Studies on Sex-Organ Development

CHANGES IN THE OESTROGENIC RESPONSE OF THE CHICK MÜLLERIAN DUCT AS MEASURED BY CHROMATIN TEMPLATE AND RIBONUCLEIC ACID INITIATION CAPACITY

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Assays of transcription *in vitro*, with *Escherichia coli* RNA polymerase or wheat-germ RNA polymerase II, were used to characterize chromatin templates isolated from the left Müllerian duct of the chick embryo during normal development, and during development in the presence of diethylstilboestrol. Control Müllerian-duct template capacity with *E. coli* RNA polymerase decreased from 6.42% on day 10 to 4.34% by day 15 of development. Similar results were found with wheat-germ RNA polymerase II. In the presence of rifampicin and heparin, the prokaryotic enzyme transcribed a number-average RNA chain of 670 nucleotide residues, at an average rate of 110 nucleotide residues/min, from Müllerian-duct chromatin of all developmental stages. From day 10 to day 15 there was a 44% decrease in the number of initiation sites for *E. coli* RNA polymerase on Müllerian-duct chromatin. A 47% decline was observed when these chromatins were transcribed with excess RNA polymerase II in the presence of rifamycin Af/013. Signs of increasing responsiveness to oestrogen developed between days 10 and 16. Embryos exposed to maximally responsive doses of diethylstilboestrol for 2 days showed increases in Müllerian-duct chromatin template capacity, RNA-chain initiation sites, wet weight, protein and RNA. The changes seen in the oviduct of the 1-week-old chick injected for 2 days with diethylstilboestrol were defined as 100% responses. By comparison, the Müllerian duct, after exposure to diethylstilboestrol from day 10 to day 12, from day 13 to day 15 or from day 16 to day 18, showed a 15%, 39% and 72% template response respectively, and a 42%, 56% and 85% initiation-site change respectively. A similar developmental trend was observed in all parameters. It is concluded that oestrogenic responsiveness in the developing Müllerian duct increases from day 10 to nearly maximal values by day 16 of development, and that this transition is paralleled by a progressive restriction of genomic activity.

The study of steroid-hormone responsiveness during organogenesis of the chick Müllerian duct has the potential of revealing molecular mechanisms involved in the attainment of competence of the target organ, and in addition may provide insight into the role of hormones in the normal or abnormal development of the sex tract.

The chick embryo has been used as a standard experimental organism in studies related to urogenital system differentiation. Both male and female 5- or 6-day chick embryos possess undifferentiated pairs of Müllerian ducts (Wolff, 1959). In the male embryo the right and left Müllerian ducts begin to involute on day 8 of incubation and disappear by day 13. In the female embryo the right Müllerian duct starts to regress on day 9 of incubation and remains as a rudimentary stump at hatching. The left Müllerian duct continues to develop and eventually becomes the

functional hen oviduct. This pattern of development is common to several avian and reptilian species (Swift, 1915; Brode, 1928; Raynaud, 1970). In the immature chick oviduct, oestrogen administration induces oviduct differentiation, development and growth. This morphogenesis is accompanied by increases in oviduct RNA, protein and DNA, and results in the differentiation of three distinct epithelial cell types from a primitive mucosa (Oka & Schimke 1969; O'Malley *et al.*, 1969; Palmiter & Gutman, 1972). This tropic response is accompanied by changes in genomic structure and function, and results in the expression of genes coding for the egg-white proteins ovalbumin, lysozyme, conalbumin and ovomucoid (Spelsberg *et al.*, 1973; Schwartz *et al.*, 1975; Sullivan *et al.*, 1973; Tsai & O'Malley, 1977). However, little is known about the effects of oestrogen on the development of the chick-embryo Müllerian duct. In

previous studies of this sex organ the characterization and ontogeny of oestrogen receptors, oestrogen-receptor nuclear translocation and changes in chromatin structure and oestradiol-binding capacity were reported (Teng & Teng, 1975a,b, 1976, 1978a). The present paper reports on the development of responsiveness to oestrogen during embryogenesis of the chick left Müllerian duct as measured by (1) changes in the transcription *in vitro* of Müllerian-duct chromatin during normal and oestrogen-influenced development and (2) changes in Müllerian-duct growth and accumulation of protein, RNA and DNA during normal and oestrogen-influenced development. These findings are discussed in terms of developmental changes in oestrogen receptors and chromatin oestradiol-binding capacity, and provide the basis for the following paper (Andrews & Teng, 1979), which focuses on developmental changes in the effects of oestrogen on ovalbumin-gene expression and tubular-gland cell differentiation.

Materials and Methods

Animals and chemicals

White Leghorn chicks (embryos or 1-week-old chicks) were obtained as described previously (Teng & Teng, 1975a). [^3H]UTP (specific radioactivity 17 Ci/mmol; Schwarz/Mann, Orangeburg, NY, U.S.A.) was dried *in vacuo* to remove ethanol, then redissolved in water before use.

The following chemicals were obtained from the sources indicated: ultra-pure Tris and sucrose (ribonuclease-free) were from Schwarz/Mann; EDTA, diphenylamine and $(\text{NH}_4)_2\text{SO}_4$ were from Mallinckrodt (St Louis, MO, U.S.A.); ribonucleoside 5'-triphosphates, heparin, tRNA (yeast), rifampicin and calf thymus DNA were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); three-quarters exponential phase *Escherichia coli* (K-12) paste was from Grain Processing Co. (Muscatine, IO, U.S.A.); proteinase K was from EM Laboratories (Elmsford, NY, U.S.A.); raw wheat germ was obtained from General Mills (Kansas City, MO, U.S.A.); Polymyxin P was kindly given by BASF, Ludwigshafen, Germany; rifamycin Af/013 was a gift from Dr. G. Lancini (Gruppo Lepetit, Milano, Italy). All other chemicals were of analytical grade.

Exposure of Müllerian duct to the oestrogenic hormone diethylstilboestrol

Embryos were exposed to oestrogen by a technique developed in this laboratory (Teng & Teng, 1979); this consists of a 10s partial immersion (dip) in diethylstilboestrol dissolved in 70% ethanol maintained at 50–55°C. The bottom half of the egg comes in contact with the solution. Unless otherwise indicated, a solution of 10mg of diethylstilboestrol/

ml was used in all experiments, and treated eggs were returned to the incubator for 2 days before removal of the left Müllerian duct by mid-ventral dissection of the embryos. Chicks were treated with oestrogen by the daily injection (subcutaneous) of 1.5mg of diethylstilboestrol in 0.2ml of propylene glycol for 2 days. Ethanol treatment alone had no effect on Müllerian-duct development. Injection of chicks with propylene glycol alone causes no discernible change in control oviducts. All treated groups were compared with control groups of the same age.

