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CHANGES IN CHEMICAL COMPOSITION AND OESTRADIOL-BINDING CAPACITY IN CHROMATIN DURING THE DIFFERENTIATION OF CHICK MÜLLERIAN DUCTS

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Biochemical and immunochemical techniques were used to probe the changes in composition of the chromatin of differentiating Müllerian ducts. The non-histone protein increases gradually in the left duct and reaches a constant amount at day 15 of incubation, then remains at the same value until after birth. In the regressing right duct, the non-histone protein increases and then decreases. Gel electrophoresis indicated an increased heterogeneity in the composition of the non-histone protein corresponding to Müllerian-duct differentiation. Little variation in quantity and quality of the histone was observed; however, immunochemical assay confirmed the structural change of Müllerian-duct chromatin during development. An antibody against the chromatin of the newborn-chick oviduct was produced in the rabbit. The chromatin of Müllerian ducts from the early embryonic stage showed a small affinity with the antibody; the affinity increased during the late embryonic stages. The affinity was greatly decreased in the regressing right duct. Oestrogen-binding sites were present in the chromatin of the left and right Müllerian ducts during differentiation, with more sites in the left duct than in the right one during the late stages of development. After oestrogen treatment *in vivo*, the oestrogen-binding sites on the chromatin of both the left and the right ducts were increased, with a greater increase in the left duct than in the right. In the developing left duct the binding sites reach a maximum on day 15 of incubation, and remain constant at that value until birth.

Differentiation of the genital tracts of the female chick embryo starts at day 9 of incubation when the right Müllerian duct begins involution and the left duct develops into the future functional oviduct. This pattern of development has been observed to be common in several avian and reptilian species (Swift, 1915; Brode, 1928; Wolff, 1949; Raynaud *et al.*, 1970).

Genetic programming and gonadal secretions are considered to be the major factors responsible for this asymmetrical development of the embryonic organ (Wolff, 1959; Hamilton, 1963; Jost, 1970; Maraud *et al.*, 1970). It is not known how these factors exert their influence. Undoubtedly the sex steroids released from the left and right gonads could initiate, directly or indirectly, the signal for differentiation. Our previous research emphasized (1) the responses of steroid secretion in both gonads to gonadotropin (gonadotropic hormone) during the early stages of development and (2) the nature of steroid-hormone–tissue interaction in the cytoplasmic and nuclear fractions of the embryonic sex tract (Teng & Teng, 1975 *a,b*, 1976, 1977). Overall we are interested in gaining insight into the development of hormone responsiveness in the embryonic target organ and in discovering the control mechanism that determines the growth, as well as the death, of the embryonic organ: both of these phenomena occur in the same organism.

Since the differentiation of an embryonic genital organ is influenced by its hormonal environment and ultimately is determined by its own genetic makeup, it is important to understand the nature of the genomic apparatus and its capacity for receiving steroid hormones during the course of organogenesis. The present paper describes (1) the changes in the chemical composition of the chromatin of the left and right Müllerian ducts and (2) the changes in the chromatin binding sites for the oestrogenic hormone. The significance of these changes to the differentiation of the sex tract is briefly discussed.

Materials and Methods

Animals and chemicals

Fertilized White-Leghorn-chick eggs were obtained from Rich-Glo Farms, Houston, TX, U.S.A., and were incubated at 38°C in a humidified Petersime model 5 incubator, equipped with an egg-turning device that rotated the eggs every 4h. The age of the embryos was determined by the criteria of Hamburger & Hamilton (1951). [6,7-^{3}H]Oestradiol-17β (sp.

radioactivity 45Ci/mmol) was obtained from New England Nuclear Corp., Boston, MA, U.S.A. It was evaporated to dryness under N_2 and redissolved in ethanol before use.

The following chemicals and hormones were obtained from the sources indicated. A 50% suspension of sheep erythrocytes in Alsever's solution was obtained from Colorado Serum Co. (Denver, CO, U.S.A.). Guinea-pig complement, haemolysin and Freund's complete adjuvant were from Gibco (Grand Island, NY, U.S.A.). Tris, bovine serum albumin, ovalbumin, myoglobin and ultra-pure sucrose were obtained from Schwarz/Mann (Orangeburg, NY, U.S.A.); EDTA, diphenylamine, ammonium persulphate, propylene glycol (99% pure) were from Mallinckrodt (St. Louis, MO, U.S.A.). Aldolase, Blue Dextran and chymotrypsinogen were obtained from Pharmacia (Uppsala, Sweden). Acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were from Eastman Kodak Co. (Rochester, NY, U.S.A.); sodium dodecyl sulphate and Coomassie Blue were from Bio-Rad Laboratories (Richmond, CA, U.S.A.); barbital, barbital sodium and oestradiol-17β were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); collagenase was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); and Amido Black 10B was from Calbiochem (San Diego, CA, U.S.A.). All other chemicals were of analytical grade.

Exposure of Müllerian duct to oestradiol (oestrogenization) and the preparation of nuclei

Oestradiol was dissolved in propylene glycol and warmed to 38°C before injection. A single dose of oestradiol (40μg/0.1ml per embryo) was applied to the allantois of the chick embryos as described by Teng & Teng (1976). The control group embryos received the same volume of propylene glycol without oestradiol. After the injection, the embryos were dissected mid-ventrally and the left and right female Müllerian ducts (or other organs) were removed for preparation of nuclei as described by Teng & Teng (1976).

Chromatin isolation

The procedure for chromatin isolation was originally designed by Dingman & Sporn (1964) and modified by Mezquita & Teng (1977). The nuclear pellet was washed with 2×10vol. of 0.14M-NaCl and then with 0.08M-NaCl/0.02M-EDTA and collected by centrifugation at 600*g*. The nuclear pellet was washed with 2×10vol. of 0.05M-Tris/HCl and then with 0.01M-Tris/HCl, pH7.6, by homogenization with 21 strokes in a Dounce homogenizer. After sedimentation at 1500*g* for 10min the nuclei were suspended in 0.002M-Tris/HCl, pH7.5, and allowed to swell overnight at 0°C. After swelling, the chromatin in the nuclei was liberated into solution by homogenization with 200 strokes in Dounce homogenizer. The chromatin in the solution was obtained by filtering through one layer of nylon cloth (30μm pore size).

