Studies on Sex-Organ Development

PRENATAL EFFECT OF OESTROGENIC HORMONE ON TUBULAR-GLAND CELL MORPHOGENESIS AND OVALBUMIN-GENE EXPRESSION IN THE CHICK MÜLLERIAN DUCT

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The effects of diethylstilboestrol on morphogenesis and cyto-differentiation of the chick-embryo left Müllerian duct were examined. Embryos were treated at different stages of development with maximal-responsive doses of diethylstilboestrol over a 5-day interval. The shell gland and magnum regions of the Müllerian duct were then assayed for growth and histological morphogenesis. The results were correlated with diethylstilboestrol-induced ovalbumin-gene expression as measured by ovalbumin-mRNA (mRNA_{ov}) accumulation and the relative rate of ovalbumin synthesis. Treatment of the embryo from day 10 to day 15 of incubation induces morphogenesis of tubular-gland cells in the Müllerian-duct magnum. Although these cells constitute 10% of the total cell population and contain an average of 8000 molecules of mRNA_{ov} per cell, ovalbumin synthesis is only 0.85% of total magnum protein synthesis. The Müllerian-duct magnum of embryos treated from day 13 to day 18 of incubation contains about 30% tubular-gland cells, which have accumulated an average of 7000 molecules of mRNA_{ov} per cell, but ovalbumin synthesis is only 3.25% of total magnum protein synthesis. The Müllerian-duct magnum of embryos treated from day 16 to day 21 of incubation contains about 50% tubular-gland cells, which have accumulated an average of 6500 mRNA_{ov} molecules per cell, and ovalbumin synthesis is 10% of total magnum protein synthesis. Oestrogen responsiveness develops simultaneously in the Müllerian-duct magnum and shell-gland regions. Compared with the rate of diethylstilboestrol-induced oviduct growth, the relative rate of diethylstilboestrol-induced Müllerian-duct growth increases with embryonic age, from 20-fold lower in the 10-day embryo to only 3-fold lower in the 16-day embryo. All results are discussed in comparison with the responses to oestrogen of the immature chick oviduct, and in terms of the ontogeny of hormone-competent epithelial and stromal components of the Müllerian duct. It is concluded that the development of oestrogenic competence in the embryonic Müllerian duct is a multiphasic phenomenon. A dramatic increase in hormone responsiveness in the Müllerian duct occurs between days 10 and 16 of development, and a less dramatic final maturation of oestrogen responsiveness occurs between day 16 of development and 1 week after hatching.

Cell differentiation implies a reprogramming of the cellular genome that produces a specialized state within the cell. This is reflected as a change in the types of RNA and protein synthesized by the cell. Highly differentiated cells are specialized in function, and this specialization often then takes the form of specific protein products that are synthesized preferentially in large amounts by one cell type.

Studies on the control of genomic expression in eukaryotic organisms have taken great steps forward in the last decade. The isolation of reverse transcriptase, an RNA-virus enzyme that has the ability

under specific conditions to copy mRNA templates into single-stranded DNA complementary to the template used (Baltimore, 1970), has provided a means of producing [^3H]DNA copies complementary to specific purified mRNA species. The [^3H]cDNA can then be used to measure quantitatively the content of a particular mRNA in RNA populations by virtue of its ability to hybridize specifically with its template mRNA. The use of [^3H]cDNA probes provides a means of assessing the degree of differentiation of a specific tissue or cell type in terms of its expression of the genes coding for its unique protein products. Several steroid-hormone-dependent mRNA species have been demonstrated in their respective

Abbreviation used: cDNA, complementary DNA.

target tissues. Of particular interest in this study is the oestrogen-inducible ovalbumin mRNA in the chick oviduct (Means *et al.*, 1972; Schimke *et al.*, 1973).

Oestrogen administered to immature chicks induces morphogenesis and cyto-differentiation within the oviduct (Brant & Nalbandov, 1956; Kohler *et al.*, 1968). Three distinct cell types differentiate from the primitive mucosa. Two of these cell types, the tubular-gland cells and the goblet cells, synthesize cell-specific proteins that can be measured readily by biochemical and immunochemical techniques and therefore can be used as markers for the differentiation process (Means & O'Malley, 1974). The tubular-gland cell, induced by oestrogen in the oviduct magnum, has been shown to be the site of synthesis of the egg-white proteins ovalbumin, conalbumin, ovomucoid and lysozyme (Kohler *et al.*, 1968; Oka & Schimke, 1969; Palmiter, 1972a; Palmiter & Gutman, 1972).

The present study was undertaken in order to define more clearly the effects of oestrogen on the chick-embryo left Müllerian duct. This organ is the embryonic precursor of the functional hen oviduct. We decided to investigate oestrogen-induced ovalbumin-gene expression in the embryonic Müllerian ducts, as measured by the accumulation and translation of ovalbumin mRNA. Correlation of these findings with the appearance of tubular-gland cells in the Müllerian-duct magnum provides a means of following oestrogen-induced differentiation of a specific highly specialized cell type within this embryonic organ. The results can then be correlated with existing evidence on the development of oestrogen responsiveness in the Müllerian duct presented in the preceding paper (Andrews & Teng, 1979), and thus provides a detailed description of the ontogeny of the acquisition of hormone competence by the embryonic sex tract.

Materials and Methods

Chemicals

The following chemicals were obtained from the sources indicated: L-[³H]leucine (62 Ci/mmol) and [³H]dGTP (17 Ci/mmol) were purchased from Schwarz/Mann (Orangeburg, NY, U.S.A.); [³H]-dCTP (24 Ci/mmol) and [³H]dATP (13 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.); avian-myeloblastosis-virus reverse transcriptase was a gift from J. W. Beard of Life Sciences (St. Petersburg, FL, U.S.A.); S₁ nuclease was from Sigma Chemical Co. (St. Louis, MO, U.S.A.); proteinase K was from EM Laboratories (Elmsford, NY, U.S.A.); high-titre antisera against ovalbumin and bovine serum albumin were from Antibodies Inc. (Davis, CA, U.S.A.); bovine serum albumin, conalbumin and ovalbumin (5×crystallized) were

purchased from ICN Chemical Co. (Irvine, CA, U.S.A.). All other chemicals were of the best commercially available grade.

Animals

Eggs and chicks were maintained and treated with oestrogen as described previously (Teng & Teng, 1978c; Andrews & Teng, 1979). Müllerian ducts were harvested after 5 days of exposure to diethylstilboestrol *in ovo*. This was accomplished by an initial treatment of the eggs with diethylstilboestrol followed 72 h later with a second treatment. The methodology, optimal dosage and length of time between exposures to diethylstilboestrol have been described previously (Andrews & Teng, 1979).

Isolation of ovalbumin mRNA

Ovalbumin mRNA (mRNA_{ov}) was generously given by Dr. Savio Woo of the Baylor College of Medicine (Houston, TX, U.S.A.). This mRNA was purified as described by Woo *et al.* (1975). The purity of the ovalbumin mRNA was assessed by acid/urea/agarose-gel electrophoresis, polyacrylamide-gel electrophoresis in 98% formamide, sucrose-gradient centrifugation in 70% formamide, translation *in vitro* in a heterologous protein-synthesizing system and enzymic digestion followed by homochromatographic analysis of the resultant oligonucleotide 'maps'. By these methods the mRNA was determined to be at least 90% pure.

Preparation of complementary DNA

The ³H-labelled DNA species complementary to mRNA_{ov} ([³H]cDNA_{ov}) were generously given by Dr. Christina Chang of the Baylor College of Medicine (Houston, TX, U.S.A.). For use in hybridization experiments in the presence of RNA excess a [³H]cDNA_{ov} probe was synthesized as described previously (Harris *et al.*, 1975); the reaction mixture contained, in a final volume of 0.5 ml: 50 mM-Tris/HCl (pH 8.1), 20 mM-dithiothreitol, 10 mM-MgCl₂, 36 µg of actinomycin D/ml, 150 µM-[³H]dATP, 83 µM-[³H]dCTP, 120 µM-[³H]dGTP and 0.5 mM-dTTP. Also included in the reactions were: 2.5 µg of oligo-(dT)₁₂₋₁₈, 50 µg of mRNA_{ov} and 150 units of avian-myeloblastosis-virus reverse transcriptase. Under these conditions the [³H]cDNA_{ov} had a specific radioactivity of approx. 5.5 × 10⁷ c.p.m./µg.