Preparation of nuclei and chromatin

The procedures for preparation of Müllerian-duct (or oviduct) nuclei were as described by Teng & Teng (1976). The chromatin was prepared from the nuclei as originally described by Dingman & Sporn (1964) and modified by Mezquita & Teng (1977). After two washes with 10vol. of 0.14M-NaCl the nuclear preparation was suspended in 80mM-NaCl/20mM-EDTA, pH6.0, and collected by centrifugation at 1000g. This step was repeated once. The crude chromatin pellet was washed twice with 10vol. of 50mM-Tris/HCl buffer, pH7.6, then twice with 10vol. of 10mM-Tris/HCl buffer, pH7.6, by homogenization with 12 strokes in a loose-pestled Dounce homogenizer. After sedimentation at 3000g for 10min the chromatin was suspended in 2mM-Tris/HCl buffer, pH7.6, and allowed to swell overnight at 0°C. After swelling, the chromatin was gently sheared by homogenization (200 strokes) with a tight-pestled Dounce homogenizer. The chromatin solution (approx. 500µg/ml) was clarified by centrifugation at 300g for 5min. The chemical composition of this chromatin has been studied and reported by Teng & Teng (1978a).

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).

Isolation of RNA polymerases

E. coli RNA polymerase containing the σ -subunit was isolated as described by Burgess & Jendrisak (1975). The final enzyme preparation was precipitated by the addition of saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 7.2) to a final concentration of 60% (w/v). The precipitate was stored at -70°C in storage buffer [10mM-Tris/HCl (pH 7.9)/10mM-MgCl₂/0.1mM-EDTA/0.5mM-dithiothreitol/50% (v/v) glycerol] at a concentration of 17mg/ml. The *E. coli* RNA polymerase was

assayed by the procedure of Burgess (1969) and found to have an activity of 1000 units/mg of protein.

Wheat-germ RNA polymerase II was prepared by the method of Jendrisak & Burgess (1975). The enzyme, eluted from the phosphocellulose column, was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (50% saturation) and stored at -70°C in the same buffer used for the *E. coli* enzyme plus 2 mg of bovine serum albumin/ml. The enzyme specific activity, assayed with single-stranded calf thymus DNA, was 500 units/mg of protein.

Conditions for RNA synthesis with re-initiation

Unless otherwise indicated, chick DNA ($1\mu\text{g}$) or chromatin ($5\mu\text{g}$ as DNA) was incubated with $10\mu\text{g}$ of *E. coli* RNA polymerase or $10\mu\text{g}$ of wheat-germ RNA polymerase II (both were diluted in 1 mg of bovine serum albumin/ml) at 37°C for 10 min in a final volume of $250\mu\text{l}$ containing $12.5\mu\text{mol}$ of Tris/HCl buffer, pH 7.9, $0.25\mu\text{mol}$ of MnCl_2 , $12.5\mu\text{mol}$ of $(\text{NH}_4)_2\text{SO}_4$, $0.50\mu\text{mol}$ of β -mercaptoethanol and 37.5nmol each of ATP, GTP, CTP and $[^3\text{H}]\text{UTP}$ (75c.p.m./pmol). Triplicate samples were assayed for RNA synthesis by the addition of $50\mu\text{l}$ of bovine serum albumin (3mg/ml) followed by the addition of 3 ml of 5% (w/v) trichloroacetic acid containing 0.01M -sodium pyrophosphate. After 30 min at 0°C , the solution was centrifuged for 20 min at 2500g in a Beckman model JS 7.5 rotor. The pellet was dissolved in 0.2ml of 0.2M -NaOH and immediately reprecipitated with 5ml of 5% (w/v) trichloroacetic acid at 4°C . The precipitate was collected on a fibre-glass filter (Reeve Angel 934-AH) and washed with 30ml of cold 5% (w/v) trichloroacetic acid at 4°C . The filters were dried for 20 min under heat lamps, then transferred to counting vials containing 4ml of scintillation fluid [6g of 2,5-diphenyloxazole and 0.15g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene]. The radioactivity was determined at 24% counting efficiency in a Beckman model LS-250 scintillation spectrometer.

RNA synthesis in the absence of re-initiation

The methods of M.-J. Tsai *et al.* (1975, 1976) were adapted for the study of RNA-chain initiation on chromatin. Unless specifically indicated, chromatin ($5\mu\text{g}$) was preincubated with *E. coli* RNA polymerase for 15 min at 37°C in a volume of $200\mu\text{l}$ containing all ingredients described above minus ribonucleoside 5'-triphosphates and inhibitors. RNA synthesis was initiated by the addition of 37.5nmol each of ATP, CTP, GTP and $[^3\text{H}]\text{UTP}$ (70c.p.m./pmol) in a volume of $50\mu\text{l}$, and incubation was continued for another 15 min at 37°C . Rifampicin ($20\mu\text{g}$) and heparin ($200\mu\text{g}$) were added simultaneously with the nucleotides and $[^3\text{H}]\text{UTP}$. The number of RNA initiation sites on chromatin and the rate of chain elongation were calculated by the methods of M.-J. Tsai *et al.* (1975)

by using the following equation:

$$\frac{\text{No. of initiation sites}}{\text{pg of DNA}} = \frac{\text{c.p.m.} \times 10^{-12} \times 4 \times N}{\text{sp. radioactivity} \times \text{DNA} \times \text{size}}$$

where c.p.m. is c.p.m. of UMP incorporated at break point in the titration curve, N is Avogadro's number, sp. radioactivity is specific radioactivity of $[^3\text{H}]\text{UTP}$ (70c.p.m./pmol), DNA (pg) is amount of chromatin DNA used and size is number of nucleotides in the average chain length of DNA. To assess the rate of RNA chain elongation, samples were taken 1 min after the initiation of RNA synthesis. Wheat-germ RNA polymerase II ($25\mu\text{g}$) was preincubated with chromatin ($5\mu\text{g}$) for 15 min at 37°C in $200\mu\text{l}$ as described above. RNA synthesis was initiated by the addition of $50\mu\text{l}$ containing 150nmol each of ATP, GTP and CTP, 15nmol of $[^3\text{H}]\text{UTP}$ (478c.p.m./pmol) and $50\mu\text{g}$ of rifampicin Af/013. Incubation was continued for 15 min at 37°C .