Determination of chemical composition and template activity of chromatin

Chromatin was extracted twice with 0.4M-H_2SO_4 for 30min in 0°C and sedimented at 1500*g* for 15min. The supernatant containing histone was neutralized by 0.2M-NaOH and used for protein determination. The pellet containing non-histone protein was dissolved in 0.2M-NaOH by heating at 100°C for 10 min and then incubated at 25°C for 30min. After centrifugation at 700*g* for 10min, the supernatant was kept for protein determination. For the detection of the template activity of chromatin, chromatin (5μg of DNA) from Müllerian ducts of different stages of development was assayed by the procedures described by Mezquita & Teng (1977). DNA from 12-day chick embryo was used as the standard.

Gel electrophoresis of chromatin proteins

Chromatin-associated proteins, comprising a mixture of histone and non-histone proteins, were resolved by electrophoresis in sodium dodecyl sulphate/polyacrylamide gel according to their molecular weight as described by Laemmli (1970). Basic proteins were analysed by electrophoresis originally developed by Panyim & Chalkley (1969) and modified by Ruiz-Carrillo *et al.* (1974). The molecular weights of the proteins were determined by the method of Weber & Osborn (1969) with the following standard proteins as markers: myoglobin, mol.wt. 17200; chymotrypsinogen, mol.wt. 23240; aldolase, mol.wt. 40000; ovalbumin, mol.wt. 45000; bovine serum albumin, mol.wt. 68000; collagenase, mol.wt. 109000; myosin, mol.wt. 212000.

Immunochemical analysis of chromatin changes

Antiserum to the chromatin of the 4-day-old chick oviduct was produced as follows. Chromatin (400μg of DNA) was suspended in 0.5ml of 10mM-Tris/HCl, pH7.4, and then homogenized with an equal volume of Freund's complete adjuvant and injected intradermally into three areas on the back of a male New Zealand White rabbit. Three rabbits were immunized with 400μg of chromatin DNA in 1.0ml on days 1, 7 and 15 of immunization. An intramuscular boost, consisting of 220μg of DNA (in chromatin form),

was given 1 week later. Post-boost bleedings were taken 1 week after the booster. Antisera pools were made by combining the sera from the three individual rabbits and heat-treated at 56°C for 40min to inactivate the rabbit complement present and then stored at −10°C before use. The immunoactivity of antiserum was tested with chromatin by the method of quantitative micro-complement fixation (Wasserman & Levine, 1961) in a total volume of 3.5ml per reaction.

Assay for hormone binding in chromatin

Assay of specific binding of the oestradiol in the chromatin of the Müllerian duct or other organ was modified from the procedures previously published (Teng & Teng, 1976). Chromatin isolated from the control or oestrogen-treated embryonic organ was used for the binding assay. In each experiment, two series of chromatin (each consisting of 30–45μg of DNA in 0.125ml final assay volume in buffer containing 10mM-Tris/HCl and 1.5mM-EDTA, pH7.4) were prepared in separate tubes, A and B. Tube A contained 7.5μM-[^{3}H]oestradiol (or other concentrations specified) and was used to determine the total amount of [^{3}H]oestradiol exchange. Tube B contained the same concentrations of [^{3}H]oestradiol as tube A plus a 100-fold excess of unlabelled oestradiol. A further control tube, containing all the ingredients except chromatin and except the 100-fold excess of oestradiol, was prepared. This control detects the non-specific radioactive oestrogen binding on the filter membrane. After incubation at 37°C for 1h (or other temperatures and time periods specified) in an Aquatherm model G-86 water-bath shaker (New Brunswick Scientific Co., New Brunswick, NJ, U.S.A.), the assay tubes were placed immediately in ice and 0.01ml of 0.125M-$CaCl_2$ was added to precipitate the chromatin (final concentration 10mM-$CaCl_2$). This caused a complete precipitation of chromatin in the medium (results not shown) and confirmed the observation described by Hemminki (1976). After 10min the chromatin was absorbed on a glass-fibre filter (Reeve/Angel 934AH). Each filter was washed with 60ml of Tris/EDTA buffer (containing 10mM-$CaCl_2$). Filters were dried and assayed for radioactivity. After the assay of radioactivity, the filters were removed from the vial, dried and cut into small pieces (3mm×3mm). The DNA on the filters was extracted with 10% (v/v) $HClO_4$ at 85°C and the concentration was determined. The radioactivity obtained from tubes A and B was individually corrected by subtracting the non-specific radioactive oestrogen binding on the filter membrane and was designated as total [^{3}H]oestradiol binding and non-specific [^{3}H]oestradiol binding respectively. Specific binding by the chromatin-associated receptor was obtained by subtracting the non-specific binding from the total binding.

Incubation of embryonic sex tracts

Freshly excised left or right Müllerian ducts (approx. 150–200mg of tissue) were incubated in a model 3001 tissue-culture dish (Falcon, Oxnard, CA, U.S.A.) containing 1ml of Hanks medium (Hanks & Wallace, 1949) in an atmosphere of O_2/CO_2 (19:1) at 41°C in a model CO-20 incubator (New Brunswick Scientific Co.). The time for organ culture and the addition of oestrogenic hormones to the incubation medium are indicated in the legends of the Figures.

General procedures

Protein was determined by the procedure of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as a standard. RNA was determined by the procedure of Munro & Fleck (1966). DNA was determined by the diphenylamine reaction (Giles & Myers, 1965) with calf-thymus DNA (Schwarz/Mann) as a standard. The embryonic DNA was isolated by the procedure of Marmur (1961).