The reaction was carried out at 46°C for 15 min and then the solution was adjusted to an EDTA concentration of 2 mM. Sheared *Escherichia coli* DNA was added as a carrier at a concentration of 100 µg/ml; the solution was adjusted to 0.5% sodium dodecyl sulphate and the entire sample was placed on a Sephadex G-50 column (80 cm × 1.5 cm) equilibrated with 0.1 M-NaCl/2 mM-EDTA. The excluded fractions were pooled and precipitated with 2 vol. of ethanol. The precipitate was then dissolved in 0.2 M-NaOH/

10mM-EDTA and incubated at 68°C for 30 min to hydrolyse the RNA. The solution was then neutralized with 2M-sodium acetate buffer, pH 4.5, and the [³H]cDNA and carrier were precipitated with 2 vol. of ethanol at -20°C for 18 h. The final precipitate was dissolved in distilled water to a concentration in which 2.5 µl represented about 3000 c.p.m. or about 50 pg of [³H]cDNA_{ov}.

A [³H]cDNA_{ov} probe for use in hybridization experiments in the presence of cDNA excess was synthesized as previously described (Monahan *et al.*, 1976b). The reaction mixture contained, in a final volume of 0.1 ml: 20 mM-Tris/HCl, pH 8.1, 2 mM-dithiothreitol, 8.5 mM-MgCl₂, 100 µg of actinomycin D/ml, 0.3 mM each of dCTP, dATP, dGTP and dTTP, and 0.1 mM of [³H]dCTP. The reaction mixtures also included: 0.5 µg of oligo(dT)₁₂₋₁₈, 20 µg of mRNA_{ov} and 10 units of avian-myeloblastosis-virus reverse transcriptase. Under these conditions the [³H]cDNA_{ov} has a specific radioactivity of approx. 5.5×10^6 c.p.m./µg. The reaction and purification of the probe were carried out as described above. The final precipitate was dissolved in distilled water to a concentration in which 2.5 µl represented about 10 500 c.p.m. or about 1.9 ng of [³H]cDNA_{ov}.

Isolation of total RNA

Total cell RNA was isolated as described by Rosen *et al.* (1975). Müllerian ducts or oviducts were frozen in liquid N₂ immediately after removal. The frozen tissue was broken into small pieces and homogenized in 5 vol. of SDS buffer (0.5% sodium dodecyl sulphate/25 mM-EDTA/75 mM-NaCl, pH 8.0) and 5 vol. of redistilled phenol saturated with SDS buffer. The aqueous phase and protein interphase were collected and re-extracted with 0.5 vol. of phenol saturated with SDS buffer and 0.5 vol. of chloroform/3-methylbutanol (24:1, v/v). The aqueous phase was then made 0.2M with respect to NaCl, 2 vol. of ethanol was added and nucleic acids were precipitated at -20°C for 18 h. The removal of DNA was according to the procedure of Palmiter (1973). The nucleic acid pellet was completely dissolved in distilled water (1 ml/g of tissue), 3 vol. of 4M-sodium acetate buffer, pH 6.0, was added and the solution was cooled for 30 min at 0°C. The RNA precipitate was collected by centrifugation at 16000 g_{max} for 30 min at 0°C in a Beckman model J-21 centrifuge with the JS-13 rotor. This process was repeated three times. The resultant RNA pellet was suspended in SDS buffer (1 ml/g of tissue), proteinase K was added to a final concentration of 30 µg/ml and the mixture was then incubated at 37°C for 30 min. The mixture was extracted with an equal volume of phenol/chloroform (1:1, w/v), the aqueous phase was made 0.25M with respect to NaCl and the RNA was precipitated with 2 vol. of ethanol at -20°C for 18 h. The resultant RNA pellet was dissolved in Millipore-filtered glass-distilled

water (100–300 µl/g of tissue). This process recovers 100–400 mg of total cell RNA/g of tissue. The RNA had an A₂₆₀/A₂₈₀ ratio greater than 2.0 and contained less than 1% DNA as judged by the diphenylamine reaction (Giles & Myers, 1965).

Hybridization in the presence of RNA excess

Hybridization reactions in the presence of RNA excess were carried out essentially as described by Harris *et al.* (1975). The reaction was performed in sealed glass conical vials that were completely submerged to prevent condensation. The final volume of 50 µl contained: 0.6M-NaCl, 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.6, 2 mM-EDTA, 1.0 ng of [³H]cDNA_{ov}/ml (5.5×10^7 c.p.m./µg) and RNA samples ranging from 0.05 µg/ml to 2000 µg/ml depending on the sample being analysed. All reactions also contained 400 µg of poly(rU) carrier (P-L Biochemicals, Milwaukee, WI, U.S.A.)/ml. The mixtures were sealed, heated for 2 min at 100°C, then incubated at 68°C for 0.5–96 h. Samples were taken at each time point, including zero time, for analysis of total trichloroacetic acid-precipitable radioactivity and S₁ nuclease-resistant radioactivity in the presence and absence of RNA sample. mRNA_{ov} could protect 86–90% of the total radioactivity from S₁-nuclease digestion; 10–12% of the total trichloroacetic acid-precipitable radioactivity was resistant to S₁ nuclease at zero time and in the absence of added RNA.

Hybridization in the presence of [³H]cDNA excess

Hybridization reactions in the presence of [³H]-cDNA_{ov} excess were carried out essentially as described by Harris *et al.* (1976). The validity of this procedure has been rigorously justified by Young *et al.* (1974).

Reactions were performed under the conditions described above for hybridization in the presence of RNA excess. In this case, however, 38 ng of [³H]-cDNA_{ov} (5.5×10^6 c.p.m.)/ml was incubated at 68°C with various concentrations of sample RNA to a C₀t [cDNA concentration (mol/l) × time (s)] of 0.1 (48 h) to ensure completion of all hybridization reactions. Purified mRNA_{ov} was added to certain reaction vials in the concentration range 3.8–100 ng/ml. RNA samples were added to the reaction vials in the concentration range 3.8–2000 µg/ml. At the end of 48 h all vials were quickly frozen in acetone/solid CO₂. With this probe 3–6% of the total trichloroacetic acid-precipitable radioactivity was resistant to digestion with S₁ nuclease at zero time or in the absence of RNA. mRNA_{ov} could protect 90–95% of the total radioactivity from S₁-nuclease digestion.

S₁-nuclease treatment of hybrids

After incubation samples were assayed for hybridization by resistance to digestion with S₁

nuclease (Ando, 1966). S_1 nuclease was stored in 50% (v/v) glycerol/100 mM-NaCl/100 mM-sodium acetate buffer (pH 4.5)/1 mM-ZnCl₂ at 1000 units/ml (Sigma).

Hybridization reactions in the presence of RNA excess (50 μ l) were assayed for hybridization by the addition of 450 μ l of S_1 buffer [100 mM-NaCl/100 mM-sodium acetate buffer (pH 4.5)/1 mM-ZnCl₂/denatured calf thymus DNA (50 μ g/ml)] followed by 5 μ l of S_1 nuclease (5 units). The reaction was allowed to proceed at 37°C for 1 h.

Hybridization reactions in the presence of cDNA excess (50 μ l) were assayed for hybrid formation exactly as described above, except that 20 μ l of S_1 nuclease (20 units) was included.

At the end of the incubation period 100 μ g of bovine serum albumin was added to the vials followed by 3 ml of cold 20% (w/v) trichloroacetic acid. After 30 min at 4°C the vial contents were collected and washed on Millipore filters. The filters were dried, then dissolved in 3 ml of methylCellosolve (methoxyethanol). After the addition of 10 ml of Aquasol (New England Nuclear) the radioactivity of the samples was counted in a Beckman model LS-250 scintillation spectrometer at 35% efficiency of counting for ³H.

The data from hybridization experiments in the presence of RNA excess are expressed as the percentage of [³H]cDNA_{ov} that was resistant to S_1 -nuclease digestion (% hybrid) versus the product of the RNA sample concentration (mol/l) $\times$ time (s) or R_{0t} . All data are reported as the equivalent R_{0t} that one would observe if the reactions were carried out in 0.12 M-sodium phosphate buffer rather than 0.6 M-NaCl (Britten & Smith, 1969). In addition, the graphed data have been corrected for the non-hybridizing components of the [³H]cDNA_{ov} probe. These manipulations in no way alter the conclusions reported.

The data from hybridization experiments in the presence of cDNA excess are expressed as the % hybrid versus the RNA/cDNA (ng/ng) ratio (Gilmour & Paul, 1973). No change in the extent of hybridization was observed if samples were incubated to a C_{0t} of 0.15 instead of 0.10.

