STUDIES ON THE MECHANISM OF ESTROGEN-MEDIATED TISSUE DIFFERENTIATION: REGULATION OF NUCLEAR TRANSCRIPTION AND INDUCTION OF NEW RNA SPECIES

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Communicated by James A. Shannon, June 10, 1968

Examination of the informational macromolecules responsible for transfer of genetic information and synthesis of specific proteins in a developing cell may lead to a better understanding of the molecular basis of growth and differentiation. The embryonic development of amphibians and echinoderms has been most extensively studied in an attempt to define the mechanisms of differentiation.[1] Evidence exists which indicates that the onset of protein synthesis in early embryogenesis represents the activation of pre-existing messenger RNA (mRNA) molecules rather than the *de novo* synthesis of new messages.[2-6] However, other investigators[7, 8] have reported that new mRNA molecules arise during late developmental stages of the sea urchin. Similarly, Denis[9] compared the mRNA population of the mature amphibian egg and early cleaving embryo by means of RNA-DNA hybridization and competition methods and demonstrated that egg RNA was ineffective as a competitor, and thus appeared to contain no mRNA sequences in common with those synthesized late in development.

The chick oviduct model system[10] appeared particularly suited for the study of mRNA populations during different stages of hormone-induced biochemical and cytological differentiation in higher animals. Growth and differentiation of the oviduct can be markedly stimulated by administration of diethylstilbestrol (DES) or estradiol to a newborn chick. After estrogen stimulation, three distinct types of epithelial cells differentiate from the homogeneous-appearing cells of the mucosa.[11] Two of these cell types synthesize cell-specific proteins which can be measured biochemically and used as markers for differentiation.[12] We do not believe that DNA-RNA hybridization studies measure only mRNA; however, the technique should be feasible for indirectly assessing gene function by qualitatively identifying the appearance of new RNA transcriptions from different genetic loci during oviduct differentiation. In the present study, we have examined nuclear RNA populations from various stages of hormone-induced oviduct differentiation by measuring the ability of unlabeled RNA from earlier stages to compete for chick DNA binding sites with rapidly labeled nuclear RNA from differentiated tissue.

Materials and Methods.—Materials were purchased from the following sources: All labeled nucleotides, Schwartz Biochemical Co.; phosphoric acid-P^{32}, Tracerlab; Subtilisin ("Nagase"), Enzyme Development Corp.; Pronase, Calbiochem Inc.; DNase (electrophoretically pure) and RNase (5× recrystallized, pancreatic), Worthington Biochemicals; sodium dodecyl sulfate (SDS), K and K Laboratories; Millipore filters (47 mm HAWP), Millipore Corp.

Preparation of nucleic acids for hybridization: Three-day-old female Rhode Island Red chicks were injected subcutaneously with 5 mg DES in sesame oil daily for various periods

up to 20 days. Oviducts from each time point were removed and incubated under sterile conditions in medium 199 for 30 min. Only the 20-day DES oviducts were incubated in the presence of 25 μc/ml of H^3-uridine (spec. act. = 20 c/mM) and 12 μc/ml of H^3-adenine (8 c/mM). Incubations were terminated by freezing. Nuclei were isolated as described previously.[10] The RNA was extracted according to a modification of the method described by Torelli *et al.*[16] and was suspended and stored at $-30°$ in 2 $\times$ SSC + 0.05% SDS (1 $\times$ SSC = 0.15 *M* NaCl, 0.015 *M* Na_3 citrate, pH 7.0). All RNA samples were required to have an OD 260:280 ratio of at least 1.94. The specific activity of the labeled nuclear RNA ranged from 1800 to 8500 cpm/μg RNA.

DNA was extracted from nuclei isolated as above and suspended in saline ethylenediaminetetraacetate(EDTA) (0.15 *M* NaCl, 0.1 *M* EDTA, pH 8.0) by a modification of the Marmur[13, 16] method.