Radioactivity was determined by adding the filter membrane to 6ml of scintillation fluid [6g of 2,5-diphenyloxazole and 0.15g of 1,4-bis-(5-phenyloxazol-2-yl)benzene per litre of toluene] in a mini counting vial (ISO Tex Co., Houston, TX, U.S.A.). The radioactivity was determined at 60% efficiency in a Beckman model LS-250 liquid-scintillation spectrometer.

Results

Analysis of the changes in chemical composition of the chromatin

The observed changes in chemical composition in the chromatin during sex-tract differentiation are shown in Table 1. Change in the content of DNA indicates that the left and right Müllerian ducts developed concurrently from day 8 to day 10 of incubation. The left duct continued to grow after day 10, but the DNA in the right duct started decreasing between days 15 and 18. The ratio of RNA to DNA and the ratio of basic protein to DNA in both ducts remained constant from day 8 to day 18. The chromatin non-histone protein/DNA ratio remained about the same in both ducts, but started to decrease in the right duct from day 12 to day 18 of incubation. Compared with the other four stages of development, the non-histone protein content in the chromatin of the 8-day-embryonic Müllerian duct is significantly lower. From day 15 to day 18 of incubation, the ratio of non-histone protein/DNA in the chromatin of the left duct was 15–32% higher than in the right duct. The template activities of chromatin *in vitro* assayed in the presence of *Escherichia coli* RNA polymerase

Table 1. *Chemical composition and template capacity of chromatin of Müllerian duct from different stages of development*

The isolation of chromatin, the determination of chemical composition and the template-capacity assay were as described in the Material and Methods section. Each value presented is the mean ± S.D. for three determinations.

Embryonic age (days)	DNA (μg/duct)		RNA/DNA		Basic protein/DNA		Non-histone protein/DNA		Non-histone protein/basic protein ratio		Template activity (% of DNA)	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
8	1.9 ± 0.15	1.9 ± 0.14	0.21 ± 0 02	0.21 ± 0.02	1.23 ± 0.13	1.24 ± 0.14	0.53 ± 0.05	0.54 ± 0.05	0.43	0.44	8.3 ± 0.4	8.4 ± 0.4
10	2.5 ± 0.46	2.3 ± 0.45	0.21 ± 0.02	0.19 ± 0.01	1.24 ± 0.12	1.25 ± 0.13	1.64 ± 0.07	1.60 ± 0.07	1.32	1.28	6.7 ± 0.3	6.9 ± 0.3
12	5.2 ± 0.44	4.1 ± 0.35	0.21 ± 0.01	0.20 ± 0.01	1.22 ± 0.11	1.23 ± 0.07	1.65 ± 0.07	1.53 ± 0.06	1.32	1.24	5.5 ± 0.3	5.7 ± 0.2
15	10.0 ± 1.25	3.8 ± 0.32	0.20 ± 0.02	0.19 ± 0.02	1.20 ± 0.12	1.23 ± 0.13	1.92 ± 0.06	1.52 ± 0.06	1.60	1.24	4.0 ± 0.2	4.2 ± 0.2
18	18.0 ± 1.15	2.5 ± 0.34	0.20 ± 0.01	0.17 ± 0.01	1.28 ± 0.13	1.21 ± 0.12	2.09 ± 0.05	1.50 ± 0.06	1.63	1.23	4.4 ± 0.2	4.8 ± 0.2
Immature chick (4-day-old)	27.0 ± 1.15	—	0.21 ± 0.02	—	1.28 ± 0.12	—	2.10 ± 0.05	—	1.64	—	4.4 ± 0.2	—

as compared with the open template of chick DNA are highest in the 8-day embryonic duct. The template activities of the chromatin preparations isolated from the left and right ducts of various developmental stages are basically identical.

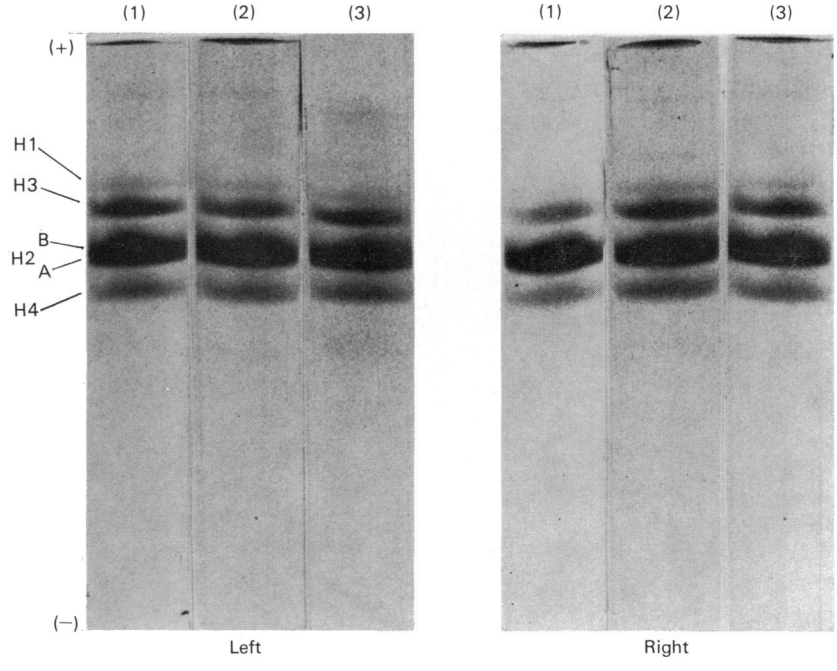
Analysis of the change in chromosomal proteins during development

Electrophoretic patterns of total histone from the isolated chromatin of Müllerian ducts from various stages of development are shown in Plate 1. The relative quantities and mobilities of each of the five histone fractions are similar and constant for both ducts throughout the different stages of development. The total chromatin-associated proteins were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The electrophoretic separation of non-histone protein subcomponents was in a molecular-weight range of 40000–200000 as presented in Plate 2. Qualitatively, the non-histone proteins of molecular weight greater than 46000 from the left and right ducts were similar. In the left duct, however, there were three proteins of mol.wt. 80000–120000 (bands 1, 3 and 4), at a higher density than in the right duct. In the late stage of development, about four protein components (bands 5–8, in the molecular-weight range 120000–200000) were increased in the left duct; a smaller increase was observed in the right duct.

