Effect of Estrogen on Gene Expression in the Chick Oviduct

EFFECT OF ESTROGEN ON THE SEQUENCE AND POPULATION COMPLEXITY OF CHICK OVIDUCT POLY(A)-CONTAINING RNA*

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Total cellular RNA preparations were isolated from chicken oviducts at three different development stages: (a) immature chicks which were chronically stimulated with estrogen; (b) estrogen-stimulated chicks which were then withdrawn from hormone for 12 days; and (c) laying hens. Total cellular RNA containing 3'-poly(A) sequences (poly(A)-RNA) were then isolated from these preparations using oligo(dT)-cellulose chromatography. The number average nucleotide length of the poly(A)-RNA preparations in each case was ~2000 nucleotides. The number average nucleotide length of the poly(A) residues at the 3'-terminal end of each RNA preparation was approximately 70 adenylate residues.

Complementary DNA (cDNA) copies to each preparation of poly(A)-RNA were synthesized using avian myeloblastosis virus RNA-directed DNA polymerase. These cDNApoly(A) preparations were then utilized in DNA excess hybridization experiments to analyze the complexity of the DNA sequences from which these RNAs were transcribed. Approximately 22% of each of the total cellular poly(A)-RNAs were transcribed from repeated DNA sequences (average repeat frequency of 35 copies/genome) while the remaining majority were transcribed from single copy or unique sequence DNA.

It was possible to estimate the number of different poly(A)-RNA sequences per cell by analyzing the kinetics of hybridization of these cDNApoly(A) preparations to total cellular poly(A) RNA extracts under conditions of RNA excess. The results revealed that 41% of the poly(A)-RNA from laying hen oviduct consisted of, on the average, three different sequences/cell, each of which was present in approximately 25,000 copies/cell. The remainder of the poly(A)-RNA in this tissue consisted of approximately 25,000 different sequences/cell, which were present largely in only two or three copies/cell. A somewhat similar sequence complexity was found for oviduct cells prepared from estrogen-stimulated chicks. We estimated that there were approximately 20,000 different poly(A)-RNA sequences/cell, each represented in only one to two copies/cell. However, there were five sequences which were present, on the average, in a concentration of 5600 copies/cell. The poly(A)-RNAs from hormone-withdrawn tissue, on the other hand, had a lower sequence complexity. There were only approximately 10,000 different poly(A)-RNA sequences/cell, each present in about three copies/cell. Furthermore, the few sequences present in a great abundance in hen and hormone-stimulated tissues were apparently absent in oviduct tissue from hormone-withdrawn chicks, suggesting that the intracellular concentrations of these high frequency RNA sequences are dependent on estrogen.

Numerous studies from this and other laboratories have shown that estrogen induces increased gene transcription during hormonal stimulation of growth and differentiation in the chick oviduct (1–11). Daily injection of diethylstilbestrol to immature chicks leads to a dramatic growth in oviduct tissue, resulting in the formation of three differentiated cell types. The predominant cell type is the tubular gland cell, which actively produces the major egg white proteins. If chicks are subsequently withdrawn from estrogen after 14 days of daily administration, the tubular gland cells undergo regression and no longer secrete egg white proteins (6). We have recently monitored changes in the amount of ovalbumin mRNA in immature chick oviduct tissues during primary stimulation with diethylstilbestrol, after hormone withdrawal, and again following a secondary stimulation with diethylstilbestrol (6). The initial stimulation of the immature tissue resulted in a corresponding increase in the concentration of ovalbumin mRNA in tubular gland cells. Withdrawal of estrogen from the chick was followed by a dramatic decrease in the level of cellular ovalbumin mRNAs to almost undetectable levels after

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12 days. Finally, restimulation produced a rapid reinduction of ovalbumin mRNA, which increased significantly within 30 min after hormone administration. We felt that it would next be of interest to examine also the total cellular mRNA population and define its response to estrogenic stimulation relative to ovalbumin mRNA.