Hybridization technique: Hybridization was carried out with a modification of the liquid-liquid annealing membrane filter separation system as modified by Torelli *et al.*[14-16] The nucleic acids were suspended in 2 $\times$ SSC, 0.05% SDS in heat-sealed ampoules, boiled at 100° for 15 min to denature the DNA, cooled, and incubated from 4 to 16 hr at 67°. The ampoule contents were then suspended in Hy-buffer (0.1 *M* Tris, pH 7.3, 0.5 *M* NaCl, 0.01 *M* $Na_4P_2O_7$), collected on Millipore filters, and washed twice with cold and once with hot (60°) Hy-buffer. The filters were then incubated with RNase (0.04 mg/ml) in 2 $\times$ SSC for 120 min. The filters were washed twice with Hy-buffer and finally with 95% ethanol. The filters were dried and counted in toluene-phosphor scintillation counting solution. Saturation of DNA with RNA was more readily obtained when the RNA existed in small pieces or was first sonicated. This also improved the specificity of competition experiments. Our hybridization results were similar using the Gillespie and Spiegelman[17] technique, where the denatured DNA is applied to a membrane filter prior to being annealed with RNA. Thirty per cent of the RNA in the initial hybrid was sensitive to the RNase digestion step. The ratio of RNA/DNA was also quite critical for competition experiments; a ratio of 2:1 was generally satisfactory but had to be determined for each separate preparation. The most efficient incubation volume for annealing was found to be 0.1 ml for 20 μg DNA. One hundred per cent control hybridization for competition reactions was generally equivalent to 1.5 per cent of the total labeled RNA added at a ratio of RNA:DNA of 3:1.

Nearest-neighbor-frequency (NNF) analysis and base composition of RNA synthesized in vitro with chromatin template: Chromatin was prepared by the procedures of Marushige and Bonner,[18] modified by Barker and Warren.[19] RNA polymerase was prepared from the early log phase cell of *E. coli* strain B by the method of Chamberlin and Berg.[20]

A final incubation volume of 0.5 ml contained tris(hydroxymethyl)aminomethane (Tris)-HCl, 0.05 *M*, pH 8.0; unlabeled ribonucleoside triphosphates, 0.5 mM; α P^{32}-labeled ribonucleoside triphosphates, 5.0 μc (0.05 mM); $MgCl_2$, 1.0 mM; β-mercaptoethanol, 12 mM; excess bacterial RNA polymerase; and 10–25 μg of DNA chromatin. Each chromatin preparation was used to direct the above incubation mixture with the use of α P^{32}-labeled adenosine, cytidine, guanosine, and uridine 5′-triphosphates in separate experiments performed simultaneously. Tubes were incubated 15 min at 37°C and the reaction was terminated by the addition of excess unlabeled ribonucleoside triphosphates and 0.5 ml of cold 6% perchloric acid. The acid-insoluble product was washed three times with cold 2% perchloric acid and once with 95% ethanol, potassium acetate 0.02 *M*, pH 7.2. The precipitates were hydrolyzed in 0.2 *N* KOH for 18 hr. The mononucleotides were separated by column chromatography by the method of Katz and Comb[21] and the results calculated by the method of Josse *et al.*[22]

Measurements of proteins: Ovalbumin was measured according to Kabat and Mayer[23] and total protein was quantified by the method of Lowry *et al.*[24]

Results.—Specificity of hybridization reaction: Labeled chick oviduct nuclear RNA hybridized specifically with chick DNA and annealed poorly to bacterial, human, or mammalian DNA (Table 1). The amount of hybridization was in-

TABLE 1. *DNA specificity for oviduct labeled RNA.*

DNA	Homologous Reaction (%)
Chick oviduct	100*
Chick liver	100
Human lymphocytes	4
Calf thymus	0.8–1.2
E. coli	<0.5
B. subtilis	None

Three hundred μg of chick oviduct RNA (labeled as in the text) were incubated with 80 μg of various DNA's at 67°C for 14 hr as described in *Methods*.
* 100% homologous reaction represents 3,600 cpm.