Incorporation of $[^3\text{H}]\text{UTP}$ was assayed by counting the radioactivity of trichloroacetic acid precipitates as described above. The efficiency of counting the ^3H radioactivity was typically 21% in the presence of rifampicin or rifampicin Af/013.

Sizing of transcripts formed in vitro

A sample of chromatin ($50\mu\text{g}$) was transcribed in the absence of re-initiation with $200\mu\text{g}$ of *E. coli* RNA polymerase. After incubation for 15 min at 37°C the solution was made 0.5% with respect to sodium dodecyl sulphate, and proteinase K was added to a final concentration of $20\mu\text{g/ml}$. The mixture was further incubated at 37°C for 30 min. Nucleic acids were extracted by the addition of 0.5 vol. of redistilled phenol saturated with extraction buffer (10mM -NaCl / 10mM -EDTA / 10mM -sodium acetate, pH 5.0) and 0.5 vol. of chloroform/3-methylbutanol (24:1, v/v). The mixture was shaken at room temperature (21°C) for 30 min. After centrifugation at 10000g for 10 min at 6°C , the aqueous phase was removed. Phenol and chloroform were added and the process was repeated. The aqueous fraction was removed and $50\mu\text{g}$ of purified tRNA was added as a carrier. The RNA was precipitated with 2.5 vol. of 100% ethanol at -20°C for 18 h. RNA was spun down at 4500g for 15 min at 0°C in a Beckman model JA-20 rotor and subsequently freeze-dried. The RNA (approx. 50000c.p.m. of radioactivity) was suspended in 0.1ml of glass-distilled water, heated at 70°C for 1 min, cooled quickly, layered on top of a linear 5–20% (w/v) sucrose gradient (4.9ml) and centrifuged at 189000g in a Beckman SW 50.1 rotor at 4°C for 2.5 h. Thirty fractions (0.17ml/fraction) were collected, and the amount of 5% (w/v) trichloroacetic acid-pre-

Fig. 1. *Transcription in vitro* of chick DNA and chick oviduct chromatin in the absence of inhibitors of RNA chain initiation. Chromatin from oestrogen-stimulated chick oviduct and pure chick DNA were transcribed *in vitro* with *E. coli* RNA polymerase under the conditions described in the Materials and Methods section. The effects of varying time of incubation, enzyme concentration and template concentration on the synthesis *in vitro* of RNA were examined in order to establish a standardized system for the comparison of template capacities *in vitro* of chromatin from different experimental groups. (a) Template capacity of 1 μg (●) or 2 μg (○) of chick DNA, transcribed with increasing amounts of *E. coli* RNA polymerase for 10 min at 37°C. (b) Transcription by 10 μg of *E. coli* RNA polymerase of increasing amounts of chick DNA (○) or oviduct chromatin (●); incubation was for 15 min at 37°C. (c) Transcription of 5 μg (as DNA) of oviduct chromatin by 25 μg (○) or 10 μg (●) of *E. coli* RNA polymerase for various times. Transcription was measured as incorporation of [^3H]UTP into RNA (trichloroacetic acid-precipitable radioactivity).

Fig. 2. *Transcription in vitro* of chick oviduct chromatin in the presence of inhibitors of RNA chain initiation. Chromatin from oestrogen-stimulated chick oviduct was transcribed *in vitro* with *E. coli* RNA polymerase in the presence of rifampicin and heparin or with wheat-germ RNA polymerase II in the presence of rifamycin Af/013. The reaction mixture described in the Materials and Methods section was used in all experiments unless specifically indicated. Chromatin was preincubated with enzyme for 15 min at 37°C in 200 μl of the reaction mixture before the addition of 50 μl containing nucleotide triphosphates and inhibitors. (a) Increasing concentrations of inhibitors of RNA chain initiation were added, along with substrates, to chromatin (5 μg) that had been preincubated with 20 μg of wheat-germ RNA polymerase II (○) or *E. coli* RNA polymerase (●) as described above. The reaction was continued for 15 min at 37°C, then the amount of trichloroacetic acid-precipitable radioactivity was measured; 100% activity was determined in the absence of inhibitors. Heparin alone (800 $\mu\text{g}/\text{ml}$) inhibited *E. coli* RNA polymerase activity by 50%. (b) Oviduct chromatin (5 μg) was preincubated with 25 μg (○) or 10 μg (●) of *E. coli* RNA polymerase before the simultaneous addition of nucleotide triphosphates, rifampicin (20 μg) and heparin (200 μg). The reaction was terminated at the indicated times. (c) Various amounts of oviduct chromatin were preincubated with 10 μg of *E. coli* RNA polymerase before the addition of substrates, rifampicin (20 μg) and heparin (200 μg). The reaction was continued for 15 min at 37°C before precipitation with trichloroacetic acid.

Fig. 3. Titration of chromatin with increasing amounts of RNA polymerase

(a) Chromatin ($5 \mu\text{g}$ as DNA) isolated from chick-embryo Müllerian ducts was preincubated with various amounts of *E. coli* RNA polymerase for 15 min at 37°C , as detailed in the Materials and Methods section, before the addition of nucleotides, rifampicin ($20 \mu\text{g}$) and heparin ($200 \mu\text{g}$). The reaction was terminated after 15 min at 37°C by the addition of trichloroacetic acid, and the amount of trichloroacetic acid-precipitable radioactivity was determined. ●, Enzyme alone; △, 15-day Müllerian-duct chromatin; ○, 10-day Müllerian-duct chromatin. (b) Chromatin ($5 \mu\text{g}$ as DNA) from oestrogen-stimulated chick oviduct was preincubated with various amounts of wheat-germ RNA polymerase II for 15 min at 37°C under the conditions described above. The synthesis of RNA was initiated by the simultaneous addition of substrates plus rifamycin Af/013 ($50 \mu\text{g}$) as detailed in the Materials and Methods section. The reaction was continued for 15 min at 37°C , and then the amount of trichloroacetic acid-precipitable radioactivity was measured. ●, Enzyme alone; ○, oviduct chromatin. Chromatin incubated without enzyme served as a control for background activity (200 c.p.m.) in all experiments.

cipitable radioactivity was determined. Purified cytoplasmic RNA (consisting of 28S, 18S and 4S RNA from the hen oviduct) was used as a molecular-weight marker to standardize the gradients. The number-average chain length of the synthesized RNA was determined as indicated by Spirin (1963) and Cedar & Felsenfeld (1973).

