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# Effect of Estrogen on Gene Expression in the Chick Oviduct. II. Transcription of Chick Tritiated Unique Deoxyribonucleic Acid as Measured by Hybridization in Ribonucleic Acid Excess†

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ABSTRACT: The extent of unique sequence DNA gene transcription was measured in undifferentiated and estrogen-differentiated chick oviducts. The technique of saturation hybridization of chick tritiated unique DNA with a large excess of nuclear RNA, cytoplasmic RNA, or total cell RNA was used to estimate the amount of unique sequence DNA transcribed. Appropriate controls were run to prove the formation of true, extensively base-paired, species-specific DNA–RNA hybrids. Nuclear RNA stability and hybridization reaction kinetics were also investigated in two different reaction media. Data from these experiments indicate a greater amount of unique sequence DNA transcription in the estrogen-differentiated chick oviduct than in the immature oviduct. A possible explanation of the observed increase in unique DNA transcription accompanying oviduct differentiation is discussed.

Previous studies from this laboratory have suggested that estrogen significantly affects gene transcription during the hormone stimulation of the undifferentiated chick oviduct (O'Malley *et al.*, 1969). Nearest neighbor frequency analysis and competition hybridization experiments using only the repeating sequences in total cell DNA have indicated a qualitative change in the nuclear RNA species synthesized during estrogen-mediated oviduct differentitation (O'Malley and McGuire, 1968; Hahn *et al.*, 1968). Quantitative changes in RNA polymerase and in the capacity of oviduct chromatin to function as a template for bacterial RNA polymerase have also been observed (O'Malley and McGuire, 1968; Spelsberg *et al.*, 1971). Finally, an estrogen-dependent increase in the amount of specific, translatable, ovalbumin mRNA has been demonstrated (Rosenfeld *et al.*, 1972; Means *et al.*, 1972; Rhodes *et al.*, 1971). Despite these advances, the mechanism of steroid action in the regulation of gene transcription is not well understood.

Several basic questions relevant to the hormonal effect on oviduct transcription remain to be answered. (1) Are unique DNA sequences transcribed in the chick oviduct, and, if so, to what extent? (2) How much of the transcribed nuclear RNA is processed into the cytoplasm and presumably translated as mRNA? (3) What effect does estrogen have on the extent of unique sequence DNA transcription and processing of oviduct RNA? To answer these questions, we have employed the technique of saturation hybridization in solution under conditions of vast RNA excess (Grouse *et al.*, 1972). Using

† From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77025. *Received February 21, 1973.* During the period this work was done, one of us (C. D. L.) was a predoctoral fellow supported by National Institutes of Health Training Grant 5RO1AM05441 at the Department of Biochemistry, Vanderbilt University, Nashville, Tenn. 37232. This work was also supported by National Institutes of Health Research Grant HD-04473, American Cancer Society Grant BC-101B, and Ford Foundation Grant 630-0141.

this method, we should theoretically be able to determine the percentage of DNA transcribed at a given state of oviduct development. In practice, however, the presence of repeated sequences of related base composition in the DNA of higher organisms (Britten and Kohn, 1968) produces extremely rapid DNA reannealing in solution and sequence cross-reaction in hybridization experiments. These problems have been avoided by separating operationally defined unique DNA sequences from repeated DNA sequences and reacting the purified unique DNA in solution with a vast excess of RNA. Tritium labeling and fractionation of chick DNA with subsequent purification of unique sequences are described in the accompanying manuscript (Rosen *et al.*, 1973).

In the following study, we have investigated the hybridization of tritiated unique sequence DNA to nuclear or cytoplasmic RNA extracted from undifferentiated or estrogen-differentiated chick oviducts. Rigorously purified RNA was utilized under reaction conditions designed to maximize either RNA stability or hybridization reaction rate. Thermal denaturation profiles of hybrids on hydroxylapatite were performed to assess the fidelity of base pairing. Preliminary estimates of the saturation values obtained with nuclear RNA from immature or fully differentiated oviducts indicate that estrogen stimulation results in a significant increase in the amount of experimentally detectable unqiue sequence DNA transcription.

## Experimental Procedures

*Preparation of Tissue.* Rhode Island Red chicks (7-day old) were subcutaneously injected with 5 mg of diethylstilbesterol per day for the number of days specified, and the magnum portion of the oviduct was removed. Oviducts were also obtained from 28-day-old unstimulated chicks and frozen at −80°. Freshly removed or frozen oviducts were minced and suspended in 2 vol (w/v) of homogenization medium [0.32 M RNase-free sucrose, 0.001 M $CaCl_2$, 0.05 M Tris-HCl, pH 7.5 (Ultra Pure, Schwarz/Mann), and heparin, 100 μg/ml]. Homogenization was performed for 30 sec at a 3.2 setting on a Polytron PT-10 tissue disintegrator. The homogenate was filtered through two layers of sterile gauze and centrifuged at 1000*g* for 10 min. The supernatant fraction was carefully separated from the crude nuclear pellet and centrifuged at 16,000*g* for 15 min to remove mitochondria and any contaminating nuclei. The original crude nuclear pellet was resuspended in 2 vol of homogenization medium and centrifuged at 800*g* for 10 min. The resulting nuclear pellet was resuspended in homogenization medium containing 0.2% Triton X-100 and centrifuged at 1000*g* for 10 min, and the supernate was discarded. All the preceding steps were performed at 0–4°. The postmitochondrial supernatant fraction and the Triton-washed nuclear pellet were then extracted for cytoplasmic and nuclear RNA, respectively. There appeared to be little nuclear breakage during homogenization as determined by diphenylamine analysis (Burton, 1968) of the postmitochondrial fraction. Only 3–8% of the total cellular DNA was found in this fraction.