Determination of protein, RNA and DNA

Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as a standard. DNA was determined by the diphenylamine reaction (Giles & Myers, 1965), with calf thymus DNA (Schwarz/Mann) as a standard. RNA was determined by the procedure of Munro & Fleck (1966). Purified RNA samples were measured quantitatively by reading the A_{260} of a 1:500 dilution; 1 mg of RNA/ml has A_{260} 25.

Gel electrophoresis of nucleic acids and protein

RNA samples and [³H]cDNA samples were electrophoresed in 2% agarose/6 M-urea/25 mM-

sodium citrate buffer (pH 3.5), with 5S, 18S and 28S rRNA samples as reference standards for approximating molecular weights (Rosen *et al.*, 1975).

Protein samples were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described by Laemmli (1970), with conalbumin (mol.wt. 76000), ovalbumin (mol.wt. 45000) and lysozyme (mol.wt. 14500) as reference standards (Warner, 1954).

Radioactive gels were frozen on solid CO₂ and then sliced, with a multiple razor-blade slicer, into 1 mm discs. Agarose-gel slices were melted at 85°C in 0.5 ml of water, and then their radioactivities counted in 7 ml of Aquasol as described above. Polyacrylamide-gel slices were extracted with 0.5 ml of 30% (v/v) H₂O₂ at 85°C for 30 min and then their radioactivities were counted in 7 ml of Aquasol.

Labelling of Müllerian-duct and oviduct proteins and preparation of tissue homogenates

Müllerian-duct and oviduct samples were cultured *in vitro* as described by Palmiter *et al.* (1971). Pieces of tissue (5–10 mg) were incubated (50 mg/2 ml), in Hanks balanced salt solution (Hanks & Wallace, 1949) containing 25 mM-Hepes buffer, pH 7.3, streptomycin (50 mg/ml) and penicillin (500 units/ml) at 41°C for 60 min in the presence of 15 μ Ci of L-[³H]leucine (62 Ci/mmol)/ml. The explants were washed with cold Hanks solution and then frozen.

Labelled explants were blotted and weighed, and a 10% (w/v) homogenate was prepared, in 25 mM-Tris/HCl (pH 7.6)/25 mM-NaCl/5 mM-MgCl₂/1% Triton X-100/1% sodium deoxycholate, in a motor-driven Teflon/glass homogenizer (Palmiter *et al.*, 1971). The homogenate was diluted to 5% with the above buffer less detergents and centrifuged in 600 μ l cellulose nitrate tubes [4.8 mm $\times$ 41.3 mm ($\frac{3}{16}$ in $\times$ 1 $\frac{1}{8}$ in); Beckman] at 100000g for 60 min at 4°C in a Beckman model L5-65 ultracentrifuge with the SW-50.1 rotor equipped with adaptor model 305527 (Beckman Instruments, Palo Alto, CA, U.S.A.).

Determination of the relative rate of ovalbumin synthesis

Total trichloroacetic acid-precipitable and specific immunoprecipitable radioactivity were determined as described by Palmiter *et al.* (1971)

Immunoprecipitation reactions were performed in 400 μ l Microfuge tubes (Beckman) in a final volume of 330 μ l of buffer [25 mM-Tris/HCl (pH 7.6)/25 mM-NaCl/5 mM-MgCl₂/1% Triton X-100/1% sodium deoxycholate] containing 5–200 μ l of sample cytosol, enough anti-ovalbumin serum to precipitate 35 μ g of ovalbumin and 10 μ g of ovalbumin carrier. Control reactions contained enough anti-(bovine serum albumin) serum to precipitate 35 μ g of bovine serum albumin and 10 μ g of bovine serum albumin carrier. The mixtures were left at 4°C for 18 h, and then centrifuged for 1 min in a Beckman model 152

Microfuge at $15000g_{\max}$. The precipitates were washed twice in $340\mu\text{l}$ of buffer less detergents, and then dissolved in $200\mu\text{l}$ of NCS (Amersham/Searle, Arlington Heights, IL, U.S.A.). The bottom half of the tube was cut off and placed in 13 ml of Aquasol. After this had been mixed, $200\mu\text{l}$ of 0.6M-HCl was added, and the vials were remixed and then their radioactivities counted as described above. The amount of radioactivity trapped in the bovine serum albumin immunoprecipitate was subtracted from the amount precipitated by anti-albumin serum to yield a final value for ovalbumin radioactivity. Approx. 0.2–0.4% of the total radioactivity in a given sample was trapped by the control precipitate. Several immunoprecipitations were performed with different amounts of sample to ensure antibody excess.

Trichloroacetic acid-precipitable radioactivity was determined on samples of the homogenate by the addition of an equal volume of cold 10% (w/v) trichloroacetic acid. The volume was adjusted to $340\mu\text{l}$ with 5% (w/v) trichloroacetic acid and the samples were left for 30 min on ice. Precipitates were collected by centrifugation for 1 min at $15000g_{\max}$, washed twice with $340\mu\text{l}$ of 5% (w/v) trichloroacetic acid and their radioactivities counted as described above for the immunoprecipitates. Explants cultured in these conditions generally incorporated 3×10^4 – 5×10^4 c.p.m./mg of tissue with a protein specific radioactivity of 300–500 c.p.m./ μg .

Fig. 1. Acid/urea/agarose-gel electrophoresis of chick Müllerian-duct total cell RNA

A $50\mu\text{g}$ sample of total cell RNA from the 21-day Müllerian duct was electrophoresed in 3 mm disc gels containing 2% agarose/6 M-urea, pH 3.5, at 1 mA/gel for 1 h followed by 2 mA/gel for 3 h. The gel was removed and scanned at 270 nm in a Pye-Unicam model SP.1800 spectrophotometer equipped with a linear recorder. The migrations of 5S, 18S and 28S rRNA from hen oviduct are indicated.

Histology

Müllerian-duct samples were removed, washed in 0.9% NaCl and then fixed for 24 h in Bouin's fixative. Explants were rinsed in 70% (v/v) ethanol before being embedded in paraffin wax. Sections ($5\mu\text{m}$) were stained with Haematoxylin and Eosin and viewed by phase-contrast microscopy (Conn, 1961).

Results

Characterization of total cell RNA isolates and [^3H]cDNA probes

The profile of total cell RNA isolated from the Müllerian duct and electrophoresed in 2% agarose/acid/urea is shown in Fig. 1. Total cell RNA extracted from tissues by direct homogenization into phenol/0.5% sodium dodecyl sulphate (1:1, v/v), a process that avoids problems with ribonuclease degradation, appeared to be intact by gross electro-

Fig. 2. Acid/urea/agarose-gel electrophoresis of [^3H]cDNA probes

[^3H]cDNA_{ov} (80000 c.p.m.), synthesized as described in the Materials and Methods section, was mixed with $20\mu\text{g}$ of carrier RNA and electrophoresed as described in the legend to Fig. 1. The gels were removed and sliced and their radioactivities counted as described in the Materials and Methods section. The relative migrations of 5S, 18S and 28S rRNA from hen oviduct are indicated. (a) [^3H]cDNA used in hybridization experiments in the presence of RNA excess (5.5×10^7 c.p.m./ μg); (b) [^3H]cDNA used in hybridization experiments in the presence of cDNA excess (5.5×10^6 c.p.m./ μg).

phoretic examination, displaying sharp peaks of 4-5S, 18S and 28S rRNA as well as some other distinct RNA peaks.

The [^3H]cDNA_{ov} synthesized with a specific radioactivity of 5.5×10^7 c.p.m./ μg and used in hybridization experiments in the presence of RNA excess had a mobility of between 5S and 9S compared with rRNA standards (Fig. 2a). This probe was estimated to be between 300 and 900 nucleotide residues in length and thus probably represented DNA complementary to the 3'-end of ovalbumin mRNA and about 25% as long as the message itself (Woo *et al.*, 1975). At this high specific radioactivity it is possible to use as little as 50 pg (3000 c.p.m.) of [^3H]cDNA per assay tube during hybridizations. Hybridization experiments in the presence of cDNA excess were also performed with total cell RNA samples. This type of analysis requires much less RNA sample and avoids the effects of mRNA size on the rate of hybridization (Young *et al.*, 1974). In addition, all samples were incubated for the same length of time and problems of maintaining conditions of RNA excess were eliminated. The electrophoretic migration of the [^3H]cDNA probe used in hybridization experiments in the presence of cDNA excess is shown in Fig. 2(b). A distinct 21S peak of cDNA appeared in acid/urea/agarose gels, and this is indicative of full-length copy of the ovalbumin mRNA (Woo *et al.*, 1975; Kacian & Myers, 1976; Monahan *et al.*, 1976b,c).