Results

Transcription in vitro of chick DNA and oviduct chromatin in the absence of inhibitors of initiation

The system utilized to study the transcription *in vitro* of chromatin is shown in Fig. 1, which illustrates the transcription of chromatin and DNA by *E. coli* RNA polymerase. Under the reaction conditions employed, the transcription of chick DNA occurred in an enzyme-limited reaction (Fig. 1a). Chick DNA was in template excess at an enzyme/DNA ratio of 10:1 (w/w), and chromatin under these conditions showed a template saturation point at an enzyme/chromatin ratio of 2:1 (Fig. 1b). Transcription of chromatin at an enzyme ratio of 2:1 or 5:1 was linear for 10 min at 37°C (Fig. 1c). The conditions chosen for the comparison of template activities in the remainder of this paper were to transcribe chromatin at an enzyme/DNA ratio of 2:1 for 10 min at 37°C and to compare this activity with that of chick DNA transcribed at an enzyme/DNA ratio of 10:1 for the same period of time.

Transcription in vitro of oviduct chromatin in the presence of inhibitors of initiation

The effects of inhibitors of initiation on the transcription of oviduct chromatin are illustrated in Fig. 2. The ribonuclease inhibitor heparin at a concentration of $800 \mu\text{g}/\text{ml}$ inhibited by 50% the activity of *E. coli* RNA polymerase (Fig. 2a). Rifampicin ($80 \mu\text{g}/\text{ml}$) in the presence of heparin ($800 \mu\text{g}/\text{ml}$) inhibited its activity by 70%. This concentration of rifampicin caused maximal inhibition of RNA synthesis and was thus chosen for use in the remainder of the study. Transcription of chromatin directed by wheat-germ RNA polymerase II was completely inhibited by this concentration of heparin, but was insensitive to rifampicin (results not shown). The rifamycin derivative rifamycin Af/013 (Meilhac *et al.*, 1972), however, was maximally effective at inhibiting wheat-germ RNA polymerase II activity at a concentration of $200 \mu\text{g}/\text{ml}$. This concentration resulted in a 90% decrease in RNA synthesis and was used in the remainder of the study. Rifampicin ($80 \mu\text{g}/\text{ml}$) and heparin ($800 \mu\text{g}/\text{ml}$) altered the kinetics of transcription of chromatin by *E. coli* RNA polymerase (Fig. 2b). Under these stringent conditions the reaction was 50% complete 4 min after the addition of ribonucleotides and inhibitors and was essentially completed by 15 min at 37°C (cf. Fig. 1c).

These kinetics did not depend on the enzyme/chromatin DNA ratio, as similar results were observed at ratios of 5:1 and 2:1. The total incorporation of nucleotides was decreased to approx. 10% of that observed in the absence of rifampicin and heparin. Transcription of chromatin with wheat-germ RNA polymerase II in the presence of rifampicin Af/013 (200 µg/ml) was completed within 2 min of the addition of substrates and inhibitor (results not shown). Transcription of chromatin with *E. coli* RNA polymerase in the presence of rifampicin and heparin was dependent on the amount of chromatin available to the enzyme during the preincubation period (Fig. 2c), and was not an enzyme-limited reaction at an enzyme/chromatin ratio of 2:1, as it was in the absence of inhibitors (Fig. 1b).

Titration of chromatin with increasing amounts of RNA polymerase

Fig. 3(a) shows the rifampicin-resistant transcription of Müllerian-duct chromatin titrated with increasing amounts of *E. coli* RNA polymerase. A typical titration consisted curve of two phases. In the initial part of the transcription curve was linearly dependent on the amount of enzyme mixed with chromatin during the preincubation period. At an enzyme/chromatin ratio of approx. 3:1, depending on the sample tested, a break point in the titration curve occurred after which transcription was not significantly stimulated by increasing enzyme concentration. The amount of RNA synthesized at the

break point is an indication of chromatin template capacity and was used to calculate the number of RNA chains synthesized/pg of chromatin DNA. In all experiments control tubes containing no exogenous RNA polymerase showed that chromatin alone had no endogenous or enzyme-independent activity (see the legend to Fig. 3). *E. coli* RNA polymerase did exhibit some template-independent activity (Fig. 3a). This activity did not contribute significantly to the titration-curve data. Fig. 3(b) shows the titration of chromatin with increasing amounts of wheat-germ RNA polymerase II in the presence of rifampicin Af/013. The titration curves were similar to those described for the prokaryotic enzyme (Fig. 3a). In this case, however, the actual amount of RNA synthesized was 6–7-fold lower. The specific radioactivity of the [³H]UTP in the reaction mixture was adjusted to yield incorporation values (c.p.m.) in a range similar to that found during transcription with the prokaryotic enzyme. Chromatin alone had no enzyme-independent activity under these conditions, and wheat-germ RNA polymerase II had only a small template-independent activity (Fig. 3b).

In the remainder of this study the titration of chromatin from different developmental groups with *E. coli* RNA polymerase was carried out as illustrated in Fig. 3(a). For comparison, saturating amounts of the eukaryotic enzyme were used (enzyme/chromatin ratio 5:1) to measure the amount of rifampicin Af/013-resistant transcription in chromatin preparations.

Table 1. *Changes in template activity of chromatin from chick Müllerian duct during embryogenesis*

Müllerian-duct chromatin (5 µg as DNA) from different developmental stages was transcribed *in vitro*, as described in the Materials and Methods section, with 10 µg of *E. coli* RNA polymerase or wheat-germ RNA polymerase II. All groups were assayed in parallel for comparison of activities. Different preparations of chromatin were used for each enzyme tested. The percentage template capacity equals:

$$\frac{\text{c.p.m. incorporated/10 min per 5 } \mu\text{g of chromatin as DNA}}{\text{c.p.m. incorporated/10 min per 1 } \mu\text{g of chick DNA}} \times 100$$

as discussed in the text. Each value for template capacity represents the mean $\pm$ s.d. for six experimental determinations. Incorporation values are the average of triplicate experimental tubes with background values (300–500 c.p.m.) subtracted. These background values were due to trapping of [³H]UTP on the filter (200 c.p.m.) and to some template-independent activity of *E. coli* RNA polymerase contaminants (300 c.p.m./10 min per 10 µg). Each chromatin was found to be inactive (200 c.p.m./10 min per 5 µg as DNA) in the absence of RNA polymerase. Wheat-germ RNA polymerase II had no detectable template-independent activity.

