and the receptors will bind estrogen in a normal manner.

Thus, we found that oviduct tissue that had been repeatedly stimulated with DES yet was withdrawn *in vitro* from hormone before digestion maintained normal levels of estrogen receptors in the monolayer of cells. Removal from hormone permitted the receptors to attain some configuration that was not susceptible to degradation by the enzyme treatment. To determine if withdrawal was important immediately before digestion or could take place at any time in the procedure, a repeat of the experiments also included minces that were withdrawn *in vitro* for 5 days, restimulated 5 days *in vitro* with 10^{-7} M estradiol, then digested and grown in 10^{-7} M E_2. These latter cells had 10,251 estrogen binders per cell but the K_D was 13.27 nM, which is somewhat weaker binding than expected. It may be that the receptors were

Table 1. Nuclear estrogen receptor determinations for monolayer oviduct cultures established following various days of withdrawal from hormone before the digestion procedure

Days of withdrawal	Receptors per cell*	K_D (fM)	Correlation coefficient
4	7046	2.18	0.997
3	8495	2.35	0.920
2	4105	4.32	0.890
1	3714	3.45	0.996
0	4006	4.04	0.966

*Nuclear receptors per cell were determined using 5 point saturation analysis and DNA determinations on replicate nuclear pellets.

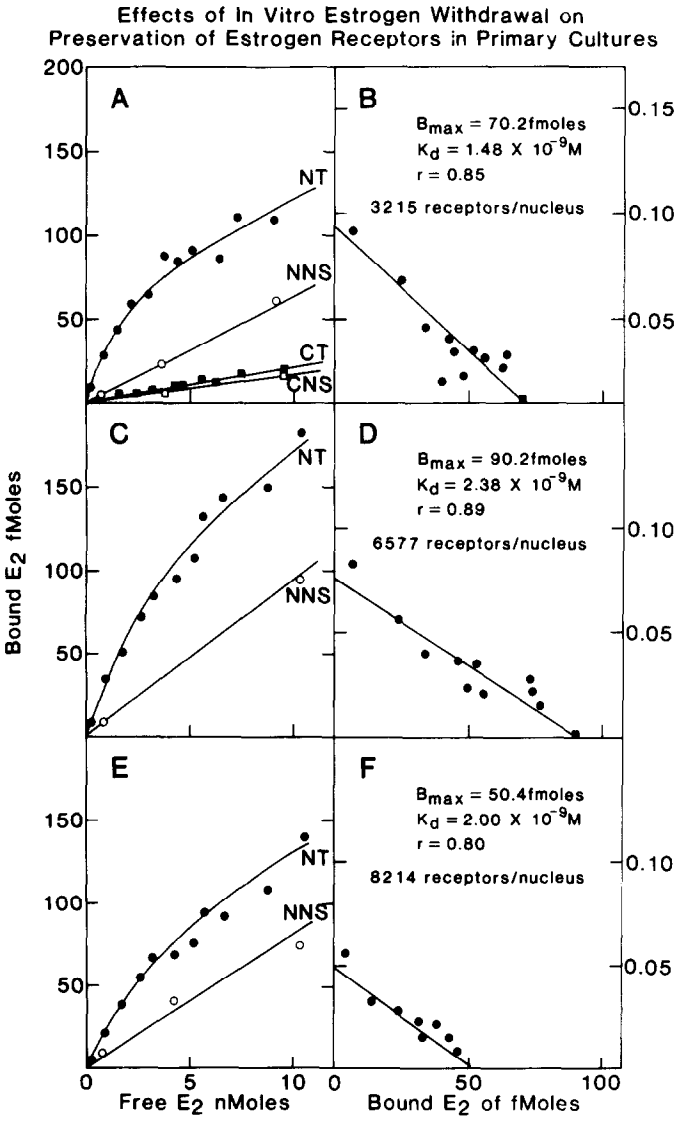

Fig. 2. Panels A & B: Nuclear and cytosol saturation binding curves, and nuclear Scatchard of minced oviduct tissue maintained in estrogen withdrawal medium 5 days. NT = nuclear receptor total binding; NNS = nuclear nonspecific binding; CT = cytosol receptor total binding; CNS = cytosol non-specific binding. Panels C & D: Nuclear saturation binding curve and Scatchard of the same withdrawn minced oviduct tissue in panels A & B, after restimulation with 10^{-7} M estradiol for 5 days. Panels E & F: Nuclear saturation binding curve and Scatchard of the same withdrawn minced oviduct tissue in Panels A & B, after enzyme digestion, differential plating of tubular gland cells and restimulation of monolayers with 10^{-7} M estradiol for 5 days.

slightly damaged and had lower affinity constants. Tissue withdrawn and digested, but given no further hormone, had no receptors, while those re-supplemented with 10^{-7} M E_2 following digestion had 7552 receptors per cell and a normal K_D. Oviduct fibroblasts or stromal cultures established from differential platings of the epithelial cultures were negative for nuclear and cytoplasmic estrogen receptors.