*Isolation of Cytoplasmic RNA.* The postmitochondrial fraction was diluted with 2 vol (v/v) of cold acetate buffer (0.01 M sodium acetate, pH 5.2, 0.01 M $Na_2EDTA$, and 0.05% washed bentonite) and 10% sodium dodecyl sulfate was added to a final concentration of 0.5%. This solution was shaken vigorously for 60–90 sec at room temperature and then extracted by vigorous shaking for an additional 5 min at room temperature with an equal volume of acetate buffer saturated, redistilled phenol–*m*-cresol (7.8:1, v/v) containing 1% 8-hydroxyquinoline. The resulting emulsion was rapidly cooled to 4° in a Dry Ice–isopropyl alcohol bath and centrifuged for 10 min at 19,600*g* in a refrigerated swinging bucket rotor. The upper aqueous layer plus the interphase were collected and extracted a second time for 5 min at room temperature with 10% sodium dodecyl sulfate added to a final concentration of 0.25%, 0.1 vol of 5 M NaCl, and 0.5 vol of phenol–*m*-cresol–8-hydroxyquinoline. After rapid cooling and centrifugation as before, RNA was precipitated overnight at −20° from the upper aqueous layer by the addition of 2 vol (v/v) of 90% ethanol–10% *m*-cresol. The RNA was collected by centrifugation at 1000*g* for 10 min and reprecipitated from 0.01 M Tris-HOAc (pH 7.0)–0.15 M NaCl with 2 vol of 100% ethanol at −20° for at least 1 hr. This RNA was collected as before, dissolved in 5.0 ml of 0.01 M Tris-HOAc, pH 7.0, containing 10 μl of 1 M $Mg(OAc)_2$, and incubated for 1 hr at 37° with 0.5 mg of RNase-free DNase I (Worthington) followed by 10 min at 37° with 0.5 mg of subtilisin (Nagase, Japan). The reaction was stopped and the RNA precipitated by the addition of 50 μl of 10% sodium dodecyl sulfate, 100 μl of 0.01 M $Na_2EDTA$, 0.25 ml of 3 M NaCl, and 2 vol of cold 100% ethanol. After standing at −20° for at least 1 hr, the RNA was collected as before and dissolved in 10–15 ml of 0.01 M Tris-HOAc, pH 7.0. This solution was extracted for 3 min at room temperature with 7–10 ml of phenol–*m*-cresol–8-hydroxyquinoline, rapidly cooled to 4°, and centrifuged at 19,600*g* for 10 min. RNA was precipitated from the upper aqueous layer at −20° by the addition of 1/9 vol of 20% KOAc, pH 5.1, and 2 vol of cold 100% ethanol. The RNA was collected as before and reprecipitated three–five times at −20° from 2 M KOAc, pH 5.1, by the addition of 1/3 vol of cold 100% ethanol. The RNA was collected after each precipitation by centrifugation at 1000*g* for 10 min and was lastly washed successively with cold 70, 80, and 95% ethanol. The RNA was finally reprecipitated from 0.01 M Tris-HOAc (pH 7.0)–0.15 M NaCl as described above, collected by centrifugation at 16,100*g* for 10 min, dried under gentle vacuum, and dissolved in deionized water.

*Isolation of Nuclear RNA.* The Triton-washed nuclear pellet was suspended in 5 vol (v/v) of cold acetate buffer and 10% sodium dodecyl sulfate was added to a final concentration of 0.5%. This mixture was vigorously shaken for 90 sec at room temperature and then extracted with an equal volume of preheated (65°), acetate buffer saturated phenol–*m*-cresol–8-hydroxyquinoline. The mixture was vigorously shaken for 3 min at room temperature followed by gentle shaking for 4 min at 65°. The resulting emulsion was rapidly cooled to 4° in a Dry Ice–isopropyl alcohol bath and centrifuged at 19,600*g* for 10 min in a swinging bucket rotor. The upper aqueous layer plus the interphase were collected and reextracted for 3 min at room temperature and 4 min at 65° with 10% sodium dodecyl sulfate added to a final concentration of 0.25%, 0.1 vol of 5 M NaCl and 0.5 vol of preheated (65°) phenol–*m*-cresol–8-hydroxyquinoline. After a second rapid cooling and centrifugation as above, RNA was precipitated overnight at −20° from the upper aqueous layer by the addition of 2 vol (v/v) of 90% ethanol–10% *m*-cresol. The RNA was collected by centrifugation at 1000*g* for 10 min and reprecipitated from 0.01 M Tris-HOAc (pH 7.0)–0.15 M NaCl as described for cytoplasmic RNA. This RNA was collected as before, dissolved in 5.0 ml of 0.01 M Tris-HOAc, pH 7.0, containing 10 μl of 1 M $Mg(OAc)_2$, and incubated for 1 hr at 37° with 1.0 mg of RNase-free DNase I followed by 10 min at 37° with 0.5 mg of subtilisin. The enzyme reaction was stopped by the addition of 50 μl of 10% sodium dodecyl sulfate and 100 μl of 0.01 M $Na_2EDTA$ and the

solution was extracted for 3 min at room temperature with an equal volume of phenol–*m*-cresol–8-hydroxyquinoline. The emulsion was rapidly chilled to 4° and centrifuged at 16,900*g* for 10 min. RNA was precipitated from the upper aqueous layer at −20° by the addition of 0.05 vol of 3 M NaCl and 2 vol of cold 100% ethanol. The RNA was collected as before, dissolved in Tris-$Mg(OAc)_2$ as described above, and incubated for 20 min at 37° with 0.5 mg of RNase-free DNase I followed by subtilisin digestion as described above. The reaction was stopped and the RNA precipitated by the addition of 50 μl of 10% sodium dodecyl sulfate, 100 μl of 0.01 M $Na_2EDTA$, 0.25 ml of 3 M NaCl, and 2 vol of cold 100% ethanol. Subsequent purification of nuclear RNA from this point is identical with the corresponding steps for the purification of cytoplasmic RNA described above.

Both nuclear and cytoplasmic RNAs prepared by these methods had $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios greater than 2.0 and were free of contaminating DNA. Removal of DNA was monitored by the addition of 6-[$^3$H]thymidine-labeled chick embryo DNA prior to the enzyme digestion step in each preparation. Where one enzyme treatment was sufficient for cytoplasmic RNA, two enzyme treatments and the subsequent series of precipitations from 2 M KOAc, pH 5.1, were necessary to remove [$^3$H]DNA oligonucleotides from the nuclear RNA preparation as evidenced by background levels of radioactivity in the final RNA solution.

