Effect of Estrogen on Gene Expression in the Chick Oviduct. I. Deoxyribonucleic Acid–Deoxyribonucleic Acid Renaturation Studies†

Jeffrey M. Rosen, Charles D. Liarakos, and Bert W. O'Malley

ABSTRACT: As a prerequisite for studying the effects of estrogen on the transcription of unique sequence DNA during hormone-mediated growth and differentiation of the chick oviduct, it was necessary to first study the complexity of chick DNA and secondly fractionate the chick genome into repeated and unique sequences. Approximately 30% of the chick genome consists of rapidly renaturing or repeated sequences, with the remainder being the more slowly renaturing, unique sequences. No effect of hormone treatment on the kinetics of chick oviduct renaturation was experimentally detectable, suggesting that estrogen-induced differentiation was not accompanied by major gene amplification or deletion. Identical renaturation profiles of ^{3}H-labeled chick embryo and chick liver DNA also indicate that tissue differentiation during embryogenesis did not give rise to major gene amplification or deletion. Tritium-labeled unique sequence DNA of high specific activity was isolated from chick fibroblasts and analyzed by renaturation with an excess of unlabeled chick DNA. Complete sequence homology existed between the unique sequences of chick fibroblast DNA and the unique sequences in chick liver DNA. Unique sequence DNA was also labeled chemically by an ultraviolet-catalyzed photoreduction with tritiated $NaBH_4$. Only 40% of the tritium radioactivity in this DNA renatured to form a stable duplex with an excess of unlabeled chick DNA, suggesting an alteration in the physical properties of the chemically labeled DNA. However, melting profiles on hydroxylapatite indicated little mismatching and the formation of stable duplexes between chick DNA and both chemically labeled and chick fibroblast DNAs. These results allow for the investigation of unique sequence transcription during estrogen-mediated changes in the chick oviduct.

Administration of diethylstilbesterol or estradiol to a newborn chick results in the differentiation of the immature oviduct's mucosal cells into three distinct epithelial cell types, two of which synthesize specific marker proteins (Kohler *et al.*, 1968; O'Malley *et al.*, 1969; Oka and Schimke, 1969). Previous studies from this laboratory have suggested that the estrogen-mediated growth and differentiation of the chick oviduct involve an alteration in gene expression, leading to the induction of new species of hybridizable nuclear RNA (O'Malley and McGuire, 1968.) However, hybridization of nucleic acids from higher organisms, performed under the standard conditions of low nucleic acid concentrations and short incubation times, has been shown to involve only the rapidly reassociating, repetitive sequences to the exclusion of the more slowly reassociating sequences, operationally defined as unique sequences (Britten and Kohne, 1968). Furthermore, the presence of closely related sequences in eukaryotic DNA may result in a lack of gene locus specificity, *i.e.*, mismatching of base sequences (McCarthy and Church, 1970).

Recent advances in the technology of nucleic hybridization from higher organisms have permitted the study of transcription of unique sequence DNA in eukaryotes (Gelderman *et al.*, 1971; Melli *et al.*, 1971; Grouse *et al.*, 1972). In order to investigate the effects of estrogen on the transcription of unique sequence DNA, it has been necessary to first study the kinetics of chick DNA renaturation, *i.e.*, chick DNA complexity, and secondly subdivide the chick oviduct genome on the basis of nucleotide sequence homology into unique and repeated sequences. An attempt was also made to determine whether estrogen treatment results in major gene amplification or deletion during hormone-mediated growth and differentiation of the oviduct. Selective amplification of the repetitive DNA in several eukaryotes has recently been reported (Gall, 1969; Willie, 1972). In addition, as a prerequisite for performing saturation hybridizations under conditions of vast RNA excess (Grouse *et al.*, 1972), it was necessary to prepare and characterize labeled unique sequence DNA of high specific activity. The results of DNA–DNA renaturation experiments using tritium-labeled DNA prepared by several different labeling methods are presented. Analysis of the kinetic complexity of various RNA fractions and saturation hybridizations with RNA populations extracted from the oviduct at various stages of differentiation will be reported in the subsequent papers in this series.

Experimental Procedures

DNA Preparation and Labeling. All DNAs were extracted from nuclei by a modification of the method of Marmur (1961) which consists of the addition of a polysaccharide hydrolysis step using α-amylase (Worthington) at a concentration of 50 μg/ml for 30 min (37°) and the omission of the isopropyl alcohol precipitation procedure. Routine analysis of DNA involved an optical density scan and thermal denaturation profile determination (Mandel and Marmur, 1968) using a Gilford 2400 spectrophotometer and a diphenylamine assay (Burton, 1968). *Escherichia coli* B late-log and *Bacillus subtilis* SB 19 were purchased as frozen cell pastes from Miles Laboratories. Tritium-labeled *B. subtilis* DNA was prepared from late-log cells grown in Spizizen's Minimal Salts (Sober, 1968) supplemented with 10% casein hydrolysate and 10%

† From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77025. *Received February 21, 1973.* This work was supported by National Institutes of Health Research Grant HD-04473, American Cancer Society Grant BC-101B, and Ford Foundation Grant 630-0141.