In vitro *withdrawal compared to* in vivo *withdrawal*

Experiments were conducted to compare *in vitro* and *in vivo* withdrawal using daily 1 mg injections of E_2 or DES for 10 days with *in vivo* withdrawal for 3 weeks or *in vitro* withdrawal for 5 days before digestion. The results (data not shown) demonstrated that E_2 and DES can be used interchangeably, that *in vivo* withdrawn oviducts average 2000 nuclear and 1000 cytoplasmic receptors, and that *in vitro* withdrawn oviducts recover normal levels of receptors when the monolayers are supplemented with estradiol. Of greater interest was the discovery that the control cultures, which were from oviducts given 10 days of E_2 or DES and immediately digested, had 4,400–15,000 receptors per cell (normal range). Receptors from this tissue given limited stimulation

were stable in culture 16 days. To determine the reliability of low estrogen stimulation, repeated digests were done on oviducts 1 week after animals had received 2 weekly DES-Carbowax injections or within 4 days of a third injection. Estrogen receptor assays routinely determined receptor numbers ranging from 5354 up to 16,913 per cell depending on the group of animals. These cultures were also established for 3 days in 10^{-7} M E_2, withdrawn for 5 days, then grown in E_2 or no E_2 for assays 4–7 days later. Cells withdrawn from hormone had from no detectable receptors up to 2004 nuclear receptors per cell; those later supplemented with 10^{-7} M or 10^{-8} M E_2 averaged 5000 receptors per cell. Thus it appeared that minimal DES stimulation on a daily or weekly depot basis provided material that would yield cell monolayers with intact estrogen receptors. Continued estrogen stimulation following digestions again appeared necessary to maintain the receptors. Figure 3 shows the morphology of cells established with this method.