*Isolation of Total Cell RNA.* For chick oviduct and rat or guinea pig liver, freshly excised tissue was homogenized (Polytron PT-10) in cold acetate buffer (see above) and total cell RNA extracted as outlined in the preparation of nuclear RNA. *Bacillus subtilis* cells (frozen cell paste, Miles Laboratories) were washed with cold 9% NaCl and lysed with lysozyme and sodium dodecyl sulfate. The lysed cells were extracted for RNA as outlined in the preparation of nuclear RNA. In each of these total cell RNA preparations, an additional step employing methoxyethanol extraction and subsequent RNA precipitation with cetyltrimethylammonium bromide was used (Ralph and Bellamy, 1964).

*Preparation of DNA for Hybridization.* Labeling of chick fibroblast DNA in culture with 6-[$^3$H]thymidine and subsequent fractionation and purification of tritiated unique sequence DNA (sp act. = 293,300 cpm/μg) for use in saturation hybridization studies have been described in the accompanying article (Rosen *et al.*, 1973).

*Saturation Hybridization.* Specific nuclear and cytoplasmic RNA preparations in vast excess (see text) were hybridized with chick fibroblast tritiated unique DNA in 0.4 M phosphate buffer (0.2 M $NaH_2PO_4$–0.2 M $Na_2HPO_4$, pH 6.8) at 70°, or in a buffered formamide medium [0.75 M NaCl–0.01 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Calbiochem) (pH 7.0)–0.001 M $Na_2EDTA$ in 50% formamide (Fisher, $A_{270}$ smaller than 0.150)] at 40°. For each hybridization experiment, 50-μl aliquots from the reaction mixture described above were heat sealed in 50-μl capillary pipets (Dispo). The capillaries were then heated in boiling water at 100° for 13 min to denature all nucleic acids, and either quick-chilled in ice at 0° (zero time points) or transferred immediately to a water bath at the appropriate incubation temperature (reaction time points). At zero time and subsequent reaction times, the contents of a 50-μl capillary were diluted 1:100 into 5 ml of cold 0.14 M phosphate buffer (0.07 M $NaH_2PO_4$–0.07 M $Na_2HPO_4$, pH 6.8) to stop the reaction and frozen at −20° for later analysis by hydroxylapatite chromatography. The following control experiments were also performed simultaneously with the hybridization reaction for most of the RNA preparations used. The first control experiment was designed to monitor the formation of [$^3$H]DNA–RNA hybrids as opposed to [$^3$H]DNA reannealing. The RNA was predigested for 1 hr at 37° with bovine pancreatic RNase IIA (Worthington; preheated 10–15 min at 85° to destroy contaminating DNase), and combined with chick fibroblast tritiated unique DNA in the identical hybridization buffer and at the same RNA concentration as the undigested RNA of the hybridization system. The second control experiment was used to measure background DNA reannealing over the time course and under the conditions of the hybridization reaction. In this system, chick fibroblast tritiated unique DNA was allowed to reanneal in the absence of RNA but in the identical hybridization buffer and at the same [$^3$H]DNA concentration as in the actual hybridization system. The 50-μl reaction aliquots from each type of control experiment were treated the same as in the hybridization system described above.

Each 0.14 M phosphate buffer diluted time point was thawed and immediately fractionated on a 2–3-$cm^3$ prewashed hydroxylapatite (Clarkson) column at 60° as follows. The use of excess hydroxylapatite with the low concentration of formamide in the diluted reaction time points did not necessitate prior dialysis to effect complete binding of hybrids to these columns (unpublished observation). After applying each 5-ml sample, the column was washed with five 5-ml volumes of 0.14 M phosphate buffer to remove single-stranded, tritiated unique DNA followed by six 1-ml volumes of 0.5 M phosphate buffer to remove the double-stranded, tritiated unique DNA–RNA hybrids. Each 0.14 M fraction (1.6 ml) and each 0.5 M fraction (0.5 ml) (+ 1 ml of water) were solubilized in 10 ml of 15% BBS-3 (Beckman) and counted in an LS-250 Beckman liquid scintillation counter at 37% efficiency for tritium. In some experiments, a hybrid in 5 ml of 0.14 M phosphate buffer was predigested for 18–24 hr at 50–55° with or without (control) 50 μg/ml of bovine pancreatic RNase IIA. To stop the reaction, 10% sodium dodecyl sulfate was added to a final concentration of 0.4% and the sample was then fractionated on hydroxylapatite as described above.

*Determination of $T_m$ Values of [$^3$H]DNA–RNA Hybrids.* The method of $T_m$ determination of [$^3$H]DNA–RNA hybrids on hydroxylapatite columns is identical with that for DNA–DNA duplexes and has been described in the accompanying article (Rosen *et al.*, 1973).

## Results

*Stability of RNA under Hybridization Conditions.* The stability of oviduct RNA in each of the hybridization buffers was determined by incubating oviduct tritiated nuclear RNA in 0.4 M phosphate buffer at 70° or in the buffered 50% formamide medium at 40°, and then assaying for $Cl_3CCOOH$-precipitable radioactivity as a function of time. Tritium labeling of oviduct RNA by incubation of tissue slices with tritiated nucleosides *in vitro* and subsequent purification has been described (O'Malley and McGuire, 1968). Figure 1 shows that [$^3$H]RNA was generally stable over the 10-day incubation at 40° in the formamide medium with only 10% breakdown between days 8 and 10. However, in 0.4 M phosphate buffer at 70°, [$^3$H]RNA was stable for only 6 days showing 40% breakdown between days 6 and 10. Although $Cl_3CCOOH$ precipitability may not necessarily correlate to hybridizability, hybridization reactions in 0.4 M phosphate buffer at 70° were terminated at 6 days to eliminate the possibility of false saturation plateaus resulting from the breakdown of hybridizable RNA.