dependent of the tissue of the homologous species from which the DNA was obtained. Relative saturation usually occurred at an RNA/DNA ratio of 3:1. When the RNA/DNA ratio was very high (>14), a type of linear nonspecific aggregation occurred which obliterated the saturation plateau. Competition experiments were usually carried out at a RNA/DNA ratio of between 1:1 to 2:1. Figure 1 shows that unlabeled mature chick oviduct RNA competed very well with labeled mature chick oviduct RNA for similar DNA base sequences. This competition closely approximated that which would be predicted from theoretical calculations. Less competition was noted when unlabeled RNA from bacteria or mammals was employed in the reaction. Each of the heterologous RNA's soon reached a plateau where further competition was not possible. In the present studies, we did not experience the lack of competition specificity noted by Birnboim *et al.*[27] in HeLa cell hybridization experiments. As a further control for the liquid-liquid DNA-RNA hybridization system employed in these studies, the hybrid was subjected to a cesium chloride density gradient. The hybridized H^3-

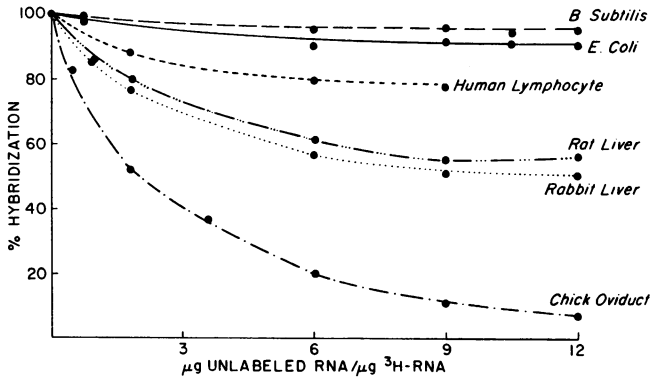

FIG. 1.—Specificity of the competition reaction between various unlabeled heterogeneous nuclear RNA's and labeled oviduct nuclear RNA. Chick oviduct DNA (40 μg) was incubated with 80 μg of H^3-labeled oviduct RNA for 14 hr at 67°C and analyzed as in *Methods*. Increasing amounts of unlabeled RNA from various heterologous species (bacteria, human, rat, rabbit) or homologous unlabeled oviduct RNA (same stage of development) were added to the initial reaction mixture to competitively hybridize to the DNA. The total H^3-RNA hybridizing to DNA in the absence of unlabeled competitor equals 100% control, hybridization.

RNA peak was reduced approximately 50 per cent by the addition of an equivalent amount of unlabeled homologous RNA to the initial hybridization reaction—these results were quite similar to those obtained with the membrane filter method.

Comparison of RNA populations from different developmental stages: We employed unlabeled oviduct RNA from various stages of DES-induced differentiation to compete with labeled RNA from 20-day DES chicks (Fig. 2). The RNA from nuclei of immature chick oviduct competed poorly with the labeled RNA of differentiated oviduct (animals treated with DES for 20 days); the extent of competition was independent of chronological age over the first 30 days of life. By two days of DES, competing species of RNA had developed. After five days of DES, competition was more apparent, and at 20 days of DES the competition reached a theoretical prediction—10 per cent hybridization of control at an unlabeled RNA:H^3-RNA ratio of 9 (Fig. 2).

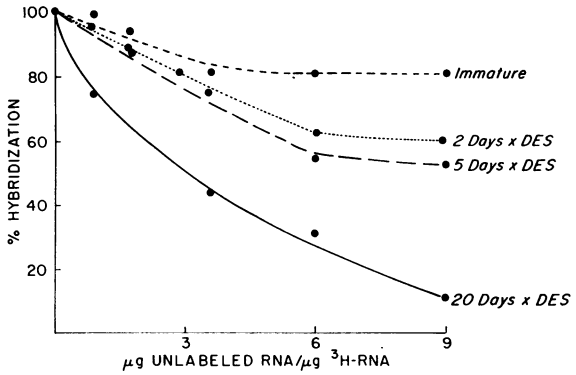

FIG. 2.—Population changes in hybridizable oviduct nuclear RNA at various stages of diethylstilbestrol (DES)-mediated differentiation. Seventy μg of H^3-labeled RNA from oviducts treated with DES for 20 days were incubated with increasing amounts of unlabeled RNA from immature, 2 days × DES, 5 days × DES, and 20 days × DES oviducts as in Fig. 1.