Fig. 3. Scatchard analysis of the RNA-driven back-hybridization reaction between mRNA_{ov} and cDNA_{ov}. The results of a back-hybridization reaction between a 50–500-fold excess of mRNA_{ov} and its complementary [^3H]cDNA were plotted according to the methods of Marsh & McCarthy (1973). The details of the hybridization reaction are presented in the Materials and Methods section. The linearity of the plot is indicative of a single-component hybridization reaction.

Determination of the amount of ovalbumin mRNA in total cell RNA by hybridization in the presence of RNA excess

Scatchard analysis of the RNA-driven back-hybridization of ovalbumin mRNA with its complementary DNA is presented in Fig. 3. This Scatchard plot, calculated as described by Marsh & McCarthy (1973), is linear and thus indicative of a single reacting component during hybridization. This conclusion is supported by the $R_0 t$ curve for this reaction presented in Fig. 4. The reaction follows pseudo-first-order kinetics, being completed within a 100-fold concentration of mRNA. The equivalent $R_0 t/2$ of this reaction was $7 \times 10^{-3} \text{ mol} \cdot \text{l}^{-1} \cdot \text{s}$, which is in close agreement with the values of $6.5 \times 10^{-3} \text{ mol} \cdot \text{l}^{-1} \cdot \text{s}$ published by Monahan *et al.* (1976a) and $4 \times 10^{-3} \text{ mol} \cdot \text{l}^{-1} \cdot \text{s}$ published by Harris *et al.* (1975) for the same reaction. In addition, the probe hybridized with RNA samples from stimulated oviduct, but only slightly with RNA samples from control oviduct, and even less with non-target tissue RNA (Fig. 4). It is therefore concluded that this probe is specific for ovalbumin mRNA.

Hybridization experiments in the presence of RNA excess were performed with total cell RNA extracts in order to examine developmental changes in the responses of the left Müllerian duct to 5 days' exposure to diethylstilboestrol, as measured by the accumulation of ovalbumin mRNA. As demonstrated in Fig. 4, the $R_0 t$ curves for all experimental samples were essentially parallel, and the rate of reaction ($R_0 t/2$) was dependent on the sample tested. The results show that throughout embryonic development and within 1 week of hatching the sex tract contained very low concentrations of ovalbumin mRNA, representing less than 1 part per 10^6 of the total cell RNA content. This was also true for non-target tissues. When, however, the Müllerian duct was exposed to diethylstilboestrol a dramatic accumulation of ovalbumin mRNA was observed. This was true for all age groups tested. The final extent of accumulation of ovalbumin mRNA, after 5 days' exposure to diethylstilboestrol, increased with embryonic age, but in no case did the Müllerian duct respond as dramatically as did the chick oviduct. The $R_0 t$ curves tended to become attenuated for samples with a low concentration of ovalbumin mRNA.

Determination of the amount of ovalbumin mRNA in total cell RNA by hybridization in the presence of cDNA excess

When the total cell RNA samples, described above, were analysed for ovalbumin mRNA content by hybridization in the presence of cDNA excess the results presented in Fig. 5 were obtained. Purified ovalbumin mRNA protected 60% of the [^3H]cDNA probe from S_1 -nuclease digestion at an mRNA/cDNA

Fig. 4. Determination of mRNA in diethylstilboestrol-stimulated and control sex tracts by hybridization in the presence of RNA excess

All eggs and chicks were treated for 5 days with diethylstilboestrol, as detailed in the Materials and Methods section, unless designated as control. Total cell RNA isolates were allowed to hybridize at 68°C for 0.5–96 h with 50 pg of [³H]-cDNA_{ov} (3000 c.p.m.) as detailed in the Materials and Methods section. The percentage of hybrid was measured as radioactivity resistant to S₁-nuclease digestion, and corrected for the non-hybridizing components of the reaction, i.e. 10% hybrid at zero time or in the absence of RNA and 86% maximal extent of hybridization. Equivalent R_0t values are corrected for the effects of salt concentration on the reaction rate (Britten & Smith, 1969). The R_0t curves represent hybridization reactions between [³H]cDNA and: curve 1, mRNA_{ov}; and total cell RNA from the diethylstilboestrol-treated animal of: curve 2, 9-day-old chick oviduct; curve 3, Müllerian duct of the 21-day embryo; curve 4, Müllerian duct of the 18-day embryo; curve 5, Müllerian duct of the 15-day embryo; curve 6, total cell RNA from the control sex tract of the respective four developmental stages previously described. Curve 7 (■), rooster liver RNA; curve 8 (□), rat liver RNA. The symbols within each R_0t curve refer to the concentration of the RNA in the sample: ○, 20 μg of total cell RNA/ml or 0.05 μg of mRNA_{ov}/ml; ●, 200 μg of total cell RNA/ml or 0.50 μg of mRNA_{ov}/ml; △, 2000 μg of total cell RNA/ml.

Fig. 5. Determination of mRNA_{ov} in diethylstilboestrol-stimulated and control sex tracts by hybridization in the presence of cDNA excess

Diethylstilboestrol was administered for 5 days as described in the Materials and Methods section. Various amounts of total cell RNA were incubated

ratio of 1:1, and could protect 90–95% of the probe at an mRNA/cDNA ratio of 2.5:1. Control sex-tract RNA contained some measurable ovalbumin mRNA, as did rooster liver RNA. The reason for this latter observation is not clear. The amounts of ovalbumin mRNA observed were less than 2 parts per 10⁶ of total cell RNA and were thus at the limits of sensitivity of the assay method. Similar obser-

at 68°C for 48 h with 1.9 ng of [³H]cDNA_{ov} (5.5 × 10⁶ c.p.m./μg) in a volume of 50 μl as detailed in the Materials and Methods section. The percentage of hybrid represents the S₁-nuclease-resistant radioactivity/total radioactivity. Titration curves represent hybridizations between [³H]cDNA_{ov} and: ●, chick oviduct total cell RNA; ■, mRNA_{ov}; △, 21-day Müllerian-duct RNA; ▲, 18-day Müllerian-duct RNA; □, 15-day Müllerian-duct RNA; ○, control, oviduct and Müllerian-duct RNA. In the absence of RNA or at zero time 5% of the total [³H]cDNA radioactivity was S₁-nuclease-resistant. At an mRNA/cDNA ratio of 2.5:1, 90–95% of the radioactivity was S₁-nuclease-resistant.

variations have been made on globin-gene expression in non-erythroid tissues (Humphries *et al.*, 1976). The results obtained by analysis of total cell RNA in the presence of cDNA excess agree well with results obtained with hybridization in the presence of RNA excess. The simplest interpretation of this finding is that treatment of embryos or chicks with diethylstilboestrol resulted in the induction of increased transcription of the ovalbumin gene within the cells of the sex tract. It must be emphasized that in fact only the accumulation of ovalbumin mRNA was being measured in these experiments. In the embryonic Müllerian duct the magnitude of accumulation of ovalbumin mRNA, after 5 days' exposure to diethylstilboestrol, was an age-related phenomenon, and in no case was this degree of response as great as that observed in the chick oviduct.

Age-dependent changes in the diethylstilboestrol-induced accumulation of ovalbumin mRNA in the Müllerian duct

From the results presented in Table 1 it is apparent that hybridization in the presence of cDNA excess yielded larger values for the content of ovalbumin mRNA than those obtained by hybridization in the presence of RNA excess. This effect was most exaggerated in samples that contained small amounts of ovalbumin mRNA. It is probable that the observed quantitative differences were due to difficulties in maintaining conditions of RNA excess during hybridization experiments, as well as to the possible

breakdown of RNA during the long incubation periods, thus making hybrids more susceptible to S_1 -nuclease digestion. In addition, a full-length cDNA probe was used in hybridization reactions in the presence of cDNA excess.

Treatment of the 10-day embryo with diethylstilboestrol resulted, after 5 days, in the accumulation of significant concentrations of ovalbumin mRNA in total cell RNA (0.0125%). By day 16 of development this 5-day response to diethylstilboestrol had increased 3–4-fold in terms of the percentage of ovalbumin mRNA in total cell RNA (0.04%). This degree of accumulation was 2–3-fold lower than that observed in the oviduct of the oestrogen-treated chick (0.095%).