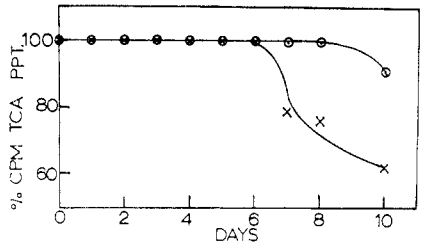


FIGURE 1: RNA stability in two different hybridization media was measured as follows: 50-$\mu$l samples of tritiated nuclear RNA in either 0.75 M NaCl–0.01 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.0)–0.001 M $Na_2EDTA$–50% formamide (⊙) or 0.4 M phosphate buffer (0.2 M $NaH_2PO_4$–0.2 M $Na_2HPO_4$, pH 6.8) (×) were incubated at 40 and 70°, respectively, for 0–10 days. At each time point, a 50-$\mu$l sample from each hybridization medium was diluted into 5 ml of cold 10% $Cl_3CCOOH$ and chilled for 30 min at 4°. This RNA was collected by centrifugation for 10 min at 2500*g*, resuspended in cold 5% $Cl_3CCOOH$, and again collected by centrifugation. The final RNA pellet was solubilized in NCS and counted in toluene–Spectrofluor at 27% efficiency for tritium. The graph illustrates the per cent of the total number of counts per minute of tritiated nuclear RNA remaining $Cl_3CCOOH$ precipitable as a function of incubation time; 100% = 40,000 cpm.

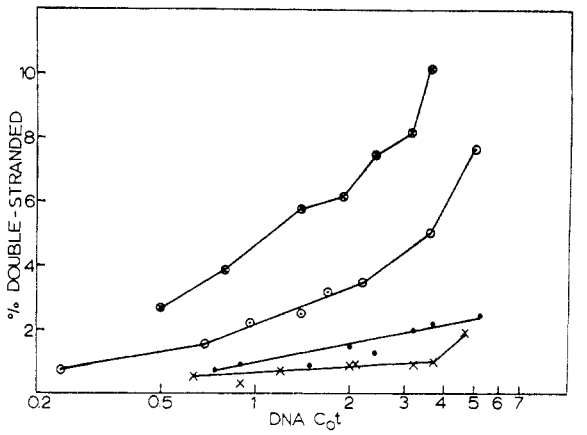


FIGURE 2: The formation of tritiated unique DNA–RNA duplexes expressed as a function of DNA $C_0t$ (see text). The formation of stable duplexes between chick tritiated unique DNA and oviduct nuclear RNA (18 days of diethylstilbesterol) is measured by hydroxylapatite chromatography as the per cent of the tritiated unique DNA in the double-stranded form. Two hybridization and two control reactions were run at 40° in 0.75 M NaCl–0.01 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.0)–0.001 M $Na_2EDTA$–50% formamide. Tritiated unique DNA specific activity = 293,300 cpm/$\mu$g: (⊗) hybridization at a nuclear RNA/tritiated unique DNA ratio of 4000/1; RNA concentration = 6.1 mg/ml; (⊙) hybridization at a nuclear RNA/tritiated unique DNA ratio of 2000/1; RNA concentration = 4.9 mg/ml; (●) control reaction between tritiated unique DNA and oviduct nuclear RNA which has been predigested for 1 hr at 37° with pancreatic RNase IIA; (×) control reaction using chick tritiated unique DNA alone.

*Hybridization of Tritiated Unique DNA to Oviduct RNA.* The ability of nuclear RNA from completely differentiated chick oviducts (18 days of diethylstilbesterol) to hybridize to chick tritiated unique DNA is illustrated in Figure 2. The hybridizations were done in the formamide medium at 40° to maximize RNA stability over extended reaction times. However, because of high viscosity, RNA insolubility, and the resulting difficulty in manipulating the RNA solutions, only RNA concentrations below 10 mg/ml were used in the formamide medium. The two hybridization curves at RNA:DNA ratios of 2000:1 and 4000:1 show similar patterns of increasing tritiated unique DNA hybridization, with the amount of tritiated unique DNA hybridized at any point in the reaction being greater at the higher RNA:DNA ratio. The data are expressed as a function of DNA $C_0t$ (*i.e.*, DNA concentration in moles per liter × time in seconds) to compare two experiments, using different DNA concentrations, with respect to the effect of RNA concentration alone on the reaction rate. Theoretically, if hybridizable RNA sequences are present in vast excess, the rate of hybridization should depend only on the RNA concentration and changes in the RNA to DNA ratio resulting from small experimental variations of the DNA concentration should not affect this rate. Therefore, although the decrease in DNA concentration (from 2.4 to 1.5 $\mu$g/ml) is reflected in the corresponding increase of the RNA to DNA ratios from 2000:1 to 4000:1, the observed higher rate of reaction at the higher ratio should result only from the increase in RNA concentration from 4.9 to 6.1 mg/ml.

Control curves utilizing RNase-digested nuclear RNA or tritiated unique DNA alone (see Experimental Procedures) in the formamide medium were performed simultaneously with both hybridization curves. These control experiments demonstrate that when the nuclear RNA used in these hybridizations is digested with RNase before reaction with tritiated unique DNA, the resulting hybridization curve is similar to that for tritiated unique DNA alone in this medium. These control curve values are less than 25% of hybridization curve values. Therefore, the hybridization observed must be due to the formation of true DNA–RNA duplexes and not to tritiated unique DNA self-annealing or to reaction of [$^3$H]DNA with DNA contamination in the nuclear RNA preparations.