Qualitative template changes following DES: The nearest-neighbor dinucleotide frequencies of RNA transcribed *in vitro* from DNA-chromatin template isolated from unstimulated oviducts and oviducts from animals treated with DES for 3, 6, and 18 days were determined (Table 2). General increases in nucleotide pairs containing adenine (ApA, CpA, GpA, ApG) and a decrease in nucleotide pairs containing cytosine (CpC, UpC, CpG, CpU) were noted in the template transcription product isolated from chicks after three to six days of DES. A change of 0.01 NNF was significant at the 95 per cent confidence limit. The base composition values at various stages of estrogen treatment reflected the above changes in the NNF spectrum (Table 3).

Induction of ovalbumin synthesis: Table 4 shows the effect of estrogen on ovalbumin production by the chick oviduct. Ovalbumin synthesis generally began after day 5–7 of DES treatment and reached a plateau at day 15–17. A less dramatic increase in soluble protein content of the tissue also occurred during estrogen administration.

Discussion.—In the newborn chick, the entire mucosal surface of the oviduct is composed simply of pseudostratified columnar epithelium supported by a compact stroma of polygonal cells. This epithelium normally remains immature until about 100 days of age, when ovarian hormones stimulate growth and differ-

TABLE 2. *Nearest-neighbor dinucleotide frequency analysis of RNA transcribed in vitro from DNA-chromatin template isolated at various stages of estrogen-mediated differentiation.*

Dinucleotide	Days of Estrogen (NNF)*			
	0	3	6	18
ApA	0.046	0.052	0.069	0.069
CpA	0.074	0.089	0.103	0.100
GpA	0.038	0.049	0.058	0.064
UpA	0.030	0.029	0.032	0.031
ApC	0.057	0.068	0.061	0.059
CpC	0.084	0.083	0.067	0.073
GpC	0.079	0.082	0.076	0.073
UpC	0.086	0.065	0.057	0.057
ApG	0.037	0.048	0.079	0.082
CpG	0.065	0.062	0.030	0.029
GpG	0.070	0.074	0.074	0.088
UpG	0.080	0.074	0.078	0.071
ApU	0.047	0.052	0.054	0.054
CpU	0.084	0.065	0.062	0.059
GpU	0.066	0.057	0.050	0.048
UpU	0.051	0.050	0.050	0.044

* Values represent a mean of four determinations from separate experiments.

entiation. After estrogen stimulation, three distinct epithelial cells develop from the primitive mucosa (Fig. 3). Tubular gland cells begin to develop at day 1 of DES and the tubular glands reach relative maturation at day 6.[25] Studies with fluorescein-labeled antibody show that these cells synthesize and store ovalbumin.[12] The major induction of ovalbumin synthesis begins at day 5–7 of DES, correlating well with the morphologic appearance of tubular gland organization at this time. Goblet cells also develop in the surface epithelium, and these cells have been shown to synthesize avidin by both fluorescein-labeled antibody and H^3-biotin radioautography techniques when the tissue is exposed to progesterone.[12] The third type of cell is a ciliated columnar epithelial cell thought to be concerned with egg motility. The morphological and biochemical development appears to reach that of the adult hen by 18 days of DES.

The results described in the present study were compatible with a major estrogen effect on oviduct nuclear transcription. The RNA from nuclei of immature chick oviduct competed very poorly with labeled RNA of differentiated (20-day DES) oviducts. This suggests that the population of hybridizable RNA from unstimulated and DES-stimulated animals differed markedly. These differences are qualitative and do not prove that there is a quantitative deficiency in oviduct mRNA in the undifferentiated oviduct. By two days of DES an increase

TABLE 3. *Base composition of RNA transcribed in vitro from DNA-chromatin templated isolated at various stages of estrogen-mediated differentiation.*

Days	A	C	G	U	$\frac{G+C}{A+U}$
0	18.9*	30.6	25.4	24.9	1.28
3	22.1	29.9	25.8	21.9	1.26
6	26.2	26.1	26.0	21.7	1.09
18	26.3	26.1	27.0	20.2	1.14

* These values represent a mean of four determinations from separate experiments.