Determination of the relative rate of ovalbumin synthesis by specific immunoprecipitation of labelled ovalbumin

The selective immunoprecipitation of ovalbumin from sex-tract proteins labelled *in vitro* provides a method for correlating the observed content of ovalbumin mRNA with the translation of this message. The validity of the assay method depends on the specificity of the antibody used. The relative rate of ovalbumin synthesis in different experimental groups can be determined, without any effect of the amino acid pool size or rate of amino acid uptake on the results obtained, assuming that a common leucine pool is used in the synthesis of all cellular proteins.

Table 1. *Age-dependent effects of diethylstilboestrol on ovalbumin-gene expression in the chick Müllerian duct*
Diethylstilboestrol was administered for 5 days as detailed in the Materials and Methods section. Ovalbumin-gene expression, as measured by accumulation of mRNA_{ov}, was determined from results presented in Figs. 4 and 5. Data from these Figures were used to calculate the percentage of mRNA_{ov} in total cell RNA from oestrogen-treated or control sex tracts. By using data for hybridization in the presence of RNA excess the percentage of mRNA_{ov} was calculated as:

$$\frac{R_{ot}/2 \text{ of pure mRNA}_{ov}}{R_{ot}/2 \text{ of sample RNA}} \times 100$$

From data for hybridization in the presence of cDNA excess the percentage of mRNA_{ov} was calculated by comparing the initial slope of the titration curve for sample RNA with that observed with pure mRNA_{ov} (Young *et al.*, 1974). N.S., Not significant (i.e. less than 0.0005%).

Sample	$R_{ot}/2$ 7×10^{-3}	$10^{-3} \times \% \text{ of mRNA}_{ov}$	
		Hybridization in the presence of RNA excess 10^5	Hybridization in the presence of cDNA excess 10^5
Oestrogen-treated			
Oviduct	10	70.0	95.0
21-day Müllerian duct	32	22.0	40.0
18-day Müllerian duct	45	16.0	33.0
15-day Müllerian duct	128	5.5	12.5
Control			
Oviduct	$\sim 30 \times 10^3$	N.S.	N.S.
21-day Müllerian duct	$\sim 30 \times 10^3$	N.S.	N.S.
Rooster liver	$\sim 100 \times 10^3$	N.S.	N.S.

Fig. 6. Electrophoresis of immunoprecipitated [³H]-ovalbumin

Diethylstilboestrol was administered for 5 days as detailed in the Materials and Methods section. Diethylstilboestrol-stimulated Müllerian-duct explants were incubated with 15 μ Ci of L-[³H]leucine/ml for 1 h as described in the Materials and Methods section. A homogenate was prepared and centrifuged as described in the Materials and Methods section. A portion of the supernatant was incubated with 5.6 A_{280} units of anti-ovalbumin serum (equivalence point 35–40 μ g) and (final concentrations) 1% sodium deoxycholate and 1% Triton X-100 in a total volume of 330 μ l for 18 h at 4°C. The precipitate was collected, washed, dissolved and electrophoresed in a sodium dodecyl sulphate/polyacrylamide gel as described in the Materials and Methods section. The relative migrations of purified conalbumin (mol.wt. 76000), ovalbumin (mol.wt. 45000) and lysozyme (mol.wt. 14500), three of the major egg-white proteins, are indicated. These standards were electrophoresed in a separate gel.

That the anti-ovalbumin serum used in this assay was highly specific is demonstrated in Fig. 6. Immunoprecipitated [³H]ovalbumin electrophoresed in a sodium dodecyl sulphate/polyacrylamide gel co-migrated with authentic ovalbumin, and no other protein peaks were observed. This cannot exclude the possibility that small amounts of some other protein may have been co-precipitated with ovalbumin, but a single contaminant representing as little as 2–4% of the total immunoprecipitate should have been detected by this method of analysis unless it co-migrated with ovalbumin.

The relative rate of ovalbumin synthesis was determined for each sample, as demonstrated in

Table 2. Determination of the relative rate of ovalbumin synthesis by chick oviduct by specific immunoprecipitation

Explants from diethylstilboestrol-stimulated (administered for 5 days) chick oviduct were incubated with 15 μ Ci of L-[³H]leucine/ml for 1 h at 41°C as described in the Materials and Methods section. Homogenates were prepared and centrifuged as described in the Materials and Methods section. Multiple portions of the supernatant were assayed for the radioactivity incorporated. Generally explants incorporated 3×10^4 – 5×10^4 c.p.m./mg of tissue, and 30–50% of the total radioactivity was trichloroacetic acid-precipitable with a specific radioactivity of 300–500 c.p.m./ μ g of protein. (a) Radioactivity precipitated with anti-(bovine serum albumin) serum; (b) radioactivity precipitated with anti-ovalbumin serum.

Sample (μ l of cytosol)	10 ⁻³ × L-[³ H]Leucine radioactivity (c.p.m./sample)		
	Trichloroacetic acid-precipitable	Immunoprecipitable	
		(a)	(b)
10	12.50	0.45	3.50
20	28.50	0.50	7.20

Table 2. Multiple portions of sample cytosol, when assayed, showed a linear precipitation of [³H]ovalbumin with increasing sample volume, thus indicating conditions of antibody excess were maintained. Anti-(bovine serum albumin) serum plus bovine serum albumin provided a control immunoprecipitate used to correct for non-specific trapping of L-[³H]leucine, which generally represented 0.2–0.4% of the total radioactivity of the sample tested.

Age-dependent changes in the diethylstilboestrol-induced synthesis of ovalbumin in the Müllerian duct

The induction of ovalbumin synthesis, by 5 days' exposure to diethylstilboestrol, is presented in Table 3. The total Müllerian duct as well as different regions within the organ were assayed. For comparison the responses of the chick oviduct are also shown. The results of this study indicate that control Müllerian ducts and oviducts were not synthesizing significant amounts of immunoprecipitable ovalbumin, at least within the limits of sensitivity of the assay. At day 10 of incubation this 5-day response (day 15) to diethylstilboestrol was very small, being only slightly above control values. The ability to respond to diethylstilboestrol increased 10-fold or more between day 10 and day 16 of incubation, but was still about 3-fold lower than the degree of response of the chick oviduct. When the sex tract was divided into magnum and shell-gland regions by morphological examination (Aitken, 1971), essentially all diethylstilboestrol-induced ovalbumin-synthesizing cells were present in the magnum region. The magnum is responsible for

Table 3. *Age-dependent effects of diethylstilboestrol on ovalbumin-gene expression in the chick Müllerian duct*

Diethylstilboestrol was administered for 5 days as detailed in the Materials and Methods section. Ovalbumin-gene expression, as measured by the relative rate of ovalbumin synthesis, was determined as described in Table 2. Explants were incubated with L-[³H]leucine and homogenates prepared as detailed in the Materials and Methods section. Several portions of cytosol were assayed for anti-ovalbumin-serum-precipitable radioactivity to ensure antibody excess. Values presented have been corrected for background trapping. The relative rate of ovalbumin synthesis was determined from homogenates of the total organ, the magnum region alone or the shell-gland region alone (Aitken, 1971). The percentage ovalbumin synthesis was calculated as:

$$\frac{\text{c.p.m. pptd. by anti-ovalbumin serum} - \text{c.p.m. pptd. by anti-(bovine serum albumin) serum}}{\text{Trichloroacetic acid-precipitable c.p.m.}} \times 100$$

All values are the means $\pm$ S.D. of nine determinations. N.D., Not detected.

Sample	% ovalbumin synthesis		
	In magnum	In shell gland	In total duct
Diethylstilboestrol-stimulated			
Oviduct	26.0 $\pm$ 3.0	0.40 $\pm$ 0.2	10.2 $\pm$ 2.0
21-day Müllerian duct	10.0 $\pm$ 3.0	N.D.	3.9 $\pm$ 2.0
18-day Müllerian duct	3.2 $\pm$ 1.0	0.90 $\pm$ 0.2	1.2 $\pm$ 0.5
15-day Müllerian duct	0.9 $\pm$ 0.2	N.D.	0.5 $\pm$ 0.2
Control			
Oviduct	N.D.	N.D.	N.D.
21-day Müllerian duct	N.D.	N.D.	N.D.

all egg-white protein synthesis in the mature hen oviduct (Gilbert, 1971). When the total duct was assayed for ovalbumin synthesis, rather than the magnum alone, the relative rate of ovalbumin synthesis decreased by about 60% in all the experimental groups tested. Therefore after exposure to hormone the contribution of the shell-gland region to the overall rate of total ductal protein synthesis was constant throughout development and after hatching.