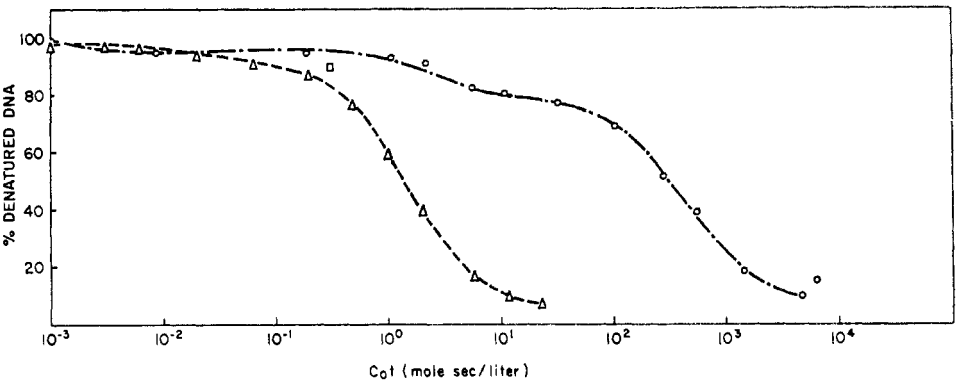

FIGURE 1: Renaturation kinetics of 20-day estrogen-treated chick oviduct DNA and *B. subtilis* DNA. Renaturation experiments were run in 0.14 M phosphate at 62° for oviduct DNA (○) and ^{3}H-labeled *B. subtilis* DNA (△). Conditions were as described under Experimental Procedures. $C_0t_{1/2}$ values were: *B. subtilis* DNA, 1.5; chick oviduct DNA, 300.

glucose, and labeled with 5 μCi/ml of 5-[*methyl*-^{3}H]thymidine (20 Ci/mmol, New England Nuclear) during log phase. *E. coli* DNA was extracted directly from the saline-rinsed cells. ^{3}H-Labeled chick fibroblast DNA was isolated from a primary monolayer culture of chick fibroblasts purchased from Microbiological Associates, and labeled from 30% monolayer to confluency with 20 μCi/ml of 6-[^{3}H]-thymidine (9.1 Ci/mmol, Swartz/Mann). For the preparation of ^{3}H-labeled chick embryo DNA 125 μCi of 6-[^{3}H]thymidine (9.1 Ci/mmol, Schwarz/Mann) was injected into the air sac of Marek negative specific pathogen-free fertile eggs (Spafas, Inc.) on days 7 and 8 after incubation at 37° was begun and the DNA was extracted on day 9. Chemical labeling of DNA with tritiated $NaBH_4$ was performed according to the procedure of Kirkegaard (1969), as modified by Vincent *et al.* (1969). Unique DNA (sheared and denatured), isolated as described in a following section, was dissolved in 0.01 M Tris-HCl, pH 8.5, at a concentration of 1.5 mg/ml. Lyophilized tritiated $NaBH_4$ (15 Ci/mmol, New England Nuclear) was then added to yield a final concentration of 100 mCi/ml and the solution irradiated at a 1-cm path length with mineralight UVS-II (Ultraviolet Products) in a 3-ml quartz cuvet with constant stirring for 6 min. The reaction was stopped by the addition of 6 drops of butanone and the solution stirred for an additional 3 min. All these procedures were carried out in a fume hood. Following three extractions with an equal volume of ether, the DNA was precipitated from ethanol several times until constant specific activity was attained. The DNA was further characterized by its binding to hydroxylapatite (Clarkson) and elution with 0.14 M sodium phosphate buffer, pH 6.8 (prepared by mixing equimolar amounts of Na_2HPO_4 and NaH_2PO_4). Treatment with RNase-free DNase I (50 μg/ml at 37° for 1 hour) rendered 94% of the radioactivity Cl_3CCOOH soluble, indicating that the tritium was incorporated primarily into DNA and not into a small amount of contaminating protein or polysaccharide.

Renaturation Kinetics of DNA. DNA renaturation was measured on hydroxylapatite columns by the method of Laird (1971), as adapted from the original procedure of Britten and Kohne (1968). DNA was sheared in 0.015 M NaCl–0.0015 M sodium citrate, pH 7.0 (0.1 × SSC), at 16,000 psi in a French pressure cell press (Aminco) to yield fragments approximately 300–400 nucleotides in length (single stranded). The 0.1 × SSC solution was then made 0.15 M in Na^+ by the addition of NaCl and the DNA alcohol precipitated. The resulting DNA pellet was dissolved in 0.14 M phosphate buffer to a concentration of between 3 and 8 mg/ml and dialyzed extensively against 0.14 M phosphate buffer. In some experiments ^{3}H-labeled *B. subtilis* DNA (56,800 cpm/μg) was added at a concentration of 10–20 μg/ml (less than 1% of unlabeled DNA) as an internal control (Laird, 1971), and the final DNA solution contained 0.001 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, pH 7.0. In some cases a dilute renaturation curve was also run at approximately 120 μg/ml to determine the early section of the renaturation curve (C_0t values <1.0). The reaction was initiated by denaturing the DNA mixture for 13 min, at 100° in boiling water, followed by incubation of 20–50-μl aliquots in sealed capillaries at 62°. At various times samples were taken, diluted 1:100 into 0.14 M phosphate, and frozen until fractionation on hydroxylapatite columns. C_0t values were calculated on the basis of the [(original A_{260} of the DNA mixture divided by 2) × the time of reassociation in hours] (Kohne and Britten, 1972). DNA was fractionated into single- or double-stranded fragments on 2–4-ml hydroxylapatite columns at 60° (maximum DNA applied = 250 μg/ml of hydroxylapatite) by elution with 0.14 and 0.50 M phosphate, respectively (usually 3 × 2 ml washes at each molarity). The A_{260} of each fraction was determined and 0.8 ml of the 0.14 M fraction and 0.2 ml of the 0.50 M fraction (+ 0.6 ml of H_2O) were each counted in 10.0 ml of 10% Biosolv BBS-3 (Beckman) and toluene–Spectrofluor (Amersham/Searle). Counting was done in an LS-250 Beckman liquid scintillation counter at 42% efficiency for ^{3}H.