TABLE 4. *Ovalbumin and total protein content of chick oviduct at various stages of DES treatment.*

Days of DES	Ovalbumin (mg/gm tissue)	Total protein (mg/gm tissue)
0	0.5 ± 0.3	16.1 ± 0.8
4	0.9 ± 0.2	19.2 ± 0.4
8	12.1 ± 1.2	31.2 ± 6.5
17	18.0 ± 2.0	43.5 ± 3.2

DES (5 mg) was administered subcutaneously to newborn chicks for up to 17 days. The animals were sacrificed at varying periods of DES treatment. Analyses were accomplished on aliquots of a 105,000 × *g* supernatant of oviduct homogenates. Each point represents the mean ± SE of six separate analyses.

in competing species of nuclear RNA was evident; this suggested induction of new species of rapidly labeled nuclear RNA which are either absent or present in very small quantities in the unstimulated chick oviduct. A further increase in competing species is seen at five days of DES. At day 5, tubular gland organization is evident and induction of ovalbumin synthesis has begun. Although the synthesis of specific proteins such as ovalbumins and lysozyme[26] follows DES and correlates with the appearance of the new hybridizable RNA's, no proof exists that these new RNA species have a messenger template capacity for these proteins or for the many additional proteins that must be needed to effect the gross structural oviduct reorganization which follows estrogen.

The changes in oviduct RNA populations may result from synthesis of many new species of RNA or may be due to relatively few new species which cross-react with a number of sites on the DNA molecule because of base sequence redundancy of similar genes.[28] Caution must also be exercised in equating hybridizable RNA with mRNA since theoretically all mRNA should hybridize but all hybridizable RNA need not be mRNA. There is good evidence for the existence in certain species of a rapidly synthesized, metabolically unstable nuclear RNA that is heterogeneous in size and DNA-like in composition.[29] Messenger RNA can be distinguished from this type of RNA by its association with functional polyribosomes, but both should hybridize to DNA. Recent evidence also exists that much of the rapidly labeled RNA of the animal cell nucleus never reaches the cytoplasm.[29, 30] Similarly, hybridization competition studies using mouse L-cells showed that some species of nuclear RNA were absent from the cytoplasm.[31] The function of this high-molecular-weight, rapidly turning-over RNA and its relationship to either nuclear mRNA or the new species of oviduct RNA produced following estrogen is not clear at this time.

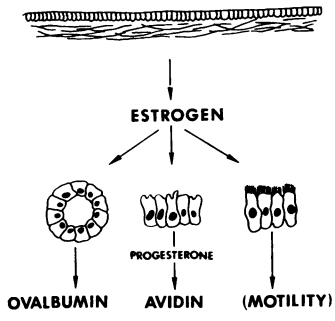

FIG. 3.—Schematic representation of sequence of steroid-induced cytological and biochemical differentiation of immature chick oviduct.

Nearest-neighbor analysis of the RNA synthesized *in vitro* by oviduct DNA-chromatin preparations also suggested a qualitative change in transcription by days 3 to 6 of the steroid-induced differentiation. *In vivo* estrogen has also

been reported to alter the nearest-neighbor nucleotides of uridine monophosphate in the nuclei of castrated rats.[32] In the present study, we do not claim that the RNA synthesized *in vitro* with bacterial RNA polymerase closely resembles the RNA *in vivo*. However, the data do suggest that there is a qualitative change in the DNA-chromatin template during estrogen-mediated differentiation; this supports the qualitative changes in transcription noted in the hybridization competition studies.

Summary.—Diethylstilbestrol markedly stimulates growth and differentiation in the immature chick oviduct. Biochemical specialization also occurs with the appearance of tissue-specific proteins. Concomitant changes in the population of hybridizable RNA occurred with the appearance of new species absent or present in only very small quantities in the unstimulated oviduct. This evidence for new genome transcriptions is supported by qualitative changes in RNA synthesized from oviduct chromatin template isolated prior to or during estrogen-stimulated differentiation. Therefore, we suggest that estrogen-mediated differentiation of the chick oviduct involves an alteration of gene expression, resulting in an induction of new species of nuclear-hybridizable RNA.

Note added in proof: Hahn *et al.*[33] have also recently noted the appearance of a new species of whole cell oviduct RNA after estrogen administration to immature chicks by use of hybridization techniques.

The authors are grateful for the advice on hybridization techniques offered by Dr. P. F. Henry, National Institutes of Health.

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