Age (days)	<i>E. coli</i> RNA polymerase			Wheat-germ RNA polymerase II	
	³ H]UMP incorporation (c.p.m./5 µg of DNA)		Template capacity (%)	³ H]UMP incorporation (c.p.m./5 µg of DNA)	
	5 min	10 min		5 min	10 min
10	9000 $\pm$ 110	17800 $\pm$ 220	6.4 $\pm$ 0.3	2900 $\pm$ 60	4800 $\pm$ 110
12	7000 $\pm$ 100	14000 $\pm$ 190	5.1 $\pm$ 0.3	2600 $\pm$ 50	4200 $\pm$ 100
15	6400 $\pm$ 90	12600 $\pm$ 210	4.3 $\pm$ 0.2	2300 $\pm$ 40	38500 $\pm$ 90
18	6400 $\pm$ 70	11900 $\pm$ 180	4.3 $\pm$ 0.2	2200 $\pm$ 50	3650 $\pm$ 80
9 (after hatching)	6200 $\pm$ 100	12000 $\pm$ 200	4.2 $\pm$ 0.2	2200 $\pm$ 60	3800 $\pm$ 100

Changes in template activity of Müllerian-duct chromatin during embryogenesis

During the development of the left Müllerian duct a restriction in chromatin template capacity was observed (Table 1). From day 10 to day 15 of incubation template capacity declined from 6.4% to 4.3%, then remained constant even after hatching. A similar trend was observed when chromatin was transcribed with wheat-germ RNA polymerase II. With this enzyme, from day 10 to day 15 a decline in template activity of 23 % was observed, compared with 33% during transcription with the *E. coli* RNA polymerase. The amount of RNA synthesized by 10µg of wheat-germ RNA polymerase II was 3.3-fold lower than that synthesized by 10µg of *E. coli* RNA polymerase under these conditions. These results were unaffected by the inclusion of the proteinase inhibitors phenylmethanesulphonyl fluoride or sodium bisulphite in the buffers used during the isolation of nuclei and chromatin, or by the inclusion of a Triton X-100 wash of the isolated nuclei.

Changes in RNA chain initiation on Müllerian-duct chromatin during embryogenesis

During development of the left Müllerian duct between days 10 and 15 of incubation, a 43% decline in the rifampicin-resistant transcriptional capacity of chromatin was observed (Table 2). After day 15 this capacity remained constant. RNA products transcribed *in vitro* were sized as described in Fig. 4. This RNA sedimented as a broad band with a peak of approx. 12S. The chain length in nucleotides (*N*) of each fraction was estimated by the method of Spirin (1963) by using the following equation:

$$\log N = 2.1 \log s_{20,w} + 0.644$$

The number-average chain length (*N*_{av.}) of this RNA was determined by the method of Cedar & Felsenfeld

(1973) by using the following equation:

$$N_{av.} = \frac{\sum_i n_i N_i}{\sum_i n_i}$$

where *n*_i is the number of RNA chains and *N*_i is the length of the RNA chains in nucleotides for each fraction. The number-average RNA chain length of chromatin transcripts varied between 600 and 800 nucleotide residues, with a mean length of approx. 670 nucleotide residues. RNA-chain elongation rates ranged from 1.5 to 2.2 nucleotide residues/s, with an average rate of 1.9 nucleotide residues/s. At this rate the average RNA chain should have been completed within 10min of initiation. Since the synthesis of RNA was allowed to continue for 15 min, small variations in chain elongation rates should have had little affect on the amount of rifampicin-resistant RNA synthesis observed.

Correlation of cellular RNA content with RNA chain initiation in vitro on chromatin during Müllerian-duct development

During development of the Müllerian duct the quantity of total RNA per cell decreased from 3.9pg on day 10 to 2.9pg on day 15 of incubation; thereafter it remained relatively constant at 3pg per cell, assuming each cell contains 2.5pg of DNA (Sober, 1968). This 25% decline in RNA content of Müllerian-duct cells during development correlates temporally with changes observed in the RNA-chain-initiation capacity *in vitro* of chromatin (Fig. 5). The number of initiation sites for *E. coli* RNA polymerase on chromatin decreased from 55000 sites/pg of DNA on day 10 to 30000 sites/pg of DNA on day 15 of development, after which it remained constant. This represents a 45% decrease in initiation-site capacity. The amount of rifamycin Af/013-resistant transcription from chromatin by wheat-germ RNA polymerase II

Table 2. *Changes in RNA chain initiation on chromatin from chick Müllerian duct during embryogenesis*

Müllerian-duct chromatin from different developmental stages was titrated with *E. coli* RNA polymerase and the amount of rifampicin-resistant transcription was measured, as described in the Materials and Methods section and illustrated in Fig. 3. The inflexion point in the titration curve was used to determine the incorporation values shown. These values are the means ± s.d. for four determinations. RNA products synthesized *in vitro* were isolated and sized as described in the Materials and Methods section and illustrated in Fig. 4. To assess the initial rate of RNA chain elongation samples were taken 1 min after the initiation of RNA synthesis. These rates were calculated, as described in the text, by the methods of M.-J. Tsai *et al.* (1975). Age refers to the day of incubation of the eggs.

Age (days)	[³ H]UMP incorporation (c.p.m./15 min per µg of DNA)	Number-average RNA chain length (nucleotide residues)	Elongation rate (nucleotide residues/s)
10	1061 ± 80	834	1.62
12	716 ± 60	607	1.50
15	594 ± 40	652	2.00
18	630 ± 30	676	2.20
9 (after hatching)	570 ± 50	653	1.97

Fig. 4. Sizing by sucrose-density-gradient centrifugation of transcripts formed *in vitro*

Chromatin from chick-embryo Müllerian ducts was transcribed *in vitro* with *E. coli* RNA polymerase in the presence of rifampicin and heparin. The transcripts were isolated, as detailed in the Materials and Methods section, by treatment with proteinase K followed by phenol/chloroform extraction and ethanol precipitation. The precipitates were dissolved in water and denatured, and then layered over a 4.9 ml linear 5–20% (w/v) sucrose gradient and centrifuged at 190000g for 2.5 h. The gradients were collected and the trichloroacetic acid-precipitable radioactivity was measured. Values shown represent sucrose gradients of RNA transcripts synthesized *in vitro* from 10-day (○), 12-day (●), 15-day (□) and 18-day (■) Müllerian-duct control chromatin. The 5S, 18S and 28S rRNA markers were used to calibrate gradients as indicated. The number-average RNA chain length was calculated by the method of Spirin (1963) and Cedar & Felsenfeld (1973).