We next attempted to determine what per cent of the tritiated unique DNA was hybridized at saturation with nuclear RNA from the estrogen differentiated oviduct. Since only moderate RNA concentrations could be attained in the formamide medium a saturation hybridization reaction was also run in 0.4 M phosphate buffer in which high RNA concentrations (greater than 15 mg/ml) were possible. Because RNA is stable in the formamide medium over long incubation periods, high RNA $C_0t$ values (*i.e.*, RNA concentration in moles/liter × time in seconds) were achieved using moderate RNA concentrations and long reaction times. Similar high RNA $C_0t$ values could be achieved in 0.4 M phosphate buffer using high RNA concentrations and shorter reaction times. In this case, the reaction was terminated at 6 days to avoid the possibility of RNA degradation (see above). The reaction curves resulting from three incubations carried out in the formamide medium are illustrated in Figure 3. Control curves (not shown) using RNase predigested nuclear RNA and tritiated unique DNA and tritiated unique DNA alone were run simultaneously with each saturation curve. Values for the per cent tritiated unique DNA hybridized to RNA were generated by subtracting control background reaction values from the corresponding hybridization curve values, and were expressed as a function of RNA $C_0t$ or RNA $C_0t$/DNA concentration ($\mu$g/ml). Figure 3A suggests that the rate of hybridization might be faster at higher RNA to DNA ratios and therefore that the concentration of *hybridizable* RNA sequences may not have been in sufficient excess to generate an overall first-order reaction dependent only on RNA concentration. Expression of hybridization as a function of RNA $C_0t$/DNA concentration tended to normalize the data from the formamide hybridizations in which changes in the RNA to DNA ratios were generated by experimental variation of both RNA and DNA concentrations (Figure 3B). As Figures 3 and 4 indicate, both hybridization systems give similar sat-

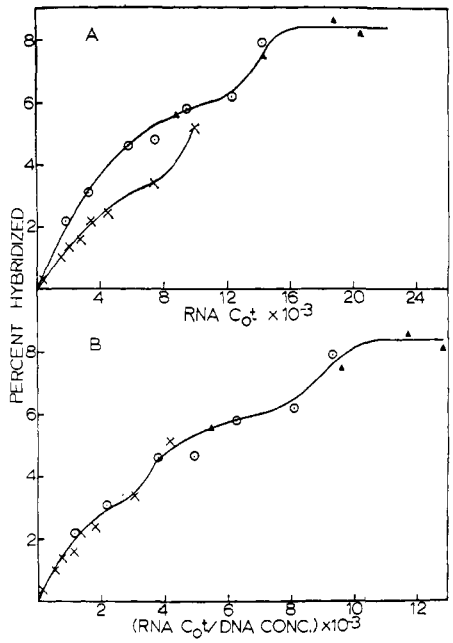


FIGURE 3: Hybridization of chick tritiated unique DNA with oviduct nuclear RNA in 0.75 M NaCl–0.01 M *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (pH 7.0)–0.001 M $Na_2EDTA$–50% formamide at 40°: (×) RNA concentration = 4.9 mg/ml; RNA/DNA = 2000/1; (▲) RNA concentration = 5.6 mg/ml; RNA/DNA = 3600/1; (⊙) RNA concentration = 6.1 mg/ml; RNA/DNA = 4000/1. (A) Hybridization expressed as function of RNA $C_0t$; (B) hybridization expressed as a function of RNA $C_0t$ divided by DNA concentration arbitrarily expressed in micrograms/milliliter.

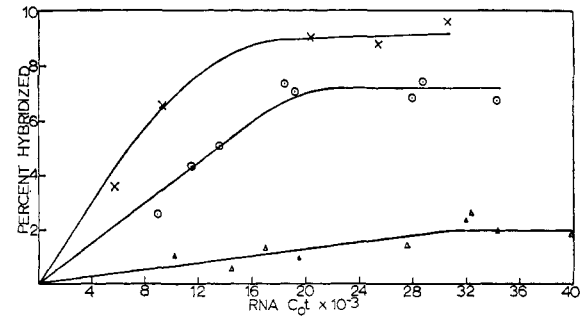


FIGURE 4: Saturation of chick tritiated unique DNA with nuclear RNA extracted from (⊙) immature or (×) estrogen-stimulated (18 days of diethylstilbesterol) oviducts, or saturated with cytoplasmic RNA from (▲) immature or (△) estrogen-stimulated (18 days of diethylstilbesterol) oviducts. Hybridizations were run in 0.4 M phosphate buffer at 70° and at RNA concentrations greater than 15 mg/ml.

uration values for tritiated unique DNA (approximately 9%); however, the formamide medium reveals the possibility of more complex kinetics in the reaction. The earlier relative plateaus observed in the formamide system may reflect gross differences in the concentrations of the various hybridizable RNA sequences present in the total RNA preparation. At the relatively low RNA concentrations used in formamide, some of these sequences may not be present in sufficient concentration to constitute the vast RNA excess required for an overall theoretical first-order reaction, thereby producing the complicated kinetics observed. In the 0.4 M phosphate buffer system, the final saturation value of 9% is attained without the appearance of earlier relative plateaus suggesting the presence of a vast excess of RNA. The relatively long reaction times and complicated kinetics associated with the low RNA concentrations in formamide precluded the use of this system in the comparative hybridizations described below.

*Effect of Estrogen on Unique Sequence DNA Transcription.* Investigation into the effect of estrogen on the extent of unique DNA transcription was conducted using both nuclear and cytoplasmic RNAs extracted from immature (no diethylstilbesterol) and differentiated (18 days of diethylstilbesterol) chick oviducts. No demonstrable biochemical effects on the oviduct from sham injections have ever been observed (O'Malley *et al.*, 1969) so this control was not deemed necessary. Saturation hybridizations were run at 70° in 0.4 M phosphate buffer using a vast excess of RNA. Control curves using RNase predigested RNA or tritiated unique DNA alone were run simultaneously with each hybridization curve and saturation curve values were again generated by subtracting these control background reaction values (not shown) from the corresponding hybridization curve values. These data are illustrated in Figure 4 as per cent tritiated unique DNA hybridized expressed as a function of RNA $C_0t$. There is no significant difference in the amount of unique DNA hybridized to cytoplasmic RNA from the immature or estrogen differentiated oviduct. However, unique DNA does appear to react to a greater extent with oviduct nuclear RNA from estrogen-stimulated animals. The 2% difference in apparent saturation values with nuclear RNA implies an increase in the amount of experimentally detectable unique DNA transcription following estrogen-mediated differentiation of the oviduct. This difference has been reproducibly demonstrated using two different estrogen-stimulated nuclear RNA preparations and three total RNA preparations, and two different unstimulated oviduct nuclear RNA preparations with the actual saturation values varying within a range of ±0.5%. Furthermore, since only 2% of the unique DNA hybridizes to cytoplasmic RNA, it appears that only 20–25% of the total unique DNA sequences transcribed in the nucleus may be represented in these cytoplasmic RNA sequences.