Isolation of Unique Sequence DNA. Unlabeled sheared chick DNA was fractionated to remove repeated sequences by renaturation to a C_0t value of 420 (0.14 M phosphate at 62°). The DNA was separated into single- and double-stranded fragments on hydroxylapatite columns as described with 45–50% of the total A_{260} material routinely present in the single-stranded fraction. Following dialysis against 0.015 M NaCl–0.001 M Na_2EDTA the DNA was recovered by alcohol precipitation. In one experiment the very rapidly reassociating sequences were separated first by incubation to a C_0t value of 0.2 (0.14 M phosphate at 62°) and passage through hydroxylapatite. The single-stranded material was reconcentrated and allowed to incubate to a C_0t of 400. The unique sequences were then isolated as described. Due to the limited quantity of ^{3}H-labeled chick fibroblast DNA, its unique sequences were separated from repeated sequences by renaturation to a C_0t of 200, yielding 65% of the total A_{260} material in the single-stranded fraction.

T_m Determination on Hydroxylapatite. Samples were placed on a 3-ml hydroxylapatite column maintained at 60° in a water bath. The single-stranded material was eluted with 4 ×

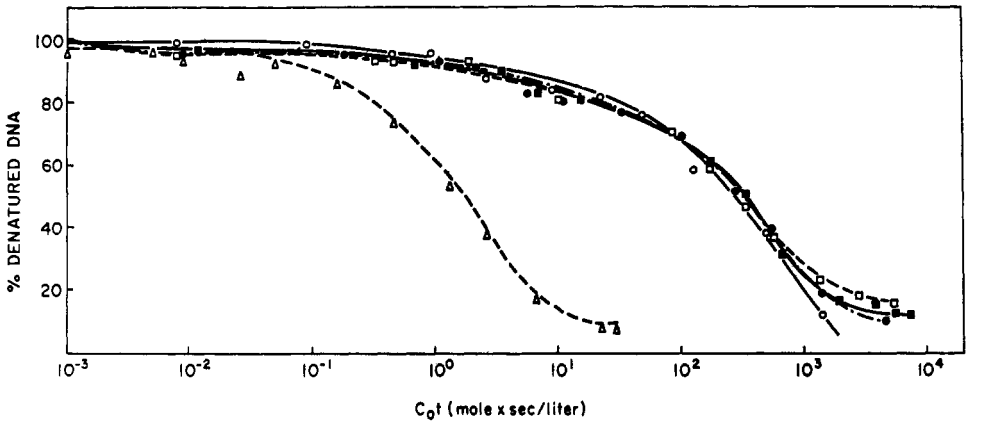

FIGURE 2: Effect of estrogen on chick oviduct DNA renaturation kinetics. DNA was extracted from unstimulated (■), 3-day diethylstilbesterol-treated (□), 20-day diethylstilbesterol-treated (●), and hen oviducts (○). Diethylstilbesterol was administered to chicks as previously described (O'Malley and McGuire, 1968). Separate renaturation experiments were run with each DNA and with ^{3}H-labeled *B. subtilis* DNA (△) used as an internal standard in each experiment. Conditions were as described under Experimental Procedures. $C_0t_{1/2}$ values were: unstimulated, 340; 3-day diethylstilbesterol, 310; 20-day diethylstilbesterol, 300; hen, 300; *B. subtilis*, 1.7.

5.0 ml washes of 0.14 M phosphate and then the temperature raised by 5° increments. When the temperature of the 0.14 M phosphate buffer in the column had equilibrated with the bath temperature, the dissociated DNA strands were eluted with a total of 3 × 2.0 ml of 0.14 M phosphate washes at each temperature. Three final washes of 2.0 ml each of 0.50 M phosphate were performed at 90–92° to remove any remaining unmelted DNA duplex. The A_{260} of each fraction was determined and when radioactivity was present samples were counted as described. Data are plotted as the cumulative per cent of the duplex DNA eluted at each temperature.

Results

Renaturation Kinetics of Chick Oviduct DNA. The complexity of chick oviduct DNA isolated from 20-day estrogen-stimulated oviducts was analyzed with respect to a bacterial DNA composed primarily of unique sequences and producing a characteristic second-order renaturation profile. As illustrated in Figure 1, under fairly stringent conditions of salt and temperature (*i.e.* 0.14 M phosphate and 62°), approximately 30% of chick oviduct DNA renatures prior to a C_0t value of 200 and can be designated as repeated sequences. The remaining 70% of the oviduct DNA is designated as nonrepetitive or unique sequence DNA, although it may well contain two or more copies of a given gene sequence (Southern, 1971; Sutton and McCallum, 1971). The oviduct genome evidently does not contain measurable amounts of the very rapidly reassociating sequences which comprise approximately 10% of the total mouse genome (Flamm *et al.*, 1967) and may represent as many as 10^6 copies of a single nucleotide sequence (Waring and Britten, 1966).