Fig. 5. Correlation of cellular RNA content with RNA chain initiation *in vitro* on chromatin during development of the chick Müllerian duct

The DNA and RNA contents of Müllerian ducts were determined on samples of a 10% (w/v) total homogenate by the procedures described in the Materials and Methods section. □, RNA/DNA ratios represent the means from nine determinations involving approximately 500 female chick embryos and chicks. ●, The number of RNA chain initiation sites/pg of Müllerian chromatin was calculated for *E. coli* RNA polymerase from the data presented in Table 2 by using the methods of Schwartz *et al.* (1977) (see the text). ○, The amount of rifamycin Af/013-resistant transcription of Müllerian-duct chromatin by wheat-germ RNA polymerase II was determined as described in the Materials and Methods section.

showed a 52% decrease during this period of development.

Dose-dependent effects of diethylstilboestrol on Müllerian-duct growth

Depicted in Fig. 6 are the results of a bioassay used in determining the dose of diethylstilboestrol maximally effective in inducing increased growth of the left Müllerian duct. Treatment of the egg with diethylstilboestrol during day 5 of incubation resulted in a dose-dependent increase in Müllerian-duct wet weight measured on day 15. Treatment with a solution of 1mg of diethylstilboestrol/ml during day 5 was ineffective in inducing an increase in day-15 wet weight. If, however, a solution of 10mg/ml was used a half-maximal response was observed, i.e. all of the Müllerian ducts removed from these day-15 embryos had increased in wet weight 50% above control values. Treatment with a solution of 20–40mg of diethylstilboestrol/ml at day 5 resulted in a maximal response, which represented a 100% increase in day-15 wet weight. These results suggest that the introduction of diethylstilboestrol into the egg occurred in a dose-dependent manner, and that the diethylstilboestrol stayed in an active form in the egg for several days. The latter conclusion is supported by the observation that after exposure to diethylstilboestrol (20mg/ml) on day 5 the first increase over control in Müllerian-duct wet weight was not observed until after day 10 of incubation. In addition,

Fig. 6. Bioassay dose-response curves depicting the effect of diethylstilboestrol on Müllerian-duct growth

Eggs were treated by dipping for 10s in solutions of diethylstilboestrol in 70% ethanol, as described in the Materials and Methods section, at day 5 (●) or day 13 (○) of incubation. Müllerian ducts were harvested at day 15 and the wet weights of eight to ten groups of 20 females each were determined. The maximal response observed represents an increase of 100% over control values. Points are the means ± S.D. for nine determinations.

Table 3. Effects of diethylstilboestrol on the transcription *in vitro* of chick Müllerian-duct chromatin
Müllerian ducts were removed from embryos 2 days after an initial treatment with diethylstilboestrol (10 mg/ml) *in ovo*. The age interval refers to the day exposure to diethylstilboestrol began and the day of harvest. Oviducts were removed from chicks injected subcutaneously with diethylstilboestrol (1.5 mg/day) for 2 days. The values for template capacity with *E. coli* RNA polymerase and for UMP incorporation with wheat-germ RNA polymerase II were determined in the absence of inhibitors of re-initiation, as described in Table 1 and detailed in the Materials and Methods section. Transcription in the presence of inhibitors of initiation was assayed as described in the Materials and Methods section. The values are means ± S.D. for five determinations. The rates of RNA chain elongation and the numbers of initiation sites were calculated by the methods of M.-J. Tsai *et al.* (1975).

Age (days)	Template capacity* (%)	<i>E. coli</i> RNA polymerase			Wheat-germ RNA polymerase II		
		[³ H]UMP incorporation† (c.p.m./15 min per µg of DNA)	Number-average chain length (nucleotide residues)	Elongation rate (nucleotide residues/s)	UMP incorporation‡ (c.p.m./10 min per 5 µg of DNA)	UMP incorporation§ (c.p.m./15 min per µg of DNA)	
10-12	5.40 ± 0.3	960 ± 80	647	1.43	N.D.	N.D.	
13-15	5.45 ± 0.3	858 ± 70	639	1.96	4670 ± 100	792 ± 50	
16-18	6.45 ± 0.3	1059 ± 80	N.D.	1.96	5240 ± 130	1232 ± 100	
9-11 (after hatching)	7.00 ± 0.3	1028 ± 80	660	1.95	5913 ± 120	1356 ± 110	

* Assay without rifampicin.
† Assay with rifampicin.
‡ Assay without rifamycin Af/013.
§ Assay with rifamycin Af/013.

treatment of the egg on day 5 or day 10 of incubation with a solution of 20mg of diethylstilboestrol/ml resulted in a nearly identical increase in day-15 wet weight (results not shown). It seems, therefore, that some of the hormone introduced into the egg on day 5 remained intact 5 days later. This observation has been confirmed by Teng & Teng (1979), who have shown that serum diethylstilboestrol concentrations remain high 5–7 days after treatment of the egg with hormone.

Treatment of the egg with diethylstilboestrol on day 13 of development resulted in a dose-dependent increase in day-15 wet weight. Exposure to a solution of 10mg of diethylstilboestrol/ml during day 13 resulted in a 100% increase in day-15 Müllerian-duct wet weight (Fig. 6). This was the maximal response observed in the day-15 embryo. Treatment with 70% ethanol alone had no effect on Müllerian-duct growth. The use of diethylstilboestrol in 70% ethanol had little effect on embryo mortality and allowed for the synchronous administration of hormone to a large population of developing embryos.

It was decided that the measurement of sex-tract responses to 2 days of exposure to diethylstilboestrol would provide a basis for the comparison of hormone responsiveness in the developing Müllerian duct with that in the 1-week-old chick oviduct. Two days of exposure to diethylstilboestrol resulted in significantly large Müllerian-duct responses to allow accurate measurements to be made. Therefore in the remainder of this study embryos were exposed to 10mg of diethylstilboestrol/ml as described in the Materials and Methods section and responses were measured 2 days later. Chicks were injected with 1.5mg of diethylstilboestrol/day for 2 days.