Immunochemical detection of changes in Müllerian-duct chromatin during development

The antisera raised against the chromatin from the newborn-female-chick (4-day-old) oviduct were tested against the chromatin of left and right Müllerian ducts of different developmental stages. This immunochemical method is used for probing changes in the chromatin components during development. Fig. 1(*a*) shows the results when the antibody was tested against chromatin from the left Müllerian duct of the 15-day chick embryo and the 4-day-old newborn-chick oviduct. The complement-fixation curve indicates the similarity of the 15-day chick-embryo Müllerian-duct chromatin to that from the newborn-chick oviduct. The value of complement fixation reached 90% as the amount of antigen increased above 10 μg of DNA in the chromatin form. The chromatin from the liver, spleen and brain also reacts with the antibody. However, the percentage of complement fixed is significantly less than in the 15-day Müllerian duct.

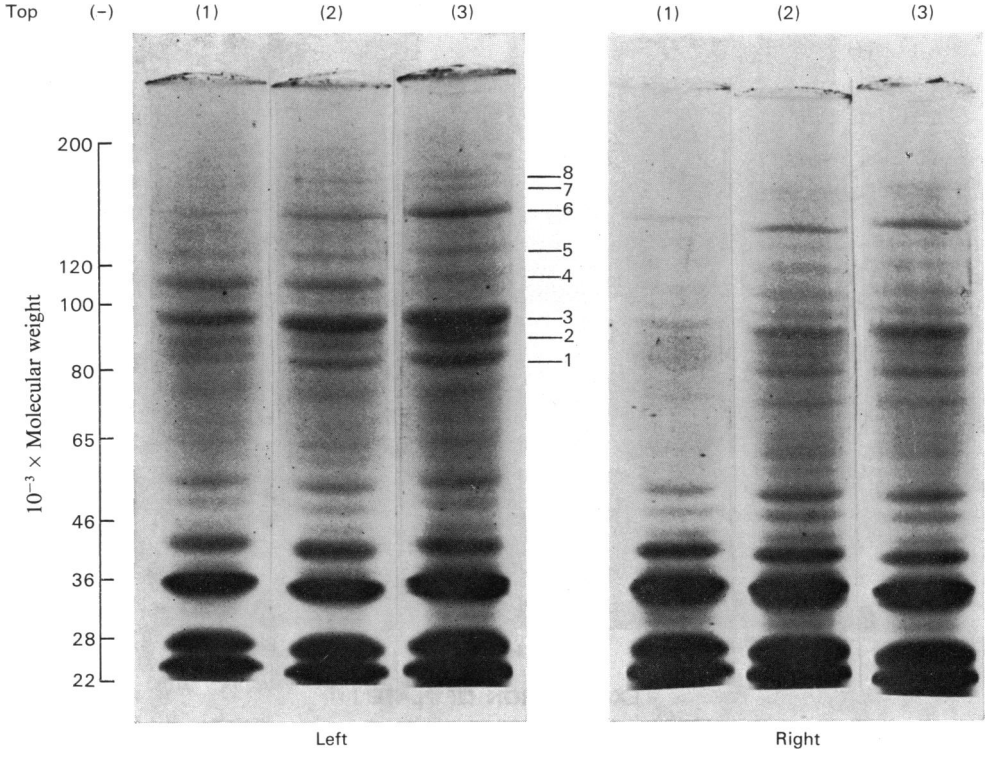
Fig. 1(*b*) shows that left and right Müllerian-duct chromatin from 8-day-old chicks reacts slightly with the antisera. The reactivity increased gradually as the age of the embryos advanced, reaching its highest value at day 15 of incubation. Furthermore, the reactivity in the chromatin of the right duct was



EXPLANATION OF PLATE 1

Polyacrylamide-gel-electrophoretic analysis of the acid-soluble proteins from chromatin of left and right Müllerian duct

Chromatin was extracted with 0.2M-H_2SO_4 containing 0.05M-$NaHSO_3$, the acid-soluble proteins were electrophoresed in 15% (w/v) polyacrylamide gels containing 2.5M-urea. Samples (18μg) of protein were run for 6h at 1mA per gel. The stages shown are left and right Müllerian-duct chromatin from (1) 10-day, (2) 15-day and (3) 18-day embryos.



EXPLANATION OF PLATE 2

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of chromosomal proteins from left and right Müllerian duct during differentiation

The chromatin proteins were solubilized with 2% (w/v) sodium dodecyl sulphate in 0.0625 M-Tris/HCl, pH 6.8, 0.7 M-2-mercaptoethanol and 5 mM-EDTA. Gels (6 mm × 85 mm) were loaded with chromatin (containing approx. 85 μg of DNA) from different stages of development as indicated in Plate 1 and were simultaneously electrophoresed in 10% (w/v) polyacrylamide gels for 17 h at 1 mA per gel and stained with Amido Black. The determination of molecular weights of the proteins was described in the Materials and Methods section. The histones are the four wide bands at the bottom of the gels with molecular weights of 22000–40000.