^{3}H-Labeled *B. subtilis* DNA is used as an internal standard in these experiments (Laird, 1971) and exhibits a $C_0t_{1/2}$ (*i.e.* the C_0t value at which 50% reassociation is reached) routinely between 1.5 and 1.7. The $C_0t_{1/2}$ for total chick DNA (0.14 M phosphate at 62°) in numerous experiments also showed limited variability and ranges from 300 to 350. The use of an internal standard and careful control of those conditions which influence renaturation rate, *i.e.* fragment size, salt concentration, and temperature, allow for the comparison of the renaturation profiles of DNA extracted from oviducts at various stages of hormone-mediated differentiation (Figure 2). In these experiments the renaturation profiles of DNAs extracted from unstimulated 3- and 20-day diethylstilbesterol treated hen oviducts are essentially superimposable. No major deviations from the characteristic oviduct DNA renaturation profile are observed, suggesting that hormone-induced growth and differentiation does not result in major gene amplification or deletion. This does not exclude the possibility that limited amplification or deletions might occur, which may not be detectable by studying the renaturation of total oviduct DNA, *i.e.* changes of $< \pm 5\%$. Furthermore, no major differences in renaturation profiles are observed between DNAs extracted from various chick tissues (data not shown). Accordingly, chick liver DNA is occasionally used instead of chick oviduct DNA for renaturation and hybridization experiments.

In the course of studies designed to prepare [^{3}H]DNA of high specific activity for use in hybridization experiments, ^{3}H-labeled chick embryo DNA of specific activity 16,900 cpm/μg was prepared as described under Experimental Procedures. In comparison, *in vivo* labeling with similar amounts of radioactive precursors yielded chick DNA (extracted from the intestine) of tenfold lower specific activity. By increasing the amount of radioactivity injected into the fertilized eggs it was possible to double the specific activity of the extracted DNA. Although this DNA is not of high enough specific activity to provide the desired sensitivity in hybridization experiments, it did allow a comparison of nucleotide sequence homology between embryo and liver DNAs (Figure 3). When greater than

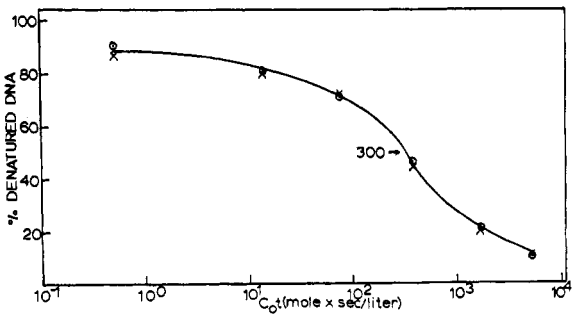

FIGURE 3: Renaturation of ^{3}H-labeled chick embryo DNA with unlabeled chick liver DNA. ^{3}H-Labeled chick embryo DNA (○), 16,900 cpm/μg, was renatured at a concentration of 6.25 μg/ml with unlabeled chick liver DNA (×), 6.82 mg/ml. The arrow designates the $C_0t_{1/2}$ value. Conditions were as described under Experimental Procedures.

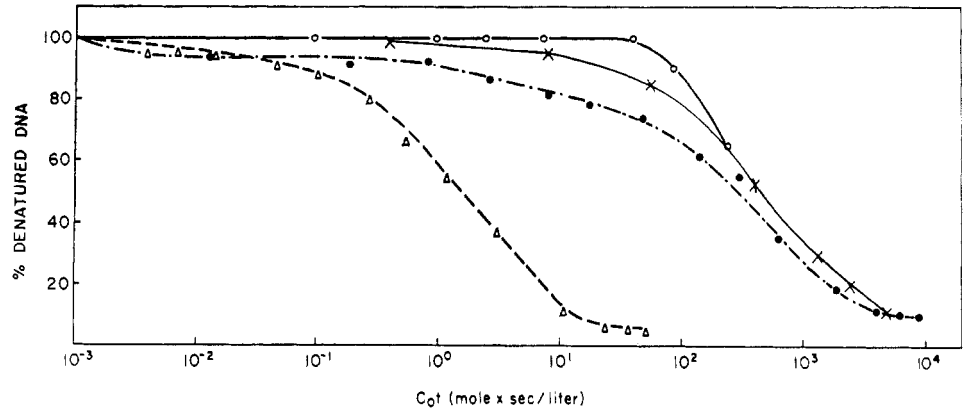

FIGURE 4: Recycling of unique sequences with total chick DNA. Two individual experiments are illustrated. In the first, unlabeled unique sequences isolated from diethylstilbesterol-stimulated chick liver DNA (○) were allowed to renature at a concentration of 1 mg/ml and ^{3}H-labeled *B. subtilis* DNA (△), 10 μg/ml, was used as an internal standard. In the second, ^{3}H-labeled unique chick fibroblast DNA (×), 293,000 cpm/μg, at a concentration of 6 μg/ml was renatured with unlabeled chick liver DNA (●), 4.75 mg/ml. Renaturation conditions and analysis of aliquots on hydroxylapatite columns were performed as described under Experimental Procedures. $C_0t_{1/2}$ values were: *B. subtilis*, 1.6; unlabeled chick liver, 350.