Effects of diethylstilboestrol on the transcription in vitro of Müllerian-duct chromatin

Embryos were exposed to hormone on day 10, 13 or 16 of incubation, then allowed to develop for 2

more days. In each case the experimental group was compared with a control group of the same age. As shown in Table 3, the template activity of Müllerian-duct chromatin increased after exposure of the embryo to diethylstilboestrol. This effect was observed in all age groups tested, with the use of either *E. coli* RNA polymerase or wheat-germ RNA polymerase II to transcribe the chromatin. The trend was towards a larger final template capacity, after exposure to diethylstilboestrol, as embryonic age increased. When chromatin was transcribed with *E. coli* RNA polymerase in the presence of rifampicin and heparin an increase in transcription occurred in all groups tested after hormone treatment. The number-average chain length of the RNA synthesized *in vitro* did not change with development or after hormone exposure, but remained at about 670 nucleotide residues. It was also found that the average initial rate of RNA chain elongation was unaffected by hormone exposure and remained at about 1.9 nucleotide residues/s. From these results it was concluded that the number of initiation sites for *E. coli* RNA polymerase on Müllerian-duct chromatin increased after hormone exposure. This occurred in all groups tested, with final values ranging between 40000 and 54000 initiation sites/pg of DNA. A similar trend was observed when the amount of rifampicin Af/013-resistant transcription of hormone-stimulated Müllerian-duct chromatin by wheat-germ RNA polymerase II was measured (Table 3).

From these results it was concluded that diethylstilboestrol affects the Müllerian duct by altering genomic activity as measured by the transcription *in vitro* of chromatin. This response of the Müllerian duct was similar to that of the 1-week-old chick oviduct to diethylstilboestrol. In each experimental group, changes in the activity of chromatin *in vitro* seemed to be mediated primarily at the level of RNA chain initiation and not by detectable changes in the length or rate of elongation of RNA chains.

Table 4. *Effects of diethylstilboestrol on chick Müllerian-duct wet weight, DNA, RNA and protein content*
Müllerian ducts were removed from the embryos 2 days after exposure to diethylstilboestrol (DES) (10mg/ml) *in ovo*. Treatment began at day 10, 13 or 16 of incubation. Oviducts were removed from chicks treated for 2 days with diethylstilboestrol (1.5mg/day). The wet weight of the organ was determined; then the amounts of protein, DNA and RNA were assayed as described in the Materials and Methods section. The values shown represent the means $\pm$ s.d. for six to eight determinations on groups of 50 each. Experimental groups were always compared with control groups of the same age.

Age (days)	Wet wt. (mg/duct)		DNA (μ g/duct)		RNA (μ g/duct)		Protein (μ g/duct)	
	Control	DES-treated	Control	DES-treated	Control	DES-treated	Control	DES-treated
10–12	1.31 $\pm$ 0.15	1.58 $\pm$ 0.14	5.3 $\pm$ 1.0	5.2 $\pm$ 1.0	7.5 $\pm$ 0.8	10.5 $\pm$ 1.0	89 $\pm$ 6	108 $\pm$ 10
13–15	2.20 $\pm$ 0.20	4.42 $\pm$ 0.48	10.4 $\pm$ 0.8	13.4 $\pm$ 1.1	12.1 $\pm$ 1.2	28.9 $\pm$ 2.5	185 $\pm$ 15	400 $\pm$ 18
16–18	4.25 $\pm$ 0.20	14.50 $\pm$ 1.21	20.0 $\pm$ 1.8	37.8 $\pm$ 1.8	22.0 $\pm$ 1.2	85.2 $\pm$ 7.0	334 $\pm$ 16	979 $\pm$ 55
9–11 (after hatching)	9.60 $\pm$ 0.70	32.80 $\pm$ 2.00	44.0 $\pm$ 3.0	75.1 $\pm$ 5.2	51.4 $\pm$ 3.0	226.0 $\pm$ 18.0	1128 $\pm$ 55	2790 $\pm$ 140

Effects of diethylstilboestrol on Müllerian-duct wet weight, DNA, RNA and protein content

The effects of exposure to diethylstilboestrol (10mg/ml) on the wet weight, DNA, RNA and protein content of the left Müllerian duct are presented in Table 4, along with control values for the appropriate age groups. Müllerian ducts were harvested 2 days after treatment with hormone. These results show that the Müllerian duct responded to diethylstilboestrol with increases in the above parameters as did the 1-week-old chick oviduct. It is clear, however, that the age of the embryo markedly affected the magnitude of the response observed. In all parameters measured the trend was towards an increased Müllerian-duct responsiveness with increasing embryonic development.

Discussion

Responses to steroid hormones are thought to be mediated by changes in gene expression at the transcriptional level (Tata, 1966; Teng & Hamilton, 1967a,b; Jensen & DeSombre, 1972; O'Malley & Means, 1974). An increase in the transcription *in vitro* of chromatin after exposure to oestrogen has been demonstrated in the rat uterus, chick liver and chick oviduct (Teng & Hamilton, 1968; Spelsberg *et al.*, 1973; Searcy, 1975). In each of these target organs oestrogen-induced changes in template activity are thought to reflect an increase in the number of initiation sites for RNA polymerase on chromatin (Schwartz *et al.*, 1975; Markaverich *et al.*, 1978; Snow *et al.*, 1978). Thus it was decided that monitoring of oestrogen-induced changes in the transcription *in vitro* of Müllerian-duct chromatin would provide a sensitive index of hormone responsiveness. With these observations in mind it was decided to measure chromatin template capacity by transcribing *in vitro* with *E. coli* RNA polymerase under conditions of template excess. In addition, the well-defined eukaryotic RNA polymerase II from wheat germ (Jendrisak & Burgess, 1975), used under similar conditions *in vitro*, provided confirmation of the results obtained with the prokaryotic enzyme. To define developmental and hormonally induced changes in Müllerian-duct chromatin template capacity further, the rifampicin-challenge technique was employed (M.-J. Tsai *et al.*, 1975). With this assay method the number of initiation sites, rate of RNA chain elongation and size of the product formed *in vitro* were monitored for each chromatin preparation.

In each chromatin preparation tested the results of the transcription *in vitro* with *E. coli* RNA polymerase were similar to those found with wheat-germ RNA polymerase II; however, the overall activity per μg of protein was 3-fold lower for the eukaryotic enzyme and the reaction tended to be less linear with time. In all cases the template changes observed

were shown to be due to apparent changes in the number of initiation sites for *E. coli* RNA polymerase on chromatin while the number-average RNA chain synthesized (670 nucleotide residues) and the rate of chain elongation (1.9 nucleotide residues/s) were constant. Chromatin from the 1-week-old chick oviduct had 1 initiation site/35000 nucleotide base-pairs of DNA. This value is 3-fold greater than the value reported by M.-J. Tsai *et al.* (1975). The reason for this discrepancy is not clear, but may reside in differences in the methods of chromatin preparation. However, their general conclusions about the effects of hormone on chromatin initiation sites agree well with those reported here (see also Schwartz *et al.*, 1977). Under the appropriate conditions the results obtained with wheat-germ RNA polymerase II confirmed the conclusion that the observed changes in chromatin template capacity were mediated by changes in the number of initiation sites.