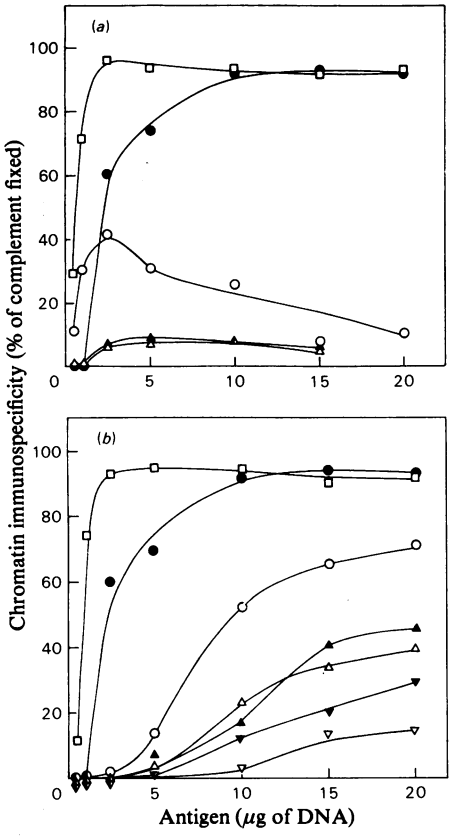


Fig. 1. *Complement fixation by various quantities of chromatin from Müllerian duct and other organs in the presence of rabbit antiserum against 4-day-old chick oviduct chromatin*

The immunospecificity of individual chromatin preparations was determined by the micro-complement fixation of Wasserman & Levine (1961). The antisera were first purified on DEAE-cellulose columns and the antigen–antiserum incubation was carried out at 37°C for 90min. (*a*) The antigens were the chromatin from: □, 4-day-old chick oviduct; ●, 15-day embryonic left Müllerian duct; ○, embryonic chick liver; ▲, embryonic chick spleen; △, embryonic chick brain. (*b*) The antigens were the chromatin from: □, 4-day-old chick oviduct; ●, 15-day left Müllerian duct; ○, 15-day right Müllerian duct; ▲, 10-day left Müllerian duct; △, 10-day right Müllerian duct; ▼, 8-day left Müllerian duct; ▽, 8-day right Müllerian duct.

significantly less than that of the left duct. Reactivity was 47, 22, 37, 38 and 42% less in the right duct chromatin than in the left for days 8, 10, 12, 15 and 18 of incubation respectively (Figs. 1*b* and 2).

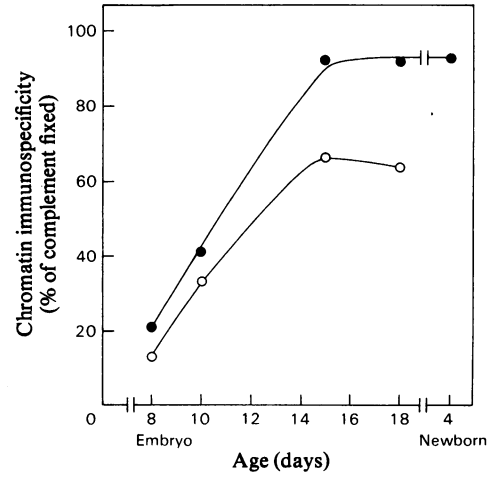


Fig. 2. *Complement fixation of chromatin from left* (●) *and right* (○) *Müllerian duct of various developmental stages of female chick embryo*

A fixed amount of antigen (18 μg of DNA in chromatin form) was fixed with the rabbit antiserum against chromatin prepared from 4-day-old chick oviduct. The procedure for micro-complement fixation was that of Wasserman & Levin (1961).

Effect of temperature and time of incubation on [^{3}H]oestradiol exchange with oestrogenized chromatin

Chromatin isolated from the Müllerian duct of the 15-day female chick embryo that had been exposed to oestradiol *in vivo* was incubated for 60min at different temperatures with a constant amount of [^{3}H]oestradiol with or without the presence of competing non-radioactive oestradiol. The specific binding of [^{3}H]oestradiol to chromatin was highest (0.45pmol/mg of DNA) at 37–41°C (Table 2). This indicated that the radioactive-hormone exchange in the chromatin binding sites is temperature-dependent. At 37°C the time required for maximal exchange is 30–60min and the radioactive hormone bound to chromatin remains at the same concentration after 120min of incubation (Table 2).

The rate of dissociation of the [^{3}H]oestradiol from the chromatin-binding sites was measured by removing excess radioactive hormone, then resuspending the chromatin in Tris buffer, and incubating at 37°C or 0°C. A typical example of the dissociation curves is presented in Fig. 3. At 37°C the half-life for the dissociation of oestradiol from the chromatin-binding sites is 14min. At 0°C there is a little dissociation during the first 3h of incubation, less than 50% dissociation having occurred after 18h.

Table 2. *Effect of temperature and time of incubation on the binding of [^{3}H]oestradiol to the chromatin of Müllerian duct*
For each 15-day female chick embryo, 40 μg of oestradiol was administered by chorioallantoic injection for 2 h; then the left Müllerian duct was removed and used for preparation of nuclei and chromatin. Chromatin was incubated with 7.5 nM-[^{3}H]oestradiol with or without a 100-fold excess of non-radioactive oestradiol at the temperature and time indicated. Each value represents the mean ± S.D. for three preparations.

Temperature of incubation (°C)	Time of incubation (min)	Total [^{3}H]oestradiol bound (pmol/mg of DNA)	Non-specific [^{3}H]oestradiol bound (pmol/mg of DNA)	Specific [^{3}H]oestradiol bound (pmol/mg of DNA)
0	60	0.095 ± 0.02	0.070 ± 0.01	0.025 ± 0.01
21	60	0.320 ± 0.03	0.110 ± 0.01	0.210 ± 0.02
37	60	0.646 ± 0.04	0.198 ± 0.02	0.448 ± 0.02
41	60	0.664 ± 0.04	0.215 ± 0.02	0.449 ± 0.03
60	60	0.180 ± 0.04	0.094 ± 0.02	0.086 ± 0.03
37	10	0.481 ± 0.03	0.162 ± 0.01	0.319 ± 0.02
37	15	0.582 ± 0.03	0.174 ± 0.02	0.408 ± 0.02
37	25	0.597 ± 0.04	0.163 ± 0.02	0.434 ± 0.03
37	30	0.643 ± 0.04	0.200 ± 0.02	0.443 ± 0.03
37	60	0.646 ± 0.04	0.198 ± 0.02	0.448 ± 0.03
37	120	0.654 ± 0.04	0.205 ± 0.02	0.449 ± 0.03