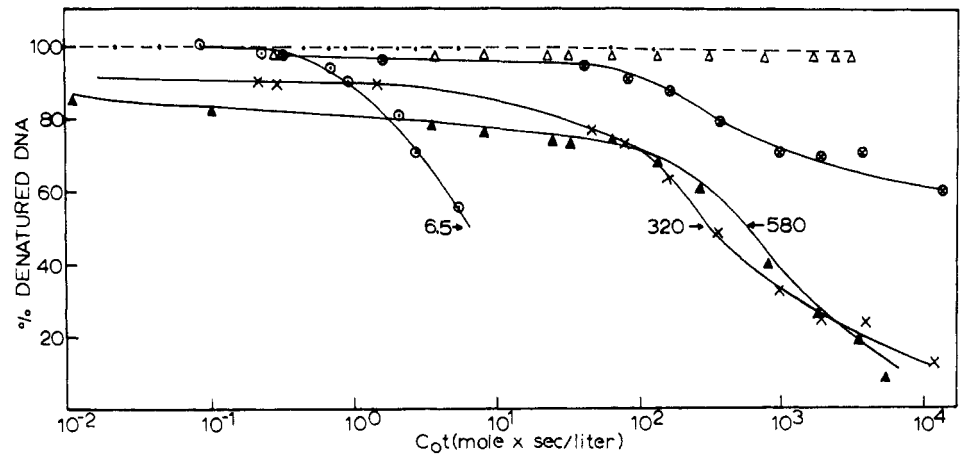

FIGURE 5: Renaturation of tritiated $NaBH_4$ labeled unique sequence DNA with *E. coli*, rat liver, and chick oviduct DNAs. Tritiated $NaBH_4$ labeled unique chick liver DNA (130,600 cpm/μg) at a concentration of 7.5 μg/ml was allowed to renature in separate experiments with: (1) *E. coli* DNA (226 μg/ml) (⊙) A_{260}, (·–·) cpm; (2) rat liver DNA (3.12 mg/ml) (▲) A_{260}, (△) cpm; or (3) chick oviduct DNA (3.76 mg/ml) (×) A_{260}, (⊗) cpm. See Experimental Procedures for additional details. $C_0t_{1/2}$ values are designated by arrows.

a 10^3-fold excess of chick liver DNA is allowed to renature with a small amount of ^{3}H-labeled embryo DNA, the renaturation rates as determined by either radioactivity or A_{260} are identical. Thus, both estrogen-induced differentiation in the oviduct and tissue differentiation during embryogenesis do not appear to result from marked gene deletion or amplification, but instead must be the result of differential gene transcription.

Isolation and Labeling of Unique Sequences. In order to achieve a physical separation of repeated and unique sequences it is critical to shear DNA to fragments of a maximum 400 nucleotides in length (Britten, 1969; Grouse *et al.*, 1972). This is necessitated by the interspersion of unique and repeated sequences throughout the eukaryotic genome (Britten and Kohne, 1968). Chick DNA in 0.1 × SSC, when sheared at 16,000 psi in a French pressure cell, is fragmented into pieces of between 300 and 400 nucleotides in length (unpublished observations; Laird, 1971). This permits adequate separation of unique and repeated sequences (Figure 4). No renaturation of recycled unique sequences (isolated following prior removal of the very rapidly reassociating sequences as described) occurs prior to a C_0t of 50, as would be expected if contaminating repeated sequences had been removed. The renaturation profiles of ^{3}H-labeled unique chick fibroblast DNA renatured with an 800-fold excess of chick liver DNA are also shown in Figure 4. In this case there is a small amount (5–10%) of repeating sequences contaminating the unique fraction. This may be related to the earlier C_0t value at which these unique sequences were fractionated, *i.e.* 200 *vs.* 400–420, or the fact that they were only passed through hydroxylapatite columns once (Grouse *et al.*, 1972). However, at the DNA C_0t values reached in saturation hybridization experiments with labeled unique sequence DNA, *i.e.* <15, little DNA–DNA reassociation would be expected. The renaturation kinetics of the ^{3}H-labeled unique chick fibroblast DNA also indicate the existence of complete sequence homology with the unique sequences in chick liver DNA. At a C_0t value of 6.4 for the ^{3}H-labeled fibroblast unique DNA almost 90% of the radioactivity is eluted as duplex DNA with the excess chick liver DNA (C_0t value = 5000). Similar results have been obtained with labeled unique mouse L cell DNA (Hahn and Laird, 1971; Grouse *et al.*, 1972).