Diethylstilboestrol-induced morphogenesis and growth of the Müllerian-duct shell gland

The shell-gland region of the Müllerian duct responded to 5 days' exposure to diethylstilboestrol by epithelial and stromal proliferation (Plate 1). The degree of the response was age-dependent, showing maximal values in the 16-day-embryonic Müllerian duct. The shell gland of the 15-day embryo, after 5 days' exposure to diethylstilboestrol starting on day 10, had undergone some morphogenesis (Plate 1a). Numerous areas of epithelial mitosis were evident, and a few small villus-like structures had developed, projecting into the lumen. The stroma was composed of mostly loose connective tissue. The general histological appearance of this region changed only slightly in response to hormone.

The shell gland of the 18-day-embryonic Müllerian duct showed a much greater morphogenic response to diethylstilboestrol (Plate 1b). The epithelia had proliferated, forming a few tubular glands in the stroma. Villus-like projections into the lumen had become prominent, and the stroma contained some

peripheral smooth-muscle layers as well as loose connective tissue.

The shell gland of the 21-day embryo exhibited the greatest relative morphogenic response after 5 days' exposure to the hormone (starting on day 16), as shown in Plate 1(c). The gland was highly convoluted, with extensive villus-like projections into the lumen. Many epithelial tubular glands were evident, and a core of smooth muscle was present within the villus-like projections. The shell gland was encased in several layers of smooth muscle. Therefore both epithelial and stromal components of the shell gland responded to oestrogen by this stage of embryonic development. However, no such pattern of differentiation was found in the shell gland of the control 21-day embryo (Plate 1d). It is clear from the results presented in Table 3 that the embryonic shell gland did not develop tubular glands active in the synthesis of ovalbumin, as would be expected. The growth of the 21-day embryonic shell gland, in terms of wet weight and DNA content, was 3-fold less than these diethylstilboestrol-induced changes in the chick oviduct shell gland.

It is noteworthy that the hormone-stimulated shell gland was very active in protein synthesis. As shown in Table 3, the shell gland was synthesizing protein at a rate approximately equal to that observed in the magnum region. In addition, the shell gland contained about 50% of the total ductal soluble protein and RNA (Table 4). Hormone responsiveness developed in the shell-gland region, in terms of increases in RNA content per cell, in conjunction with the morphogenic response.

EXPLANATION OF PLATE I

Age-dependent effects of diethylstilboestrol on morphogenesis of the chick Müllerian-duct shell-gland region

Diethylstilboestrol was administered for 5 days as described in the Materials and Methods section. Routine histological sections ($5\mu\text{m}$) from tissue fixed in Bouin's fixative were stained with Haematoxylin and Eosin and observed by phase-contrast microscopy. Photomicrographs (final magnification $\times 200$) present typical views of longitudinal sections through the shell-gland region of (a) 15-day Müllerian duct, (b) 18-day Müllerian duct and (c) 21-day Müllerian duct, all receiving 5-day diethylstilboestrol treatment, and (d) 21-day Müllerian duct without exposure to diethylstilboestrol (final magnification $\times 800$). Key: E, epithelia; S, stroma.

EXPLANATION OF PLATE 2

Age-dependent effects of diethylstilboestrol on morphogenesis of the chick Müllerian-duct magnum region

Diethylstilboestrol was administered for 5 days as described in the Materials and Methods section. Routine histological sections ($5\mu\text{m}$) from tissue fixed in Bouin's fixative were stained with Haematoxylin and Eosin and observed by phase-contrast microscopy. Photomicrographs (final magnification $\times 200$) present typical views of longitudinal sections through the magnum region of (a) 15-day Müllerian duct, (b) 18-day Müllerian duct and (c) 21-day Müllerian duct, all receiving diethylstilboestrol treatment, and (d) 21-day Müllerian duct without exposure to diethylstilboestrol. Key: E, epithelia; S, stroma.

Table 4. *Effects of diethylstilboestrol on development of the chick Müllerian-duct shell-gland region*

Diethylstilboestrol was administered for 5 days as detailed in the Materials and Methods section. After exposure to diethylstilboestrol the sex tract was removed, weighed and then divided into magnum and shell-gland regions and reweighed (Aitken, 1971). A separate 5% (w/v) homogenate of each region was prepared in 25 mM-Tris/HCl (pH 7.6)/25 mM-NaCl/5 mM-MgCl₂/1% Triton X-100/1% deoxycholate. Portions of the total homogenate were assayed for DNA content (Giles & Myers, 1965) and RNA content (Munro & Fleck, 1966) as described in the Materials and Methods section. The remaining homogenate was centrifuged at 100000g for 1 h and the protein content of the supernatant was determined. All values presented are the means $\pm$ s.d. for determinations from six groups of three to ten females each, and are expressed as the percentage of total ductal wet weight, protein and DNA found in the shell-gland region alone.

Sample	% of total ductal content			RNA/DNA ratio
	Wet weight	Soluble proteins	DNA	
Oviduct	50 $\pm$ 6	47 $\pm$ 4	51 $\pm$ 5	2.75 $\pm$ 0.30
21-day Müllerian duct	50 $\pm$ 6	49 $\pm$ 4	53 $\pm$ 5	1.75 $\pm$ 0.10
18-day Müllerian duct	51 $\pm$ 6	50 $\pm$ 4	49 $\pm$ 3	1.49 $\pm$ 0.10
15-day Müllerian duct	49 $\pm$ 6	48 $\pm$ 4	50 $\pm$ 3	1.49 $\pm$ 0.10

The results presented in Table 4 show clearly that development of oestrogen competence in the different regions of the Müllerian duct is co-ordinated, i.e. the shell gland showed the same relative rate of diethylstilboestrol-induced growth in terms of its content of total ductal wet weight, DNA, RNA, and protein throughout development.

Diethylstilboestrol-induced morphogenesis of tubular-gland cells in the Müllerian-duct magnum

The effects of 5 days' exposure to diethylstilboestrol on the histological organization of different embryonic ages of the Müllerian-duct magnum are shown in Plate 2. In all age groups tested oestrogen induced epithelial mitosis and the appearance of tubular glands. The magnum tubular-gland cells are responsible for egg-white protein synthesis in the hen oviduct (Kohler *et al.*, 1969; Palmiter & Gutman, 1972). The magnitude of the Müllerian-duct magnum response to diethylstilboestrol was age-dependent, developing between day 10 and day 16 of incubation.

The control embryonic Müllerian duct and immature chick oviduct magnum were composed of a thin layer of pseudostratified columnar epithelia surrounded by a compact stroma, with no tubular-gland cells in evidence.

The 15-day-embryonic magnum, after developing in the presence of hormone from day 10, had undergone a small morphogenic response (Plate 2a). The epithelial layer contained areas of intense mitotic activity; however, few tubular glands had formed and the stroma was still relatively compact and undifferentiated.

The 18-day-embryonic magnum, after developing for 5 days in the presence of diethylstilboestrol, had undergone morphogenesis (Plate 2b). Many tubular-gland cells were evident; the stroma had become loosely organized and stromal oedema was evident. Little smooth muscle was evident in the magnum.

Cells composing the tubular glands tended to have pycnotic nuclei, but in general appeared to be immature secretory cells.

The 21-day-embryonic magnum, after 5 days' exposure to the oestrogen, showed the greatest relative morphogenic response (Plate 2c). In this case a massive proliferation of tubular glands occurred. The tubular-gland cells appeared large, with a flocculent translucent cytoplasm and a small highly pycnotic nucleus indicative of glandular maturation (Kohler *et al.*, 1969). The stroma was loosely organized and had essentially been replaced by tubular glands. Within the stroma the organization of peripheral smooth-muscle layers was evident, but overall the stroma appeared to be undifferentiated. There is no such morphological differentiation observed in the duct of embryos not receiving oestrogen (Plate 2d).