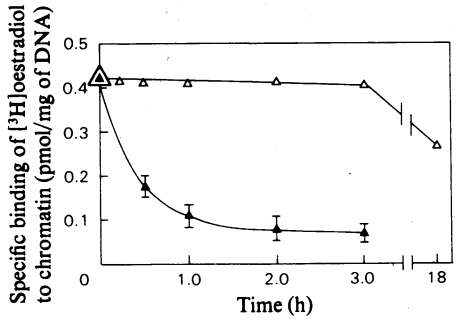


Fig. 3. *Dissociation of [^{3}H]oestradiol from chromatin at 37°C and 0°C*
Chromatin prepared from left Müllerian duct of oestradiol-treated 15-day female chick embryo was incubated with 7.5 nM-[^{3}H]oestradiol for 1 h. The chromatin was pelleted at 1500*g* for 10 min and resuspended in 0.125 ml of 0.001 M-Tris/HCl buffer, pH 7.4, and incubated at 37°C (▲) or 0°C (△) for different time periods as indicated. After incubation the chromatin was pelleted by adding 0.01 ml of 0.125 M-$CaCl_2$ and absorbed on a glass-fibre filter, washed and dried before radioactivity assay. Each value presented represents the mean ± S.D. for three determinations.

Table 3. *[^{3}H]Oestradiol binding to chromatin isolated from various embryonic organs*
An equal amount of chromatin (40 μg of DNA/0.125 ml assay volume) was assayed with 7.5 nM-[^{3}H]oestradiol as described in the legend of Table 2. (*a*) Chromatin prepared from the organ that did not receive oestradiol treatment. (*b*) Chromatin prepared from the organ receiving oestradiol administration *in vivo* for 2 h. Each value is the mean ± S.D. for three preparations. N.D., Not detectable.

Source	Specific binding of [^{3}H]oestradiol to chromatin (pmol/mg of DNA)		
	(*a*)	(*b*)	% increase
Left Müllerian duct	0.185 ± 0.02	0.448 ± 0.03	142
Liver	0.098 ± 0.01	0.122 ± 0.03	24
Brain	0.047 ± 0.01	0.045 ± 0.01	0
Heart	N.D.	N.D.	—
Skeletal muscle	N.D.	N.D.	—

Specificity of oestradiol binding to chromatin

The exchange of [^{3}H]oestradiol at 37°C with the control or oestrogenized chromatin of various organs of the 15-day embryonic female is presented in Table 3. The results indicate that oestrogen selectively binds to the chromatin of its target organ. Detectable amounts of binding were observed in the liver and brain, but no binding was detected in the heart and skeletal muscles. After oestrogenization, the binding in the Müllerian duct chromatin increased by approx. 140%, but increased only slightly (25%) in the liver. No increase of binding was detectable in the brain, heart and skeletal muscles.

Oestradiol-binding sites and the dissociation constant of oestradiol receptor in the chromatin of chick Müllerian duct

Oestrogenized chromatin was incubated with increasing concentrations of radioactive oestradiol. Maximal exchange occurred at 5–10 nM-[^{3}H]oestradiol (Fig. 4). A Lineweaver–Burk (1934) plot, based on the specific-binding curve of the chromatin recep-

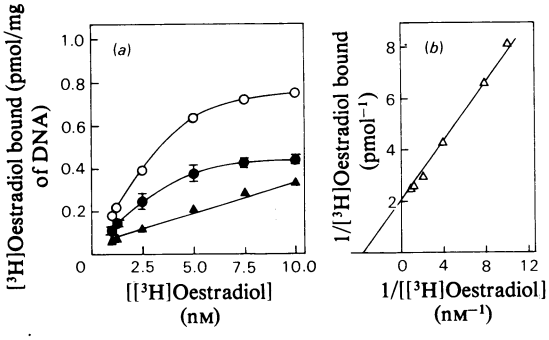


Fig. 4. *Determination of specific oestradiol binding and dissociation constant of chromatin from Müllerian duct*

Oestradiol binding to chromatin is shown in (*a*) as a function of added [³H]oestradiol for exchange, and the corresponding Lineweaver–Burk plot of the specific binding is shown in (*b*). Chromatin was prepared from left Müllerian duct of 15-day female chick embryos. All embryos were treated with unlabelled oestradiol *in vivo*. The conditions of hormone administration, nuclei and chromatin isolation, and [³H]oestradiol exchange were detailed in the Materials and Methods section. ○, Chromatin incubated with [³H]oestradiol alone (total binding); ▲, chromatin incubated with [³H]oestradiol plus a 100-fold excess of non-labelled oestradiol (non-specific binding); ●, specific binding, after the subtraction of non-specific binding from the total binding; △, Lineweaver–Burk plot of the data presented for specific binding. Each value presented represents the mean ± S.D. for three determinations.