A method for chemically labeling isolated fractions of DNA to high specific activity, that yields DNA of unaltered physical properties, would be especially useful for the preparation of labeled unique sequence DNA for saturation hybridization

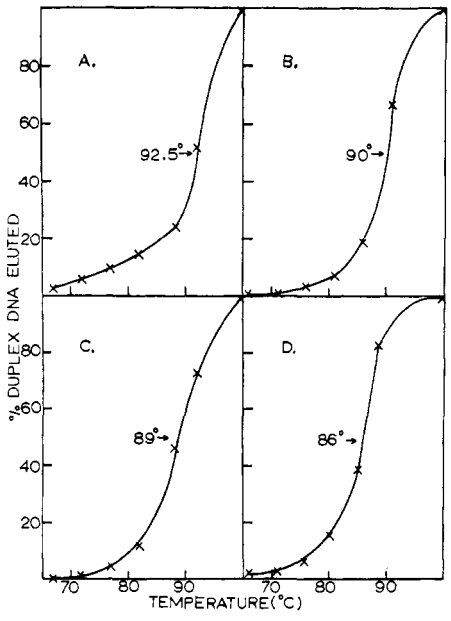

FIGURE 6: T_m determinations on hydroxylapatite. See Experimental Procedures for methods used: (A) native, sheared chick DNA; (B) ^{3}H-labeled chick embryo–unlabeled chick liver DNA duplex, C_0t = 7000; (C) ^{3}H-labeled unique chick fibroblast–unlabeled chick liver DNA duplex, C_0t = 6150; (D) tritiated $NaBH_4$ labeled unique chick liver–unlabeled chick liver DNA duplex, C_0t = 3750. All renaturations were in 0.14 M phosphate at 62°. T_m values are designated by the arrows.

experiments. Chemical labeling by methylation with [^{3}H]-dimethyl sulfate, while useful for the introduction of radioactivity into RNA (Smith *et al.*, 1967), is not satisfactory for the preparation of labeled DNA. The extremely labile glycosyl linkage in 7-methyldeoxyguanosine results in liberation of 7-methylguanine upon heat denaturation of methylated DNA (Pochon and Michelson, 1967). Introduction of tritium into nucleic acids with tritiated $NaBH_4$ appears to be a likely alternative (Cerutti *et al.*, 1965). Hybridization experiments with tritiated $NaBH_4$ labeled RNA have recently been reported (Vincent *et al.*, 1969; De Jiménez *et al.*, 1971). Unique chick DNA was isolated and labeled with tritiated $NaBH_4$ as described to yield [^{3}H]DNA of 130,600 cpm/μg specific activity. The renaturation profile of the tritiated $NaBH_4$ labeled unique chick DNA with a 500-fold excess of either unlabeled chick oviduct DNA or unlabeled rat liver DNA or a 30-fold excess of unlabeled *E. coli* DNA is shown in Figure 5. No sequence homology is evidenced between the tritiated $NaBH_4$ labeled unique chick DNA and either *E. coli* or rat liver unique sequences as would be expected (Britten and Kohne, 1966). However, quite unexpectedly only 40% of the radioactivity in the tritiated $NaBH_4$ labeled unique chick DNA renatures to form a duplex with chick oviduct unique sequence DNA. When tritiated $NaBH_4$ labeled unique chick DNA is allowed to renature with itself at 4 mg/ml in 0.14 M phosphate at 62° complete renaturation as measured by optical density is attained, but again only 40% of the radioactivity is present in the double-stranded fraction. The apparent $C_0t_{1/2}$ of this optical density profile is 900 (data not shown). Furthermore, no free radioactivity is present as a contamination of the DNA preparation, as demonstrated by hydroxylapatite chromatography and sucrose density gradient centrifugation of the labeled DNA purified to constant specific activity (data not shown).

Melting Profiles on Hydroxylapatite. At present one of the best ways to determine the fidelity of base pairing of a DNA–DNA duplex or a DNA–RNA hybrid is by its melting profile and T_m. Using the convention of Melli and Bishop (1969), a T_m 1° below that of native DNA represents approximately 1.5% mismatching of base sequences. The T_m of native, sheared chick DNA in 0.14 M phosphate as determined on hydroxylapatite columns is 92° (Figure 6A). It was not significantly altered by prior incubation at 62° in 0.14 M phosphate for 6 days (Liarakos *et al.*, 1973). The duplexes formed between ^{3}H-labeled embryo DNA and ^{3}H-labeled unique fibroblast DNA with total chick DNA have T_m values only 2.5 and 3°, respectively, below that of native chick DNA (Figures 6B and C). This indicated that there was little mismatching, only 4–5%, under these stringent incubation conditions. Since G + C content is known to influence the T_m (Mandel and Marmur, 1968) this calculation is based on the assumption that the G + C contents of unique sequence and embryo DNA are similar to that of total chick DNA. It should also be noted that the T_m of the duplex formed between tritiated $NaBH_4$ labeled unique chick DNA and total chick DNA (Figure 6D) is only 6.5° below that of native chick DNA, indicating a 90% fidelity of base pairing. Therefore, although only 40% of this labeled DNA renatured in 0.14 M phosphate at 62°, the duplexes formed under these incubation conditions contained little mismatching. It is possible that under less stringent conditions a greater percentage of the labeled DNA would have renatured.