Age-dependent changes in the functional status of diethylstilboestrol-induced tubular-gland cells in the Müllerian-duct magnum

Although similarities exist between the oestrogen-induced responses of the embryonic Müllerian duct and those of the chick oviduct, several distinct and important differences were also observed. As shown in Table 5, the relative amount of hormone-induced growth, in terms of wet weight and DNA content of the Müllerian duct, never reached the relative degree of response observed in the oviduct from the oestrogen-treated chick. The ability to respond to diethylstilboestrol by increased growth rate developed from marginal values in the 10-day Müllerian duct to a value, by day 16 of incubation, only 3-fold less than that observed in the chick oviduct. When the percentage of tubular-gland cells in the magnum was calculated for each experimental group (Table 5), it became apparent that the relative ability to differentiate tubular glands in response to oestrogen

Table 5. Age-dependent effects of diethylstilboestrol on development of the chick Müllerian-duct magnum region. Chicks and embryos were exposed to diethylstilboestrol for 5 days as described in the Materials and Methods section. The wet weight of the stimulated sex tract was compared with that of a control organ of the same age. Values are the means ± s.d. for ten determinations. RNA and DNA contents were determined, for the total organ and for the magnum region respectively, as described in the legend to Table 3 and in the Materials and Methods section. Values are the means ± s.d. for ten determinations. Three to five different females per group were assayed for the percentage of tubular-gland cells in the magnum by microscopic examination of both longitudinal (see Plate 2) and cross-sectional slides through the magnum region. At least 500 cells were counted in each determination. Values represent the means for three to five determinations. The number of ovalbumin mRNA molecules per tubular-gland cell was estimated by using the following equations:

$$a = \text{mRNA}_{\text{ov}} \text{ molecules/duct} = \text{RNA/duct} \times \% \text{ of mRNA}_{\text{ov}} \times \frac{\text{Avogadro's number}}{\text{mol.wt. of mRNA}_{\text{ov}}}$$
$$b = \text{no. of tubular-gland cells/duct} = \frac{\text{magnum DNA content}}{\text{DNA content/cell}^*} \times \% \text{ tubular-gland cells/magnum}$$
$$a/b = \text{mRNA}_{\text{ov}} \text{ molecules/tubular-gland cell}$$

Sample	Fold increase in wet weight	RNA/duct (μg)	DNA/magnum (μg)	% of tubular-gland cells in magnum	Molecules of mRNA _{ov} §/ tubular-gland cell
Oviduct	45 ± 5.0	3230 ± 50	590 ± 50	45 ± 5†‡	27 000 ± 4000
21-day Müllerian duct	13 ± 1.0	460 ± 20	133 ± 10	51 ± 4	6 500 ± 2000
18-day Müllerian duct	4 ± 0.2	107 ± 3	36 ± 2.0	31 ± 5	7 500 ± 2000
15-day Müllerian duct	2 ± 0.2	32 ± 1.8	11 ± 1.0	11 ± 4	8 000 ± 2000

* 2.5ng/cell (Sober, 1968).

† From Kohler *et al.* (1969).

‡ Values from Table 1 were determined by hybridization in the presence of cDNA excess.

§ 6.3 × 10⁵ g/mol (Woo *et al.*, 1975).

Fig. 7. Ontogeny of oestrogen-responsiveness in the chick Müllerian-duct magnum

Diethylstilboestrol was administered for 5 days as detailed in the Materials and Methods section. The age-dependent ability of the chick-embryo left-Müllerian-duct magnum to respond to diethylstilboestrol by epithelial morphogenesis and ovalbumin-gene expression are compared with the magnitude of these responses observed in the 9-day-old chick oviduct magnum. ●, Percentage of tubular-gland cells in the magnum (from Table 5); ○, relative rates of ovalbumin synthesis (from Table 3); □, percentage of mRNA_{ov} in total cell RNA (from Table 1), representing values obtained by hybridization in the presence of cDNA excess.

had developed to maximal values by day 16 of incubation, i.e. after 5 days' exposure to diethylstilboestrol the 21-day Müllerian-duct magnum contained about 50% tubular-gland cells, as did the chick oviduct magnum after 5 days' exposure to diethylstilboestrol (Kohler *et al.*, 1969). Since the relative DNA increase in the diethylstilboestrol-stimulated 21-day Müllerian-duct magnum was 3-fold less than that in the diethylstilboestrol-stimulated oviduct magnum, it seems probable that both epithelia and stroma in the embryonic magnum were less responsive to hormone.

If it is assumed that only the embryonic magnum tubular-gland cells are synthesizing ovalbumin, then it is possible to estimate the average number of ovalbumin mRNA molecules per tubular-gland cell (Table 5). The formulae used in this calculation are shown in the legend to Table 5, and are similar to calculations used by Harris *et al.* (1975) in determining the number of ovalbumin mRNA molecules per tubular-gland cell during oestrogen-induced differentiation of the chick oviduct magnum. By using results obtained in the present study and the histological evidence presented by Kohler *et al.* (1969), it was possible to estimate that after 5 days' exposure to hormone the oviduct magnum tubular-gland cells each contained on the average 27 000 molecules of ovalbumin mRNA. Tubular-gland cells induced in

the embryonic Müllerian duct seem to have contained between 6000 and 8000 molecules of ovalbumin mRNA each, after 5 days' exposure to hormone, regardless of the embryonic age group tested.

Ontogeny of oestrogen responsiveness in the chick Müllerian duct

The results presented in Fig. 7 delineate the ontogeny of responsiveness to oestrogen in the Müllerian-duct magnum as measured by the morphogenesis and functional differentiation of tubular-gland cells. These results indicate that the hormone-induced expression of the ovalbumin gene occurred coordinately with the appearance of tubular-gland cells in the magnum. The responses of the 10-day Müllerian-duct magnum to hormone were marginal. Oestrogen induced the cyto-differentiation of tubular-gland cells, which after 5 days represented about 10% of the total magnum cell population. These cells contained an average of about 8000 molecules of ovalbumin mRNA each; however, ovalbumin synthesis in the magnum was barely detectable, as less than 1% of the total magnum protein synthesis. The responses of 13-day Müllerian-duct magnum to diethylstilboestrol were much greater than those of the day-10 magnum. After 5 days' exposure to hormone the magnum (day 18) contained 30% tubular-gland cells. Each tubular-gland cell contained on average 7000 molecules of ovalbumin mRNA, and ovalbumin synthesis represented 3% of the total magnum protein synthesis. By day 16 the Müllerian-duct magnum's ability to respond to diethylstilboestrol had developed to a very high degree. After 5 days' exposure to diethylstilboestrol the magnum (21 days) contained about 50% tubular-gland cells that each contained an average of 6500 ovalbumin mRNA molecules. Ovalbumin synthesis represented about 10% of the total magnum protein synthesis.

Discussion

Our observations indicate that the finding of the presence of low concentrations of ovalbumin mRNA in control and non-target tissues must be regarded with some reservation owing to the extremely low values observed. These low values are at the limits of sensitivity of the assays employed and indicate the presence of 1–10 ovalbumin mRNA molecules per cell.

The location of ovalbumin mRNA in the control sex tract is not known. It is possible that all cells in the sex tract contain low concentrations, as is indicated by the presence of this mRNA in liver cells. It is also possible that this mRNA is restricted to certain cell types within the sex tract. Perhaps a few epithelial cells have begun to differentiate prematurely in response to the small amount of oestrogen secretion by the embryonic ovary (Teng & Teng, 1977).

However, histological examination of the control sex tract does not indicate the presence of tubular-gland cells (Kohler *et al.*, 1969; Teng & Teng, 1979). The subcellular distribution of ovalbumin mRNA in control sex tracts is also unknown. It is possible that this message is restricted to the nucleus in control cells and is not translated on the polyribosomes. No detectable synthesis of ovalbumin was observed in control sex tracts, although this could be due to the limits of sensitivity of the immunoassay method used.

Oestrogen responsiveness in the Müllerian duct is an age-dependent phenomenon, developing from marginal values on day 10 to substantial values by day 16 of incubation. This developmental trend has been observed for: (1) short-term oestrogen responses, which include (a) the retention of oestrogen–receptor complexes by chromatin isolated 2 h after hormone exposure *in ovo* and (b) the induction of increased ornithine decarboxylase activity 3 h after hormone exposure (Teng & Teng, 1978*a, b*); (2) 2-day oestrogen responses, which include an increased growth rate and an increase in the activity *in vitro* of Müllerian-duct chromatin (Andrews & Teng, 1977, 1979); (3) 5-day oestrogen responses (the present work), which include epithelial morphogenesis and cyto-differentiation of tubular-gland cells and expression of the ovalbumin gene. Most of the responses mentioned above are quantitatively smaller in the 16-day Müllerian duct when compared with those of the chick oviduct.