During organogenesis of the Müllerian duct, the development of a hormone-responsive organ is accompanied by functional and structural changes in the genome and in the biochemical status of the Müllerian-duct cells (Teng & Teng, 1978a). Results reported in the present paper show that between days 10 and 15 of development there is a restriction in the number of initiation sites for RNA polymerases on Müllerian-duct chromatin. Similar results were observed for chromatin template capacity. During this period of development a decline in total cellular RNA content occurs. It has been shown that the activity of ornithine decarboxylase in this organ undergoes a similar age-related decline, which is accompanied by a decrease in the cellular content of the three polyamines putrescine, spermidine and spermine (Teng & Teng, 1978b). High polyamine content is closely correlated with transcriptionally and translationally active cells (Jänne, 1967; Heby *et al.*, 1975). These changes in genomic function and the biochemical status of Müllerian-duct cells between days 10 and 15 of incubation can be correlated with observations on changes in chromatin chemical compositions during this period of embryogenesis. Teng & Teng (1978a) reported that between days 8 and 15 of development the high-molecular-weight non-histone proteins change in quality and increase in quantity, while the relative amounts and types of histone proteins remain constant relative to DNA. Non-histone protein changes were accompanied by alterations in the antigenicity of Müllerian-duct chromatin, which seemed to undergo a progressive maturation from day 8 to day 15, after which it remained consistent. The non-histone proteins may be responsible for the age-related decrease in chromatin initiation sites. Kostraba & Wang (1975) have demonstrated RNA-chain-initiation-inhibiting activity among the non-histone proteins from Ehrlich-ascites-tumour chromatin.

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

These graphs represent data calculated and compiled from results presented in Tables 3 and 4 and Fig. 5. (a) Developmental changes in the sex-tract response to 2 days of primary exposure to diethylstilboestrol as measured by the transcription *in vitro* of Müllerian-duct chromatin with wheat-germ RNA polymerase II in the absence (○) or presence (Δ) of rifampicin Af/013, and with *E. coli* RNA polymerase in the absence (●) or presence (▲) of rifampicin and heparin. (b) Development of the 2-day response to diethylstilboestrol as measured by changes in RNA (▲), wet weight (○), DNA (●) and protein (Δ).

The ontogeny of oestrogen responsiveness in the chick Müllerian duct was summarized from Tables 3 and 4 and Fig. 5 and is presented in Fig. 7. At day 10 of incubation a small response to hormone (less than 25% of the response of chick oviducts to hormone) was observed. By day 16 of Müllerian-duct development the genomic response to oestrogen had increased to 85% of the degree of responsiveness of chick oviduct (Fig. 7a). Oestrogen-induced responses of the Müllerian duct as measured by changes in wet weight, DNA, RNA and protein content are represented in Fig. 7(b). The results show that (1) the

magnitude of these responses increased with developmental age, and (2) the genomic response (Fig. 7a) measured *in vitro* correlates very well with the responses observed *in vivo*. Results from the present study on the development of oestrogenic responsiveness during sex-tract organogenesis support the view that the observed age-related genomic and cellular changes in the Müllerian duct have physiological relevance in that they reflect the development of an oestrogen-responsive system (e.g. receptor and acceptor for hormone) in this organ.

On day 10 of incubation, the Müllerian duct contains 2140 cytoplasmic oestrogen-binding sites per cell, and after 2h of hormone exposure *in ovo* 330 sites/cell are observed to be co-isolated with chromatin (Teng & Teng, 1975b, 1978a). At this age all 2-day responses to diethylstilboestrol are minimal, representing a response about 25% of that seen in the oviduct of the oestrogen-treated chick. In the 10-day embryo, 2 days of exposure to diethylstilboestrol did not cause an increase in Müllerian-duct DNA content. This could reflect the small degree of retention of oestrogen-receptor complexes in the nucleus and on the chromatin. It has been shown in the rat uterus that true uterine growth is related to the number of nuclear bound oestrogen-receptor complexes and to the long-term nuclear retention of these complexes (Anderson *et al.*, 1972a,b, 1973). Studies on the development of responsiveness to oestrogen in the neonatal rat also indicate that an oestrogen-inducible increase in uterine DNA synthesis is one of the last responses to appear during uterine development (Kaye *et al.*, 1972; Katzenellenbogen & Greger, 1974). Alternatively, this lack of DNA increase in the 10-day-embryo Müllerian duct could be the result of diethylstilboestrol selectively affecting certain cell types in the epithelia to differentiate while inhibiting the mitotic rate of other cell types. This phenomenon has been observed in the immature Müllerian vagina of the mouse (Forsberg, 1969, 1970).

On day 16 of development Müllerian-duct cells contain 3300 cytoplasmic oestrogen-binding sites per cell, and after 2h of oestrogen exposure *in ovo* 690 sites/cell are observed to be co-isolated with chromatin. This represents a 2-fold increase in nuclear binding capacity compared with the 10-day-embryo Müllerian duct (Teng & Teng, 1975b, 1978a). Treatment of the 16-day embryo with diethylstilboestrol resulted in a 2-day response 80% of that seen in the oviduct of the oestrogen-treated chick on a quantitative basis. On the other hand, it has been shown that Müllerian duct develops the maximal ability to respond to oestrogen, by increases in ornithine decarboxylase activity, on day 15 of incubation. The response is rapid, reaching a maximum 3h after exposure to the hormone (Teng & Teng, 1978b).

From these results it can be concluded that, during organogenesis of the Müllerian duct, a positive temporal relationship exists between observed age-related changes in chromatin structure and function, as measured by alterations in chromatin proteins, transcriptional capacity and the retention of hormone-receptor complexes and the ability of this organ to respond to oestrogens. Similar conclusions have been made from studies on the relationships between nuclear retention of hormone-receptor complexes and the number of chromatin initiation sites on chick oviduct chromatin (S. Y. Tsai *et al.*, 1975; Kalimi *et al.*, 1976). The following paper (Andrews & Teng, 1979) establishes that the Müllerian duct responds to diethylstilboestrol, as does the chick oviduct, by epithelial morphogenesis of tubular-gland cells, which accumulate and translate the mRNA coding for ovalbumin into immunoprecipitable ovalbumin protein. The magnitude of these responses is shown to be age-dependent, developing to maximal values in the 16-day embryo.

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