Oviduct cultures were also established from two

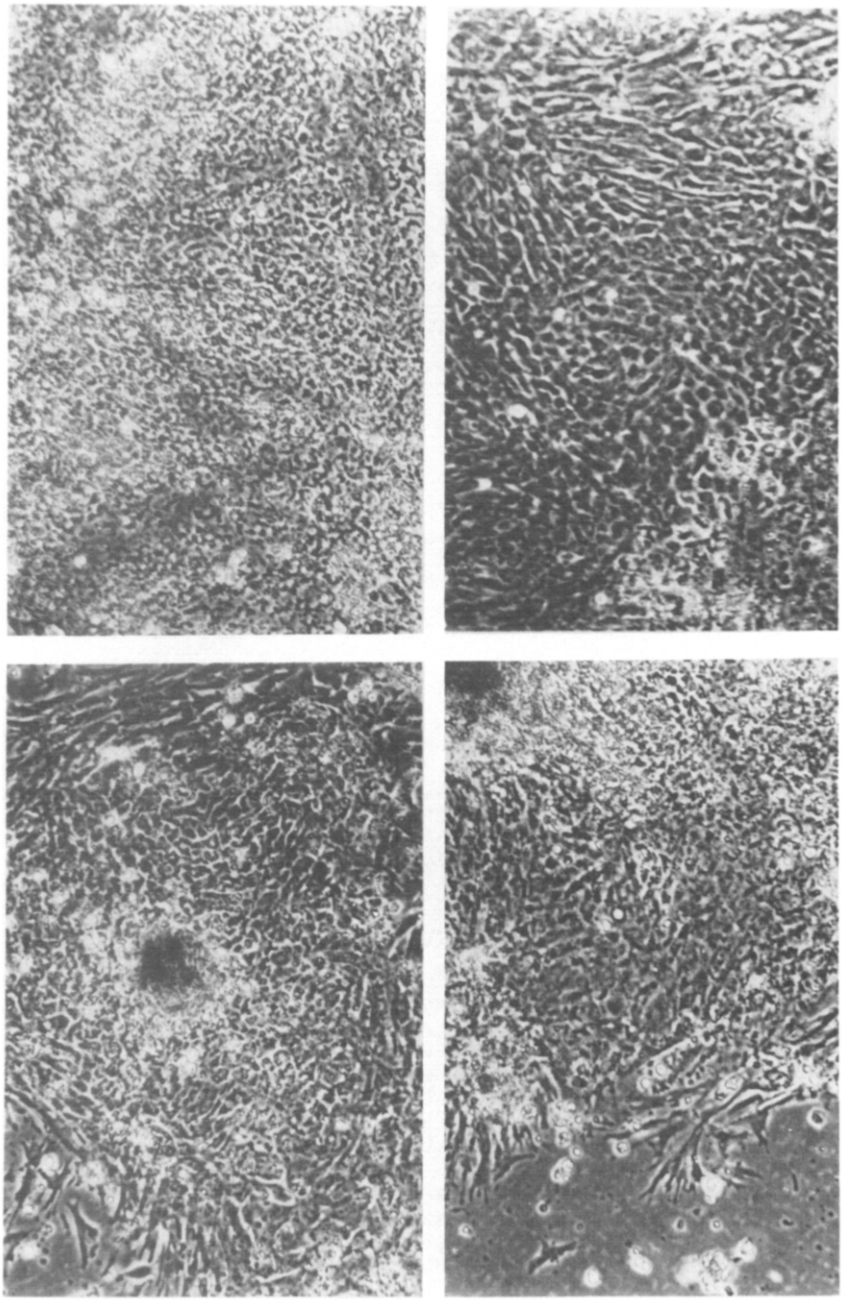

Fig. 3. Representative areas of monolayer cultures of chicken oviduct cells. The cultures were initiated 5 days earlier using collagenase, pancreatin, and BSA as described in text.

laying hens. These were birds that never received exogenous hormones. The cultures were initiated on two separate days using two separate preparations of enzyme. The cells attached to the plates but never formed the confluent monolayers seen previously. Two separate estrogen receptor assays were negative for nuclear and cytoplasmic receptors. This indicates that length of stimulation rather than amount of hormone is influential in the stability of the receptors.

Progesterone receptor levels

Progesterone receptor assays were done on several of the cultures initiated from animals given 2 DES injections, digested and maintained on 10^{-7} M E_2. One experiment for cultures on day 7 revealed 16,913 estrogen receptors and 100,000 progesterone receptors per cell which is a normal level. Another group of cultures on day 6 had 5354 estrogen receptors and 88,057 progesterone receptors per cell. One set of culture on day 10 in 10^{-8} M E_2 had estrogen receptors below our 4000 minimum requirement but had 16,800 progesterone receptors. Thus, the estrogen receptors were able to induce development of progesterone receptors to normal levels, and some induction even when estrogen receptors were below our minimum requirements.

Estrogen stimulation of ovalbumin and ov-mRNA in cultured cells

The presence of ovalbumin in the medium was quantitated using the solid phase radioimmunoassay. As shown in Fig. 4, estradiol stimulated a significantly greater production of ovalbumin compared to withdrawn cultures. This difference was seen even after the cells had been in culture for 2 weeks. The cells remained intact and capable of producing

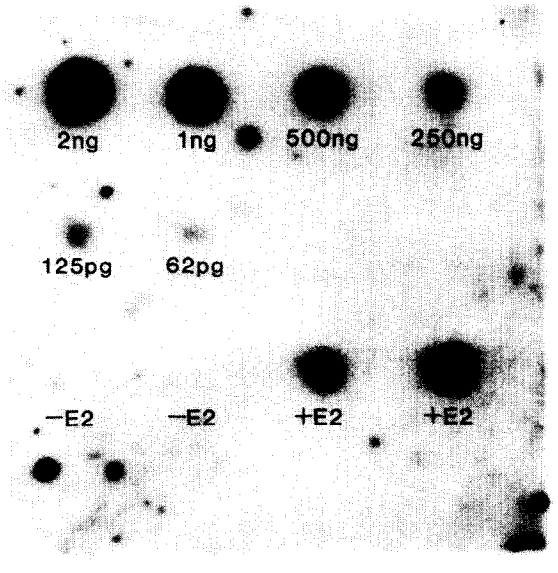

Fig. 4. Autoradiograph of a solid-phase immunoassay for ovalbumin in medium samples taken from oviduct cells cultured in the presence and absence of 10^{-7} estradiol.