The left Müllerian duct of the 10-day embryo has a poorly developed oestrogen-responsive system (see Andrews & Teng, 1979). Histological examination of the oestrogen-treated magnum from 10-day-old chick Müllerian duct shows that about 10% of the total cells are tubular-gland cells. At this point ovalbumin mRNA represents 0.012% of the total cell RNA, and a tubular-gland cell contains on the average about 7000–8000 molecules of ovalbumin mRNA. This dramatic increase in the content of ovalbumin mRNA is not accompanied by a significant induction of ovalbumin synthesis, which at this point represents less than 1% of the total protein synthesis in the magnum. This could reflect, in part, the lack of sensitivity of the assay method used, but would also seem to reflect a poor translation capacity within these tubular-gland cells, which appear histologically to be inactive secretory cells. Therefore induction of the accumulation of ovalbumin mRNA in these cells appears to precede the ability to translate this message efficiently. Similar observations were made by Harris *et al.* (1975), who showed that during primary oestrogen stimulation of the oviduct the number of molecules of ovalbumin per molecule of ovalbumin mRNA increases dramatically with continued maturation of the tubular-gland cells. Palmiter (1972*b*) showed that both peptide elongation and initiation rates are increased in the oviduct of the oestrogen-treated chick. Similarly Comstock *et al.*

(1972) reported that polyribosomal ovalbumin mRNA appears before detectable synthesis of ovalbumin during primary stimulation of the oviduct.

The shell-gland region of the 10-day Müllerian duct responds to diethylstilboestrol by morphogenesis. After 5 days' exposure to the hormone the shell gland contains 50% of the total ductal wet weight and DNA. The general histological appearance of this region changed only slightly in response to hormone. It is noteworthy that on a gross level the development of responsiveness to hormone in different regions of the sex tract is co-ordinated. The functional status of the different cells and tissues in the precociously induced embryonic shell gland is not known. However, an early study by Greenwood & Blyth (1938) showed that exposure of female chick embryos to oestrone very early in development adversely affects the differentiation of the sex tract of the mature hen and leads to the production of shell-less and membrane-less eggs.

The hormone-responsive system, in terms of the number of oestrogen-receptor sites per cell and the ability of chromatin to retain oestrogen-receptor complex, has started developing in the left Müllerian duct of the 13-day embryo. By day 16 of incubation, the responsive system in the duct appeared to be fully developed (Teng & Teng, 1975*a,b*, 1978*a*). Accordingly a drastic increase in hormone-responsiveness in the Müllerian duct occurs during this stage of development. This age-related increase in responsiveness is expressed by an increase in the relative number of tubular-gland cells induced in the epithelial cells of the magnum. The acquisition of hormone-responsiveness of epithelial cells seems to relate to their ability to bind and retain oestrogen-receptor complexes on chromatin. Within a population of epithelial cells it is reasonable to assume that a range of hormone competence exists. As development proceeds a greater complement of epithelial cells reach the threshold values of nuclear binding capacity required before morphogenesis can be induced during 5 days' exposure to hormone. A further increase in nuclear binding capacity would lead to a more rapid response of the epithelial stem cells and thus to a quicker maturation of function. On day 10 the epithelial population may contain only a few cells competent to undergo morphogenesis in response to hormone. These cells mature slowly, having just reached the threshold values of nuclear binding capacity required for response. In the next 3 days (days 10–13) the complement of cells that have acquired this minimal value of nuclear binding capacity may increase greatly. From day 13 to day 16 a few more cells reach this threshold value, but most of the responsive cells have passed this point of development and are able to mature rapidly in response to hormone. Thus the relative number of hormone-inducible tubular-gland cells would in-

crease from day 10 to day 13, but their relative rate of maturation would remain consistent. It is thus expected that the relative number of diethylstilboestrol-inducible tubular-gland cells and/or their rate of maturation would increase from day 13 to day 16 of incubation. The evidence obtained in the present work supports this hypothesis.

Both the shell-gland and the magnum regions of the 13-day Müllerian duct show enhanced responses to hormone compared with those of the 10-day Müllerian duct.

Histological examination of the 21-day Müllerian duct, after having developed for 5 days in the presence of oestrogen, shows that 50% of the magnum cells are tubular-gland cells. Ovalbumin mRNA represents 0.04% of total cell RNA. Therefore each tubular-gland cell contains on the average about 6500 molecules of this message. Ovalbumin synthesis represents 10% of the magnum protein synthesis. This indicates that these tubular-gland cells are efficiently translating ovalbumin mRNA and are more mature than tubular-gland cells induced in the 13-day magnum during 5 days' exposure to the hormone. This contention is supported by histological evidence indicating that, in the 21-day magnum, the tubular glands contain maturing secretory cells. In addition, the number of ovalbumin mRNA molecules per tubular-gland cell and the rate of ovalbumin synthesis are both 3-fold lower in the oestrogen-treated 21-day Müllerian-duct magnum than in the oviduct magnum of the oestrogen-treated chick. The efficiency of ovalbumin mRNA translation would appear to be comparable in these two groups. The percentage of hormone-inducible tubular-gland cells in the magnum and the rate of maturation of these cells increased between days 13 and 16 of incubation.

It should be emphasized that the distribution of oestrogen-receptor content and nuclear binding capacity within the different cell types of the Müllerian duct are completely unknown. It is possible that some cell types acquire hormone competence earlier in development than others. This concept is supported by evidence obtained in the present study, i.e. the 21-day Müllerian duct, after having developed in the presence of oestrogen for 5 days, contains 50% tubular-gland cells in the magnum. The chick oviduct magnum also contains about this percentage of tubular-gland cells 5 days after exposure to hormone. However, the relative rate of magnum growth (DNA content) and the rate of tubular-gland cell maturation are slower in the Müllerian duct. Therefore the Müllerian duct contains a higher percentage of tubular-gland cells than would be predicted by a comparison with the oviduct of the oestrogen-treated chick. This implies that the Müllerian-duct epithelia have acquired hormone competence earlier in development than the stroma. A greatly attenuated stomal response could, in part,

account for the relative difference in hormone-induced DNA increase observed between the Müllerian duct and the oviduct. A slightly attenuated epithelial response could account for the lower rate of gland cell maturation. Forsberg (1973) has shown that oestrogen stimulates the embryonic mouse vaginal endometrium but inhibits the vaginal epithelium. Exposure of the embryonic organ to oestrogenic hormone results in the disruption of normal development. This has been demonstrated in the chick system (Greenwood & Blyth, 1938) and in mammalian systems (Staff & Foley, 1974). Exposure of the human foetus to non-steroidal oestrogens results in abnormal development of the sex tract and predisposes the cervix and vagina to neoplastic transformation (Herbst & Scully, 1970; Greenwald *et al.*, 1971).

The ability of the Müllerian duct to respond to oestrogen undergoes a further step of maturation between day 16 of incubation and 1 week after hatching. A more exact time is unknown. This secondary phase of development results in a much less dramatic increase in responsiveness to hormone than that observed to occur between days 10 and 16 of incubation. In this later period the rates of response of both stroma and epithelia seem to increase. It would appear that this process is not due to an increase in oestrogen receptor content or to a further maturation of the genome. Receptor dynamics studies have shown that the replenishment of oestrogen receptor does occur in the Müllerian duct within a few hours of a single injection of oestradiol (Teng & Teng, 1975b). However, receptor dynamics during chronic oestrogen administration are unknown. In this regard it has been shown that treatment of the embryo with hormone very early in development (day 5) results in an alteration in the oestrogen receptor population in the 15-day Müllerian duct (Teng & Teng, 1979). This involves an apparent decrease in high-affinity binding sites with the appearance of a large population of non-saturable binding sites. The effect of these receptor changes on subsequent responsiveness to hormone is not known. A similar receptor heterogeneity has been observed in the oviduct of the laying hen (Best-Belpomme *et al.*, 1975).

The maturation of responsiveness occurring after day 16 could relate to the action of other hormones in this system. Essentially nothing is known about the effects of diethylstilboestrol on other steroid-hormone receptor species in the Müllerian duct.

Physiological changes occurring in the chick at the time of hatching and after hatching could relate to the observed maturation of responsiveness to hormone in the sex tract. For example, breathing air rather than absorption of air through the shell membranes must alter body metabolism. In addition, the switch from constant darkness to light-dark cycles may

have a profound effect on gonadal function, as has been shown in a variety of mammalian and avian species (Sadleir, 1972). It is also possible that the availability of a food supply in the embryo limits the response of the sex tract by some compensatory mechanism. It is therefore apparent that an explanation of this final maturation of hormone-responsiveness in the sex tract, occurring during this complex period of development, will require a more comprehensive study of the physiology of the chick and is likely to reflect a combination of processes and events.

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