For many of these experiments, melting profile analysis and $T_m$ determinations were used to assess the fidelity of DNA–RNA duplex formation. Figure 5 represents the typical melting profile analyses of unique DNA–RNA hybrids. Although stable hybrids were formed using chemically labeled tritiated unique DNA, these hybrids had a lower $T_m$ than those formed using tritiated unique DNA from chick fibroblasts. The chick fibroblast tritiated unique sequence DNA hybrid ($T_m$ = 86°) was slightly less stable than the corresponding reassociated DNA duplex ($T_m$ = 89°). Furthermore, both were somewhat less stable than sheared, native chick DNA ($T_m$ = 92°). These values are consistent with previously reported data (Brown and Church, 1971; Firtel, 1972) for high sequence specificity hybrids. Compared to sheared, native chick DNA melted on hydroxylapatite, these $T_m$ values may reflect either 4.5–9% mismatching (Melli and Bishop, 1969) or, more likely, a shorter length of precisely matched base pairs of the DNA–RNA duplex (Miyazawa and Thomas, 1965).

Figure 6 illustrates the reaction of chick tritiated unique DNA with total extractable cell RNAs from chick oviduct, rat or guinea pig liver, and *B. subtilis*. Total cell RNA from the estrogen-stimulated (18 days of diethylstilbesterol) chick oviduct gives a saturation curve similar to the corresponding nuclear RNA and an identical saturation value. The bacterial and mammalian total RNA preparations produce relatively insignificant reaction (less than 1%) under the same hybrid-

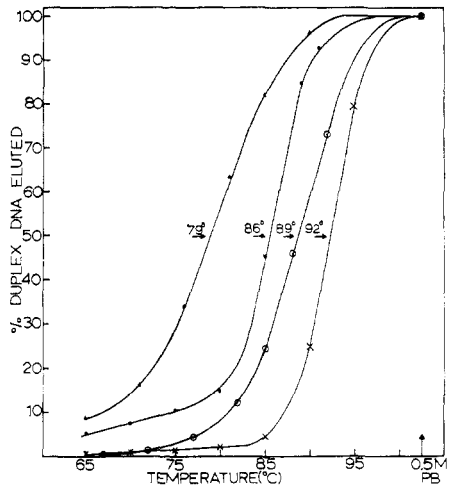


FIGURE 5: Typical melting profiles of tritiated unique DNA–oviduct RNA hybrids. Hybrid melting profiles were assayed on hydroxylapatite columns by increasing the column temperature in small increments and eluting off the resulting single-stranded tritiated unique DNA with 0.14 M phosphate buffer, pH 6.8. Each data point is an expression of the cumulative per cent of the total tritiated unique DNA–RNA hybrid melted as a function of temperature. $T_m$ values are indicated by arrows: (▲) melting profile analysis of hybrid between oviduct cytoplasmic RNA and tritiated unique DNA labeled chemically with tritiated $NaBH_4$ (Rosen *et al.*, 1973); (●) melting profile analysis of hybrid between oviduct nuclear RNA and tritiated unique DNA isolated from chick fibroblasts; oviducts were stimulated for 18 days with diethylstilbesterol prior to RNA extraction; (⊙) melting profile analysis of chick fibroblast tritiated unique sequence DNA reassociated with unlabeled chick oviduct DNA (Rosen *et al.*, 1973); (×) melting profile analysis of sheared, native tritiated chick DNA incubated 6 days in 0.14 M phosphate buffer at 62°. The cytoplasmic hybrid was formed in 50% formamide at 40° and the nuclear hybrid in 0.4 M phosphate buffer at 70°.

ization conditions, thereby indicating the species specificity of the chick RNA–unique DNA interaction. [$^3$H]DNA was recovered from the isolated oviduct RNA hybrid (at saturation) by the method of Firtel (1972). The reassociation of this "hybridized" [$^3$H]DNA with an excess of unlabeled chick oviduct DNA is illustrated in Figure 7. More than 90% of the [$^3$H]DNA reacted in the unique sequence portion of the reassociation curve (*i.e.* at a $C_0t$ value greater than 100). This indicates that the original hybrids contained predominantly unique sequences and that observed differences in saturation values with estrogen (Figure 4) cannot be attributed to a small amount of repeated sequence contamination in the unique sequence fraction. The higher $C_0t_{1/2}$ value for the re-

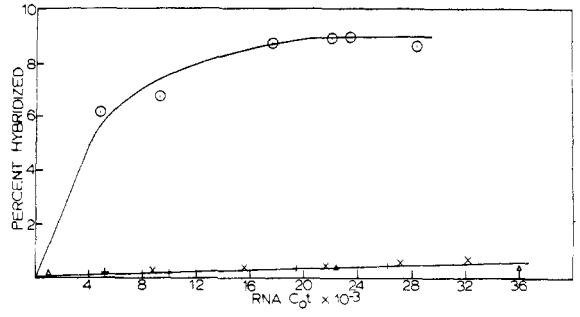


FIGURE 6: Saturation of chick tritiated unique DNA in 0.4 M phosphate buffer, pH 6.8, at 70° with (⊙) total chick oviduct RNA (18 days of diethylstilbesterol), (×) total rat liver RNA, (+) total guinea pig liver RNA, or (△) *B. subtilis* RNA.