Discussion

The data presented here establish that approximately 70% of the chick genome is comprised of slowly renaturing or unique sequence DNA. It should be mentioned that the percentage of a given genome that is designated as unique sequence DNA is operationally defined by the conditions of ionic strength and temperature (McCarthy and Duerksen, 1970) and DNA fragment size (Grouse *et al.*, 1972). Furthermore, unique sequence DNA may contain more than a single copy of a given gene sequence (Southern, 1971; Sutton and McCallum, 1971). We have utilized fairly stringent incubation conditions,, *i.e.* 0.14 M phosphate (0.21 M Na^+) at 62°, in order to minimize extensive mismatching in our renaturation studies (Figure 6). Under these conditions, it is clear that approximately 30% of the chick DNA renatures more rapidly than would be expected of entirely single copy DNA of a diploid genome size of 1.3×10^{12} (Mirsky and Ris, 1951). However, the $C_0t_{1/2}$ value of isolated chick unique sequence DNA was 460 (Figure 4). Since the average $C_0t_{1/2}$ of tritiated *B. subtilis* DNA is 1.5, a unit genome of chick DNA reassociates 307 times slower. This results in an experimentally determined mol wt of 1.22×10^{12} of a diploid chick genome in close agreement with the reported value.[1] In these experiments (Figure 1) a *B. subtilis* renaturation profile was included as a comparison to illustrate the renaturation of a bacterial DNA, composed of primarily unique sequences. The renaturation kinetics and $C_0t_{1/2}$ of this DNA are in agreement with the reported value (Laird, 1971; Britten and Kohne, 1968).

The identical renaturation profiles of DNA, extracted from oviducts at various stages of growth and differentiation (Figure 2), suggest that estrogen-induced changes are not a result of major gene amplification or deletion. Once again it should be emphasized that minor changes ($< \pm 5\%$) in gene

[1] $307 \times 2 \times 10^9$ (mol wt of *Bacillus subtilis* genome, Laird, 1971) $\times$ 2 (diploid) = 1.22×10^{12}.

amplification, which could conceivably have functional significance, might not be detectable under conditions of renaturation of total DNA. The use of an internal control in these experiments does, however, ensure that any variations in the renaturation kinetics of the different DNAs would result from sequence differences and not experimental variables, such as ionic strength and temperature.

We have demonstrated the ability to fractionate the chick genome into unique and repeated sequences and have characterized the renaturation of these highly labeled purified unique sequences (Figure 4). ^{3}H-Labeled unique chick fibroblast DNA of high specific activity is homologous to and forms stable duplexes with total chick liver DNA. It is, therefore, suitable for use in saturation hybridization experiments with excess RNA extracted from the chick oviduct at various stages of differentiation (Liarakos *et al.*, 1973).

One disadvantage of extracting labeled nucleic acids from cells in culture is the high cost and correspondingly low yields of material obtained. Chemical labeling of purified fractions of nucleic acids, *e.g.*, a specific mRNA or unique sequence DNA, is desirable, if the labeled product has unaltered biological and physical properties. We were able to adapt the method of Kirkegaard (1969) to label unique sequence DNA with tritiated $NaBH_4$ to high specific activity. This [^{3}H]DNA exhibited renaturation kinetics characteristic of unique sequence chick DNA. It showed no homology with either rat liver or *E. coli* DNA, as would be expected by the evolutionary divergence of the sequences in these DNAs (Britten and Kohne, 1967). Finally, it formed stable duplexes, with little mismatching, with total chick DNA (Figure 6) and stable unique DNA–RNA hybrids (Liarakos *et al.*, 1973). However, quite unexpectedly only 40% of the radioactivity in this DNA renatured to form duplex DNA. A calculation of the number of bases substituted, assuming a 60% A + T content for unique chick DNA and one ^{3}H substitution per ring, gives a maximum of 1.01% of the thymine moieties that may be altered. This does not appear to be sufficient to prevent duplex formation unless specific T-rich segments are present in the DNA. Such segments have been identified in mouse satellite DNA (Southern, 1970). The formation of ultraviolet-catalyzed thymine dimers or ring cleavage could also lead to the inability to form hydrogen-bonded duplexes. However, this seems improbable since the renaturation profile, as monitored by the optical density of the hydroxylapatite fractions, showed 90% duplex formation at the expected C_0t value. It may be possible that some tritium radioactivity was tightly bound to the unique DNA, but not incorporated into the thymine ring. However, this radioactivity must then have remained bound during heat denaturation, hydroxylapatite chromatography, and sucrose gradient centrifugation, which appears unlikely. It is also not clear whether the 60% of the labeled DNA, which did not form duplexes, represents a random population of gene sequences, or whether specific gene sequences have been rendered unrenaturable. This question must be answered before this chemically labeled DNA can be used for comparative DNA–RNA hybridization experiments.

While our experiments were in progress, Lee and Gordon (1971) reported that tritiated $NaBH_4$ labeled *B. subtilis* DNA had a similar renaturation profile to that of *in vivo* [^{3}H]-thymidine-labeled *B. subtilis* DNA. However, their bacterial DNA had a 10^3-fold lower specific activity than the tritiated $NaBH_4$ labeled unique chick DNA used in our experiments. In one experiment they prepared ^{3}H-labeled salmon sperm DNA of 8700 cpm/μg, which had an unaltered T_m and hyperchromicity. However, no renaturation profiles were determined for this material. McConaughy and McCarthy (1972) have also utilized the technique of Lee and Gordon (1971) to chemically label DNA isolated from chick erythrocyte chromatin fractions. Their DNA (3000 cpm/μg) hybridized to the same extent as *in vivo* labeled DNA with chick erythrocyte RNA. These differences may merely reflect a higher specific activity and an increased thermolability of the labeled chick DNA, compared with the DNA preparations used by these workers. In this regard, Lee and Gordon (1971) did report lowered thermal stabilities of their chemically labeled DNA at temperatures greater than 60°, but stated that photoreduced DNA was useful for hybridization studies run at 60°. However, their studies involved rather short incubation times and rapidly reassociating bacterial DNA. It is quite probable that the longer reaction times necessary for the reassociation of the more complex chick DNA may have led to an increased thermal lability even at 60°. Accordingly, the radioactivity present in the $NaBH_4$-labeled unique chick DNA reassociated to a greater extent when a lower temperature incubation was carried out at 37° in the presence of formamide (data not shown). The previous studies stress the necessity of thoroughly characterizing chemically labeled nucleic acids to determine if they have unchanged physical properties prior to their use in hybridization or renaturation experiments.