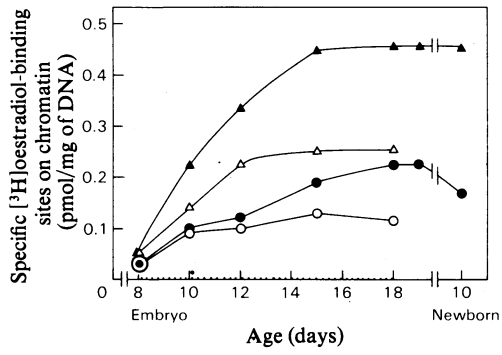


Fig. 5. *Number of specific oestradiol-binding sites on chromatin of the Müllerian duct during different stages of differentiation*

Chromatin was prepared from the control group of the embryonic female chick left (●) or right Müllerian ducts (○), at various stages of development and was incubated with 7.5 nM-[³H]oestradiol with or without a 100-fold excess of non-labelled oestradiol for 1 h at 37°C. The experimental group of chick embryos received chorioallantoic injection of oestradiol (40 μg/egg) for 2 h; chromatin was prepared from the left (▲) or right Müllerian ducts (△) and was incubated under the same conditions described for control chromatin. For the newborn female chick (10-day-old), oestradiol (2.5 mg/chick) was administered daily for 4 days starting at 6 days of age. ----, Standard binding conditions in the presence of 45 μg of DNA from the chick embryo. The preparation of chromatin and the procedure for exchange assay of chromatin-receptor–[³H]oestradiol complex were as described in the Materials and Methods section. The results presented are the specific binding of the average of triplicates from three experiments.

tor, was obtained. The number of oestradiol-receptor-binding sites in the oestrogenized Müllerian-duct chromatin of 15-day embryonic female chick was calculated to be 0.465 pmol/mg of DNA; the corresponding dissociation constant was 3.15 nM.

The specific oestradiol binding sites in the chromatin of control and oestrogenized Müllerian duct of various developmental stages were obtained by incubating the chromatin at 37°C with a constant concentration of [³H]oestradiol (7.5 nM) (Fig. 5). Oestradiol-binding sites in the chromatin of the control's left Müllerian duct (in terms of pmol/mg of DNA) increased steadily from day 8 to day 10 and then started to increase gradually from day 10 to day 19 of incubation; the sites then decreased slightly at day 10 after hatching. The oestradiol-binding sites in the chromatin of the right duct followed the same pattern as for the left side, except that the binding sites in the right duct were 18–35% less than in the left duct during days 10, 12 and 15 of incubation. A drastic decrease was found at day 18 of incubation when chromatin binding in the right duct was 108% lower than in the left.

Oestradiol-binding sites in the chromatin of oestrogenized left or right Müllerian ducts increased compared with their counterparts in the controls. The binding sites in the chromatin of the left duct increased from 0.06 to 0.45 pmol/mg of DNA during the 7 days of development (days 8–15 of incubation), a 7.5-fold increase. The number of binding sites reached a saturation value at day 15 of incubation; the number then reached a plateau from day 15 to the day near hatching. In the left duct, oestradiol-binding sites in the oestrogenized chromatin were approx. 110–164% higher than in the non-oestrogenized chromatin. The oestradiol-binding sites in the chromatin of the oestrogenized right Müllerian duct increased from 0.06 to 0.23 pmol/mg of DNA from day 8 to day 12, an increase of 3.8-fold. The number of binding sites reached a saturation value from day 12 to day 18. In the right duct, the numbers of oestradiol-binding sites in the oestrogenized chromatin were 50–125% higher than in the control chromatin.

Table 4. *Binding of [^{3}H]oestradiol to left and right Müllerian ducts in vitro*

Müllerian ducts (150–200mg) from both sides of the 15-day embryonic female chicks were incubated as described in the Materials and Methods section; 15nM-[^{3}H]oestradiol was present in all incubation media, and incubation was for 2, 3 or 5h. At the end of incubation, the tissue was removed and washed with 3×100vol. of Hanks' medium before the isolation of chromatin. Chromatin isolated from left and right ducts was precipitated with 10mM-$CaCl_2$, absorbed on glass-fibre filters (Reeve Angel 934AH) and washed with 60ml of Tris/EDTA buffer containing 10mM-$CaCl_2$. The assay for radioactivity and DNA content are described in the Materials and Methods section.

Time for culture (h)	[^{3}H]Oestradiol bound to chromatin (pmol/mg of DNA)	
	Left	Right
2	0.445	0.212
3	0.452	0.224
5	0.442	0.214

During the late stages (days 15–18 of incubation) of Müllerian-duct differentiation, the oestradiol-binding sites in the chromatin of the left duct were 80% higher than in the right duct.

In the chromatin isolated from the oviduct of the oestradiol-stimulated 10-day-old female chick (receiving 2mg of oestradiol-17β/chick daily for 4 days) the number of binding sites was 170% higher than in the control non-stimulated chick oviduct. The numbers of binding sites on the chromatin of the oestrogenized newborn-chick oviduct were equivalent to the binding sites on the chromatin of the Müllerian duct at its late developmental stages. No oestradiol binding was observed in the purified double-stranded DNA.

Oestradiol binding to chromatin during Müllerian-duct culture in vitro

The left or right Müllerian duct from the 15-day female embryo was cultured *in vitro* in the presence of [^{3}H]oestradiol for various time periods. The chromatin binding of radioactive oestradiol was maximal at 2–3h after culture (Table 4). At 3h after culture, the binding of oestradiol (in terms of pmol/mg of DNA) in the chromatin of the left Müllerian duct was 0.452, which is 102% higher than the oestradiol binding observed in the chromatin of the right Müllerian duct (0.224).

Discussion

Biochemical studies of chromatin composition during the developments of the embryonic genital tract indicate structural differentiation of the genome of the chick left and right Müllerian ducts. In the differentiating left Müllerian duct, the general pattern of change in the genome is marked by gradual increases in the quantity, as well as the diversity of the non-histone proteins, and by little variation in quantity and quality of the histones. A similar observation of increases in chromatin-associated non-histone proteins during early embryogenesis has been reported in the sea urchin (*Strongylocentrotus*), milkweed bug (*Oncopeltus*) and African toad (*Xenopus*) (Seale & Aronson, 1973; Teng, 1974; Theriault & Landsman, 1974). As judged by the changes of chromatin structure, the template for RNA synthesis of the developing left duct tends to progress from a less differentiated state toward a more differentiated state. It has been proposed that in several developmental systems, chromosomal proteins and non-histone proteins in particular are major influences on this development (Dingman & Sporn, 1964; Marushige & Ozaki, 1967; Teng, 1974; Chytil *et al.*, 1974).