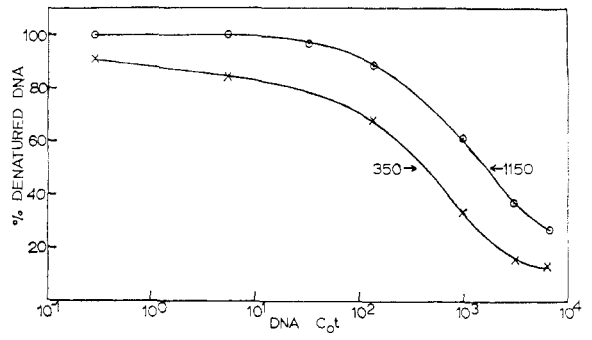


FIGURE 7: Reassociation of hybrid tritiated DNA with total chick oviduct DNA. Tritiated DNA was recovered from a hybrid (0.5 M phosphate) fraction of total oviduct RNA (18 days of diethylstilbesterol) and chick tritiated unique DNA ($C_0t$ = 23,000 in 0.4 M phosphate at 70°) by the method of Firtel (1972). Approximately 2000 cpm of [$^3$H]DNA (⊙) per point was reassociated with excess total chick oviduct DNA (×) at a concentration of 2.7 mg/ml (0.14 M phosphate at 62°). The reassociation kinetics were monitored on hydroxylapatite as described (Rosen *et al.*, 1973). $C_0t_{1/2}$ values are indicated by the arrows.

association of "hybridized" unique sequence DNA compared to total unique sequence DNA (Rosen *et al.*, 1973) may reflect either limited deamination and strand shortening during the extensive alkaline hydrolysis of the hybrid (Rosen, unpublished observation) or hybridization of a relatively more unique component of the total unique sequence DNA.

## Discussion

The data from these experiments demonstrate that unique DNA is transcribed in both the immature and estrogen-differentiated chick oviducts; furthermore, these data may be used to estimate the extent of this unique DNA transcription. At saturation, 7 and 9% of the chick unique DNA reacted with the nuclear RNAs from immature and estrogen-differentiated oviducts, respectively. Assuming only one strand of DNA is transcribed in any gene, this implies that 14 and 18%, respectively, of the unique DNA genes are transcribed in the immature and differeniated oviducts. Since the unique DNA used in these experiments represents 65% of the total DNA per haploid chick genome, in the immature and differentiated oviducts, 9 and 11.5%, respectively, of the total genes are transcribed as unique sequence genes. Therefore, there appears to be more unique sequence gene transcription after estrogen-stimulated differentiation of the chick oviduct than before.

The validity of these data rests on our ability to prove that we are in fact measuring the formation of true, species-specific DNA–RNA hybrids with a high degree of base pairing. This proof comes from three observations. (1) Predigestion of the RNA with RNase reduces the reaction level to that produced by unique DNA alone in our hybridization systems, thus implying that our hybridization reaction values result from the formation of true DNA–RNA duplexes. Furthermore, 50- to 90% of the double-stranded character of these hybrids could be destroyed by exhaustive digestion of the hybrid with RNase at 55° for 18–24 hr. (2) The inability of nonchick RNAs to react to any appreciable extent with chick unique DNA attests to the species specificity of our hybridization results. (3) Finally, the relatively high $T_m$ values of our DNA–RNA duplexes indicate a high degree of base pairing with little mismatching.

Although our control experiments indicate that we are in-

deed measuring DNA–RNA duplex formation, the interpretation of the saturation curve values is subject to a number of qualifications. The saturation values obtained for chick unique DNA with nuclear RNA from the immature (7%) or differentiated (9%) oviduct are, at best, minimum estimates of unique DNA transcription. True saturation values are difficult to determine since most RNA preparations are heterogeneous and therefore contain a distribution of base sequences at different frequencies. While RNA species present at high to moderate frequencies will change to DNA over the course of a hybridization, species present at low frequencies may not react completely, making it difficult to obtain a true saturation value. Furthermore, the analytical complexity of eukaryotic DNA results in a relatively slow hybridization reaction rate so that even though RNA remains $Cl_3CCOOH$ precipitable under our reaction conditions, some potentially hybridizable RNA species could still be sufficiently degraded to preclude formation of stable duplexes with DNA. The efficiency of extraction of hybridizable RNA may also be a function of the purification procedure or tissue changes (*e.g.*, increases in endogenous oviduct RNase) occurring during differentiation. The latter possibility appears less likely in that we have quantified both total cell and nuclear RNase levels prior to and during estrogen administration and noted no difference (Schrader and O'Malley, unpublished data). Nevertheless, these saturation values should not be interpreted as an absolute measure of unique DNA transcription. Furthermore, although the difference in saturation values for nuclear RNAs from immature and differentiated oviducts has been reproducibly demonstrated in repeat experiments, this does not necessarily imply that hiterto unexpressed genes are now being expressed as a result of estrogen administration. Alternatively, estrogen may alter the rate of transcription of a population of commonly expressed genes, thereby increasing the concentration of previously undetectable hybridizable RNA transcripts to levels experimentally measurable by saturation hybridization.

Identical saturation values (2%) were obtained with cytoplasmic RNA from the immature or differentiated oviduct. However, we hesitate to draw any conclusions about the processing of unique DNA–transcribed RNA into the cytoplasm since the cytoplasmic RNA hybridization data exhibited some variability (not shown). This suggests that the observed reaction values may be influenced by nuclear RNA contamination of cRNA preparations, perhaps as a result of leakage from nuclei or nuclei breakage during the tissue fractionation procedure. Also, we were unable to demonstrate any hybrid formation between chick unique DNA and RNA purified from oviduct polysomes. In these experiments, a low concentration of hybridizable RNA sequences may preclude their detection by saturation hybridization.

Other investigators (Grouse *et al.*, 1972) have observed unusually slow reaction rates and low saturation values for mouse liver or embryo RNA hybridized with mouse tritiated unique DNA in a formamide medium containing high salt as compared to the values obtained in the aqueous, 0.4 M phosphate buffer system. This phenomenon was especially apparent at RNA concentrations greater than 1 mg/ml. However, by using RNA concentrations below 10 mg/ml and longer reaction times in formamide, we were able to achieve a saturation value for estrogen-differentiated oviduct nuclear RNA essentially identical with that obtained with this RNA in 0.4 M phosphate buffer. In the formamide system, we have observed that high concentrations (greater than 10 mg/ml) of RNA are difficult to maintain in solution and that even when the RNA can be completely dissolved, the resulting reaction mixture is so viscous as to prevent manipulation except at high temperature. Thus, at the lower reaction temperatures associated with the formamide system, RNA insolubility or reaction mixture viscosity at high RNA concentrations may reduce the rate of hybridization either by excluding hybridizable RNA species from solution or by hindering molecular movement sufficiently to prevent the "collisions" necessary to produce DNA–RNA duplexes.