Table 2. Quantitation of ovalbumin mRNA by percent hybridization of [^{3}H]RNA labeled in cells and bound to filters containing the pOV_{230} ovalbumin plasmid

Source of [^{3}H]RNA	% Hybridization	
Control tissue minces	Expt 1	Expt 2
Directly from DES chicks	0.0617*	ND
Maintained 12 days in 10^{-8}*M* E_2	0.0279	0.1040
Oviduct cell monolayers		
No estradiol	0.0010	0.0169
10^{-8} Estradiol	0.0134	0.0615

*All values represent the average of 3 determinations and have had baseline hybridization to plasmid pBR322 subtracted. Input counts of [^{3}H] total cellular RNA = 4×10^{-6} cpm and [^{32}P] cRNA = 3×10^{-3} cpm as an internal control of hybridization. ND = not done.

ovalbumin in a controlled response to estradiol demonstrating that new functional receptors were generated in the cells and that the protein synthesis mechanism was intact.

The cells in monolayer cultures showed a positive correlation for increased levels of ovalbumin mRNA when cultured with E_2 supplementation. Experiment one of Table 2 represents the transcriptional activity in cells derived from chicks that had been given 5 weekly DES-Carbowax injections. The cells were established from minces that had been withdrawn *in vitro* 5 days prior to digestion. Following digestion, half of the cultures were restimulated with 10^{-8} M E_2 and the other half were not given E_2. The controls were derived from (1) minces maintained *in vitro* with E_2 from the same tissue pool that had been digested, and (2) tissue taken directly from DES-injected chicks at the time of the labeling. The labeling was done on culture day 12 for the *in vitro* cells. The results demonstrated that cell monolayers responded to estradiol with a much greater transcription of the ovalbumin mRNA. In this experiment there was 13 times as much ovalbumin mRNA made in monolayers stimulated with estradiol compared to non-hormone stimulated cells. Ovalbumin mRNA synthesis was greatest in the oviduct minces obtained directly from the chicks, and this rate was 4.6 times higher than that in the cell monolayers, and 2.2 times more than those maintained 12 days in culture. The minces that had been maintained for 12 days in culture had twice the amount of specific mRNA compared to the monolayer cells.

Experiment two was labeling performed on tissue and cells obtained from chicks that had received 2 weekly DES-Carbowax implants. One portion of the pooled oviducts was maintained as minces with 10^{-8} M estradiol stimulation; the rest was digested and maintained as monolayers with or without estradiol. Labeling for RNA synthesis was carried out on culture day 5. Cell monolayers supplemented with estradiol again demonstrated more synthesis of ovalbumin mRNA when compared to the withdrawn monolayer cells, and as before, the greatest amount of labeling was seen in the tissue minces. There was 1.69 times the amount of specific mRNA in the minces compared to the E_2 monolayers (comparable

to 2.08 times the amount on day 12) and 2.64 times more mRNA in the E_2 monolayers compared to the withdrawn cells. In this experiment there was more labeled mRNA in all categories, reflecting either a non-specific experimental difference, or a more efficient transcription system in cells in culture a shorter period of time, or those that have not gone through the mince withdrawal procedure. The overall result does demonstrate that ovalbumin mRNA is produced in the monolayer purified cells and increased in response to E_2.

Cellular localization of estrogen receptors

One other series of experiments was required to complete the characterization of the estrogen receptor in these cells. Recent evidence has redirected the thinking of the cellular localization of the estrogen receptor [12, 13]. Previously it was agreed that unoccupied Type I receptors were in the cytoplasm, while occupied Type I and all Type II receptors were in the nucleus. There is a recent concensus that the majority of estrogen receptors reside in the nucleus and cytoplasmic receptors are translocated during homogenization. We were therefore interested in the location of receptors in the withdrawn and stimulated minces and cells. When chicks were given DES or E_2 daily for 2 weeks there were 4000 to 13,000 nuclear receptors but none in the cytoplasm. When chicks were then withdrawn for 2 weeks, an average of 2000 nuclear and 1000 cytoplasmic receptors were detected in the oviducts. However, when minces were withdrawn and assayed every other day in culture up to 11 days, there were no detectable nuclear or cytoplasmic receptors. Neither were there any detectable receptors in minces that were withdrawn and digested but given no E_2, or in minimally stimulated oviducts that were digested but never supplemented with E_2. While the receptor exchange assay should detect occupied and unoccupied sites, it was possible there was excess DES or E_2 in the tissues that interfered with exchange equilibrium. Cytosol was treated with 1% charcoal at 42°C for 15 min to adsorb any endogenous estrogens. The cytosol was then centrifuged to remove the charcoal and standard assay procedures were used on the treated and non-treated cytosol. Temperatures and times used for saturation and exchange were 0 and 37°C, both up to 18 h. Some assays detected estrogen binding, but the binding curves and the affinities measured were not characteristic of the estrogen receptor we had measured in cells directly from the chicks. We interpret these results as indicating that cells maintained as tissue minces in withdrawal medium lacked functional estrogen receptors and that unoccupied receptors did not return to the cytoplasm.