In conclusion, we have analyzed the genetic complexity of chick DNA and studied the effect of estrogen on the renaturation kinetics of oviduct DNA. Furthermore, highly labeled unique chick DNA has been isolated and characterized for use in saturation hybridization experiments. This investigation is a necessary prerequisite for the study of unique sequence DNA transcription during hormone-mediated growth and differentiation and the analysis of various RNA populations by their kinetics of hybridization with chick DNA.

Acknowledgments

The authors wish to thank Mrs. Maryna Barnard for her excellent technical assistance and Dr. Brian McCarthy for his helpful advice.

References

Britten, R. J. (1969), *in* Symposium on RNA in Development, Hanly, E. W., Ed., Salt Lake City, Utah, University of Utah Press, p 187.

Britten, R. J., and Kohne, D. E. (1966), *Carnegie Inst. Washington Yearb. 65*, 98.

Britten, R. J., and Kohne, D. E. (1967), *Carnegie Inst. Washington Yearb 66*, 73.

Britten, R. J., and Kohne, D. E. (1968), *Science 161*, 529.

Burton, K. (1968), *Methods Enzymol. 12B*, 163.

Cerutti, P., Ikeda, K., and Witkop, B. (1965), *J. Amer. Chem. Soc. 87*, 2505.

De Jiménez, E. S., Dominguez, J. L., Webb, J. H., and Bock, R. M. (1971), *J. Mol. Biol. 61*, 59.

Flamm, W. G., McCallum, M., and Walker, P. M. B. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1729.

Gall, J. (1969), *Genetics 61*, Suppl. 121.

Gelderman, A. H., Rake, A. V., and Britten, R. J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 172.

Grouse, L., Chilton, M. D., and McCarthy, B. J. (1972), *Biochemistry 11*, 798.

Hahn, W., and Laird, C. D. (1971), *Science 173*, 158.

Kirkegaard, L. (1969), Ph.D. Thesis, University of Wisconsin.

Kohler, P. O., Grimley, P., and O'Malley, B. W. (1968), *Science 160*, 86.
Kohne, D. E., and Britten, R. J. (1972), *Proc. Nucleic Acid Res. 2*, 500.
Laird, C. D. (1971), *Chromosoma 32*, 378.
Lee, V. F., and Gordon, M. P. (1971), *Biochim. Biophys. Acta 238*, 174.
Liarakos, C. D., Rosen, J. M., and O'Malley, B. W. (1973), *Biochemistry 12*, 2809.
Mandel, M., and Marmur, J., (1968), *Methods Enzymol. 12B*, 195.
Marmur, J. (1961), *J. Mol. Biol. 3*, 208.
McCarthy, B. J., and Church, R. B. (1970), *Annu. Rev. Biochem. 39*, 131.
McCarthy, B. J., and Duerkson, J. D. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 621.
McConaughy, B. L., and McCarthy, B. J. (1972), *Biochemistry 11*, 998.
Melli, M., and Bishop, J. O. (1969), *J. Mol. Biol. 40*, 117.
Melli, M., Whitfield, C., Rao, K. V., Richardson, M., and Bishop, J. O. (1971), *Nature (London), New Biol. 231*, 8.
Mirsky, A. E., and Ris, H. (1951), *J. Gen. Physiol. 34*, 451.
Oka, T., and Schimke, R. T. (1969), *J. Cell Biol. 41*, 816.
O'Malley, B. W., and McGuire, W. L. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 1527.
O'Malley, B. W., McGuire, W. L., Kohler, P. O., and Korenman, S. G. (1969), *Recent Progr. Hormone Res. 25*, 105.
Pochon, F., and Michelson, M. (1967), *Biochim. Biophys. Acta 149*, 99.
Smith, K. D., Armstrong, J. L., and McCarthy, B. J. (1967), *Biochim. Biophys. Acta 142*, 323.
Sober, H. A., Ed. (1968), Handbook of Biochemistry, Selected Data for Molecular Biology, Cleveland, Ohio, Chemical Rubber Publishing Co., I 17.
Southern, E. M. (1970), *Nature (London) 227*, 794.
Southern, E. M. (1971), *Nature (London), New Biol. 232*, 82.
Sutton, W. D., and McCallum, M. (1971), *Nature (London), New Biol. 232*, 83.
Vincent, W. S., Halverson, H. O., Chen, H-R., and Shin, D. (1969), *Expt. Cell Res. 57*, 240.
Waring, M., and Britten, R. J. (1966), *Science 154*, 791.
Willie, J. J. (1972), *Biochem. Biophys. Res. Commun. 46*, 692.

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†

Charles D. Liarakos, Jeffrey M. Rosen, and Bert W. O'Malley*

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.