The transcription of certain essential unique sequence genes necessary for cell viability and therefore common to all cell types may be reflected in the total per cent of the genome expressed. For example, there is some evidence (Grouse *et al.*, 1972) for unique sequence RNA transcripts common to both liver and spleen cells in the mouse. Furthermore, the heterogeneity of cell types characteristic of some tissues such as the brain may be reflected in higher levels of overall (*i.e.* tissue) unique gene transcription when compared to tissues of more homogeneous cell type such as liver or spleen. Considering the different genome sizes for mouse ($2.7 \times 10^9$ base pairs) and chick ($1.05 \times 10^9$ base pairs), the actual number of unique genes transcribed in the differentiated chick oviduct is similar to that in mouse liver or spleen but less than that for mouse brain. A number of investigators (Brown and Church, 1971; Hahn and Laird, 1971; Grouse *et al.*, 1972) have offered evidence for a higher level of unique sequence DNA transcription in mouse brain (10–12%) as opposed to mouse liver or spleen (2–4%). Since estrogen stimulation is known to produce the differentiation of the chick oviduct epithelium into three distinct cell types (O'Malley *et al.*, 1969), the increase in unique gene transcription reported in this paper may likewise be a reflection of the induced heterogeneity of cell types in the differentiated oviduct. Similar results have been reported for the development of the mouse brain (Grouse *et al.*, 1972) and in the developmental cycle of the cellular slime mold *Dictyostelium discoideum* (Firtel, 1972). Thus, it may be hypothesized that in the differentiated chick oviduct, each of the three cell types may have some unique RNA sequences in common and some peculiar to each cell type, perhaps reflecting its function. The observed increase in transcription for the oviduct tissue as a whole could be the result of the appearance of those unique RNA transcripts peculiar to each cell type and function. The validity of this explanation of estrogen-induced oviduct differentiation can be determined using recycling and competition hybridization experiments.

In conclusion, we have demonstrated a specific increase in the experimentally detectable transcription of nuclear RNA from unique sequence DNA in the estrogen-differentiated chick oviduct. The mechanism by which estrogen ellicits this transcriptional change remains to be determined.

Acknowledgment

The authors with to thank Sister Penelope Wink for her excellent technical assistance.

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# Abortive Assembly of the Lactose Transport System in *Escherichia coli*†

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ABSTRACT: Induction of the lactose operon of *Escherichia coli* has been studied in an unsaturated fatty acid auxotroph grown at different temperatures with a variety of essential fatty acid supplements. Induction of a fully functional transport system, as deduced from the ratio of transport activity to the activities of either β-galactosidase or thiogalactoside transacetylase, proceeds normally with decreasing growth temperature until a critical temperature is reached. At this temperature, induction of the transport activity becomes abortive. The critical temperature at which abortive transport system induction is first observed varies with the essential fatty acid supplement employed, and is identical with the temperature at which transport has previously been demonstrated to undergo a physiological transition. The critical temperature for abortive transport induction is also identical to the temperature at which the membrane lipids undergo a change of state that can be detected by physical probes. The phenomenon of abortive transport induction is not a reflection of any gross defect in complex lipid biosynthesis, nor is it caused by any substantial increase in the lability of the transport system, since preformed transport activity is not markedly labilized below the critical temperature for abortive induction. The most likely explanation for abortive transport system induction is abortive assembly, which occurs when transport is induced at a temperature where both the preexisting and newly synthesized membrane lipids are in a nonfluid, immobile state.

The activities of numerous membrane systems are highly sensitive to the fatty acid composition of the membrane lipids (Fox, 1972; Getz, 1972). Mutants which require unsaturated fatty acids have enabled investigators to alter the fatty acid composition of membrane lipids in microorganisms (Resnick and Mortimer, 1966; Silbert and Vagelos, 1967; Silbert *et al.*, 1968; Keith *et al.*, 1969). Processes such as cellular growth, transport, and respiration have markedly different responses to temperature in mutants grown with different unsaturated fatty acid supplements (Schairer and Overath, 1969; Wilson *et al.*, 1970; Overath *et al.*, 1970; Fox *et al.*, 1970; Wilson and Fox, 1971a; Overath *et al.*, 1971; Raison *et al.*, 1971), and the morphogenesis of transport systems and the cellular respiratory apparatus is highly dependent upon the cellular ability to synthesize complex lipids containing unsaturated fatty acids (Fox, 1969; Proudlock *et al.*, 1969). Using unsaturated fatty acid auxotrophs of *Escherichia coli*, Overath and his colleagues and workers in this laboratory have demonstrated transitions in β-galactoside transport as a function of the temperature for transport assay (Wilson *et al.*, 1970; Overath *et al.*, 1970). Arrhenius plots which describe transport rate as a function of assay temperature are biphasic in slope, intersecting at a point which has been termed the transition temperature. The transition temperatures for the independent β-galactoside and β-glucoside transport systems are identical when assayed in cells grown with the same unsaturated fatty acid supplement, and are modified in parallel when the cells are grown with different unsaturated fatty acid supplements. The identical response of two transport systems which share no common functional protein to an alteration in lipid fatty acid composition was interpreted to be the result of a change of state in the membrane lipids (Wilson *et al.*, 1970). This conclusion is supported by the results of a study which compares the transition temperatures for physiological functions with the onset temperature for a change in physical properties of the extracted membrane lipids (Overath *et al.*, 1970).

† From the Department of Bacteriology and the Molecular Biology Institute, University of California, Los Angeles, California 90024. *Received March 14, 1973.* Supported in part by U. S. Public Health Service Research Grant GM-18233, Grant 569 from the California Division of the American Cancer Society, and U. S. Public Health Service Institutional Grant FR 7009. The preceding paper in this series is Wilson and Fox (1971b). A preliminary report of this work has been published (Fox, 1971). This paper is number 3 in the series: Membrane Assembly in *Escherichia coli*.

‡ Recipient of U. S. Public Health Service Research Career Development Award 42359 from the National Institute of General Medical Sciences.