In the involuting right duct, the pattern of change is parallel to some cases of terminal differentiation, e.g. the observed spermiogenesis of the male germ cell in the testes of fish (Marushige & Dixon, 1969), bird (Mezquita & Teng, 1977) and mammals (Platz *et al.*, 1975), and the embryonic development and maturation of erythrocytes (Loeb & Creuzet, 1970; Vidali *et al.*, 1973, Ruiz-Carrillo *et al.*, 1974) in which a drastic decrease in the number of most (or part) of the chromosomal proteins, especially non-histone proteins, was observed. The decrease in chromosomal proteins in the involuting embryonic organ could be related to the corresponding increase of hydrolytic enzyme activity in the tissue undergoing regression (Scheib-Pfleger & Wattiaux, 1962).

Our experiments on radioimmunomicrocomplement fixation for detection of chromatin change corroborate the view that the genomic composition is indeed subjected to a gradual change during organogenesis. Some previous experiments have indicated the same type of change in the developing rat liver tissue (Chytil *et al.*, 1974), in the developing chick oviduct (Spelsburg *et al.*, 1973) and in the liver during transformation into hepatoma by carcinogens (Chiu *et al.*, 1975). A general conclusion was drawn that the difference in antigenicity was caused mainly by the non-histone proteins. Since in our experiments intact chromatin was used as the source of antigen, we are unable to conclude whether the antisera are recognizing non-histone proteins or histones. However, in view of the relative consistency of histones in composition and quantity throughout the stages of development, this explanation is probably correct. Thus histones do not exhibit the stage specificity that would be expected for proteins involved in regulating the genomic expression. Our immunochemical

observation indicates that the genomic differentiation that distinguishes the structural difference between the left and the right duct probably starts on day 8 of incubation and reaches a significantly different state on day 12 of incubation. The composition of the chromatin of the developing left Müllerian duct becomes constant on day 15 of incubation and is maintained throughout the early stage after birth. The development of the genomic structure of the chromatin of the 15-day embryonic Müllerian duct could be considered as the initial stage toward the final maturation of the sex tract.

In the past decade, the concept of specific steroid binding to the chromatin of target organ has been demonstrated in uterine tissue (Teng & Hamilton, 1968), prostate gland (Mainwaring & Peterken, 1971) and oviduct (Steggles *et al.*, 1971). Detection of nuclear and chromatin acceptor sites for steroid-hormone binding has been difficult and controversial (Chamness *et al.*, 1974; Yamamoto & Alberts, 1974; Buller *et al.*, 1975; Jaffe *et al.*, 1975; Spelsberg, *et al.*, 1976; Hemminki, 1976). In the present paper we describe attempts to probe the chromatin oestrogen-binding sites by oestrogenizing the Müllerian duct *in vivo* and then subjecting the chromatin to radioactive oestrogen exchange at 37°C. The rationale behind this approach is on the basis of the following observations. (1) Most of the cytoplasmic oestrogen receptor can be translocated into the nucleus by a high dose of oestrogen. Eventually, portions of the oestrogen–receptor complexes bind to the chromatin (Shyamala & Gorski, 1967; Jensen *et al.*, 1967; Jensen & DeSombre, 1972). (2) The oestrogenization of the target organ could increase the oestrogen-binding capacity in the chromatin of the rat uterus and the rooster liver (Teng & Hamilton, 1968; Gschwendt & Kittstein, 1974; Gschwendt, 1976). (3) In target organs, the oestrogen-binding sites on the chromatin have high affinity with the hormone (Maurer & Chalkley, 1967; Teng & Hamilton, 1968; Puca *et al.*, 1974; Mešter & Baulieu, 1975).

The oestrogen-binding sites associated with chromatin were measured by a previously published hormone-exchange technique in the presence of radioactive oestrogen. The basic characteristics, such as temperature-dependency, dissociation constant, competibility with non-radioactive oestrogen and the saturable binding with increasing amount of oestrogen *in vitro*, are similar to the characteristics of the nuclear oestrogen-binding sites measured by the same technique (Anderson *et al.*, 1972; Mešter & Baulieu, 1975; Teng & Teng, 1976; de Boer *et al.*, 1977). The chromatin oestrogen-binding sites measured by this technique have been compared with the total nuclear oestrogen-binding sites as reported previously (Teng & Teng, 1976). The binding sites on the chromatin represent approx. 14–18% of the total nuclear-binding sites. This fraction of specific oestrogen-binding sites within the nucleus are probably similar to the high-affinity KCl-resistant and oestrogen-specific-binding sites observed in the nucleus of rat uterus and chick liver (DeHertogh *et al.*, 1973; Lebeau *et al.*, 1973; Mešter & Baulieu, 1975; Clark & Peck, 1976).

Compared with that of the developing left duct, the chromatin of the involuting right duct has less oestrogen-binding sites, with or without the oestrogenization. The small number of binding sites observed in the right duct could be due to the following possibilities: (1) the cytoplasmic-receptor concentration was low in the right duct; (2) the affinity of the receptor toward oestrogen was decreased in the right duct, and these two factors limited the amount of receptor available for translocation into the nuclei; (3) the decrease in non-histone protein in the chromatin of the right duct could influence the susceptibility of receptor–hormone complex. In eukaryotic organisms, the role of non-histone proteins in the binding of the steroid hormone to the genome of the target organ (Nyberg & Wang, 1976; Spelsberg *et al.*, 1976) and the regulation of the synthesis of these proteins by steroid hormones have been investigated (Teng & Hamilton, 1969, 1970; Shelton & Allfrey, 1970; Cohen & Hamilton, 1975).

At present the relation between the change in the chromosomal steroid-binding sites and the physiological function of the genome is still unknown. We postulate that the structural differentiation and the development of the capacity for hormone interaction in chromatin are related. These are two activities that probably determine the developmental programming for accurate genomic expression and hormone responsiveness in the target tissue.

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