The cultures derived have not been established as cell lines. Trypsin destroyed the viability of the cells upon transfer. We also tried to withdraw the cells again for 5 days before subculturing, but that did not provide viable cells to subculture. The cells were viable as monolayers for 3–4 weeks when regularly fed with medium supplemented with estradiol.

DISCUSSION

We have established methods to routinely obtain monolayers of epithelial cells from the chick oviduct. Animals in various stages of natural and induced estrogen stimulation were used as sources of material. It was possible to initiate monolayer cultures of oviduct epithelial cells using any of the estrogen-primed tissues and any dissociation protocol. However, estrogen receptor assays showed that only certain combinations of the protocols provided epithelial cells that retained receptors at levels that provided biological function and response. It was discovered that animals whose oviducts had been minimally stimulated with either E_2 or DES yielded tissues that withstood enzymatic digestion and then established monolayer cultures that maintained *in vivo* amounts of both estrogen and progesterone receptors. When the receptors were intact and kept active with E_2 supplementation, the biological activity of cells for ovalbumin synthesis was also preserved. The "minimal stimulation" term that we have used to define an oviduct that has grown to a size of 1.5–2.0 g with initial stimulation is apparently dependent upon time rather than the amount of hormone. This was evidenced by the ability to obtain similar receptors in cells from animals given 1 mg per day E_2 or DES for 2 weeks and those given 2 or 3 weekly injections of 25 mg DES in Carbowax. However, the weekly DES injections induced greater growth and therefore provided more oviduct tissues for cultures. If it were the levels of hormone rather than the length of time, then one would assume that the laying hen with natural endogenous levels of estrogen would be the best source of tissue. However, laying hen oviduct cells not only lost receptors, when cultured, but they also formed the poorest cultures in terms of the percentage of cells that attached and had the morphology of epithelial cells. The animals that had been given 4–8 weekly DES-Carbowax injections could not be withdrawn *in vivo* due to the depot characteristics of the Carbowax. However, when these oviducts were minced and cultured 72 h in a withdrawal medium before digestion, the receptors attained the ability to withstand standard digestion techniques, yielding viable cultures that maintained the normal quantitative and functional characteristics of true estrogen receptors. The length of withdrawal before digestion also appeared critical based on time course experiments and those where chronically stimulated minces were withdrawn and then restimulated in culture before digestion.

Ovalbumin mRNA levels increased in response to E_2 in the monolayer cells, but were always lower than mRNA made in tissue minces. This probably reflects the increased efficiency of differentiated cells in a three-dimensional tissue architecture. The monolayer

cells may have better specific functions when grown in collagen or biomatrix support gels. There was more mRNA labeled in the withdrawn monolayer cells of experiment two when compared to the stimulated cells of experiment one, which may be an indication that more than 5 days in culture is required to completely eliminate the estrogen or its influence on the cells. In both experiments there was clearly a stimulation by estrogen.

We have used the term "withdrawn" for tissues *in vivo* and *in vitro* when estrogen has been removed. However, this term does not fit into the classic definition because there were no cytoplasmic receptors. The receptors disappeared and were not resynthesized unless estrogen was readministered, and then they were again nuclear. Homogenization of tissue requires much more time and force using the Dounce than that needed for cells scraped off the culture dishes. The ease of disrupting the cultured cells and obtaining clean intact nuclei probably reduces loss of receptors into the "cytosol". This leads us to conclude that in the presence of estrogen the cultured cells had occupied nuclear receptors; in the absence of estrogen there were no receptors.

We have devised methods to attain large numbers of cells in culture using oviducts from chicks given minimal stimulation with E_2 or DES. There did not seem to be a difference in the quality or quantity of cells or receptors with either hormone. However, both hormones in large enough doses or for long enough times interrupted the receptor cycle so that it could not be maintained or reconstituted in individual cells established in culture with digestion enzymes. Following digestion of minimally stimulated tissue with collagenase, pancreatin, and BSA, cell monolayers of highly purified epithelial oviduct cells are obtained. The cells have proper functioning estrogen receptors which can induce progesterone receptors and stimulate increased transcription of ovalbumin mRNA. This method establishes monolayers of cells with an intact genetic apparatus and hormonal response that can be used as a homologous system to study the regulation of the ovalbumin gene.

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