We have chosen initially to study changes in the sequence and population complexities of the oviduct total cellular poly(A)-RNAs in the hope that we could correlate them with the changes in chromatin template activity and the number of chromatin-initiation sites for RNA polymerase which have been observed in this system (12, 13). Total cell poly(A)-RNA was obtained from hen, diethylstilbestrol-stimulated and hormone-withdrawn chick oviduct tissues. Complementary DNA 

EXPERIMENTAL PROCEDURE

Materials

Ten-day-old white Leghorn chicks and mature laying hens were obtained from Rich-G10 Farm, La Grange, Texas. Chicks received daily subcutaneous injections of the synthetic estrogen diethylstilbestrol (2.5 mg/kg) for 14 days and the chicks were killed 12 days later. Oviducts in all cases were isolated from estrogen-withdrawn chick oviduct tissues. Complementary DNA 

Preparations of Total Cellular Poly(A)-RNA—Total cellular RNA was prepared from chick and hen oviduct tissue by a modification of previously described procedures (21, 22). Throughout the isolation procedure described below, all glassware was prewashed in 1 N NaOH and extensively rinsed with deionized water. All stock reagents, except those containing phenol, were filtered through Millipore filters (0.45 μm) and all oviducts were crushed with a hammer and immediately homogenized in a Waring Blender with 5 volumes of 0.01 M NaOAc buffer (pH 5.0) containing 0.5% sodium saccharide and 5 volumes of phenol m-cresol mixture at room temperature (21). Homogenates were extracted for 5 min at 37°, rapidly cooled to 0°, and then centrifuged at 12,000 rpm for 10 min. The aqueous and interphase were removed and re-extracted as described above. The RNA was precipitated from the final aqueous phase with 2 volumes of ethanol, 0.1 volume of 2 M NaOAc (pH 5.5), and maintained at -20° overnight. The pellet was dissolved in 0.01 M Tris-Cl (pH 8.0)/0.5% sodium dodecyl sulfate/20 μg/ml EDTA/50 μg/ml RNase A and incubated at 37° for 30 min. The sample was then again treated with phenol, the aqueous phase removed, and the RNA precipitated as described above.

The precipitate was dissolved in 0.5 M KCl, 0.01 M Tris-Cl (pH 7.6), 2 mM EDTA, and then applied to an oligo(dT)-cellulose column. Affinity chromatography using (dT)-cellulose was performed at room temperature as described previously (23). The RNA bound to the oligo(dT)-cellulose was eluted with 0.01 M Tris-Cl (pH 7.6)/2 mM EDTA, and then precipitated with ethanol. The RNA was resuspended in 0.01 M Tris-Cl (pH 7.6) to a concentration of not less than 8 mg/ml. Poly(A) Distribution of Chick DNA—Total chick liver DNA was prepared by a modification of the procedure of Marmar (24) as described previously (23). The DNA preparations used were essentially free of RNA and protein. The final DNA solution was adjusted to approximately 1 mg/ml in 0.01 M Tris (pH 7.6) containing 0.2 M NaCl/2 mM EDTA, and sheared twice in a French press to yield DNA fragments about 400 base pairs in length. Protease K was then added to 10 μg/ml and sodium dodecyl sulfate to 0.5% and the solution was incubated for 1 hour at 37° to remove any possible trace contamination of nuclease. The solution was then vigorously shaken for 5 min with an equal volume of the same phenol-sodium dodecyl sulfate (pH 5.0) buffer which was used for the isolation of poly(A)-RNA and rinsed in boiling water. The mixture was then centrifuged, and the aqueous phase removed and re-extracted with chloroform (24) twice. The DNA in the aqueous phase was then precipitated with ethanol, redissolved to about 8 mg/ml in 0.1 M EDTA, heat-denatured by heating to 100° for 10 min, and stored as a stock solution. \( \text{RNA ladder DNA} \) was prepared essentially as described above. Short \( E. \text{coli} \) DNA fragments (3.8 |

Centrifugation of Poly(A)-RNA on Formamide Gradients—The total poly(A)-RNA fractions obtained from the (dT)-cellulose columns were analyzed on 0 to 30% (w/v) linear sucrose gradients containing 70% formamide/5 mM Tris-HCl (pH 7.4)/3 mM EDTA, as described by Suzuki et al. (25). RNA samples were incubated at 37° in the above buffer (containing no sucrose) before loading on the gradient. Centrifugation was for 48 hours at 38,000 rpm in a Beckman SW 40 rotor at 20°.

Centrifugation of cDNA in Alkaline Sucrose Gradients—Labeled \( \text{[H]} \) cDNA samples in 0.1 ml of 0.1 M NaOH, 0.9 M NaCl, and 5 mM EDTA were layered on 8 to 18% linear sucrose gradients in the same solution. The gradient was centrifuged for 24 hours at 38,000 rpm, 20°, in a Beckman SW 40 rotor. Aliquots of fractions collected from the gradient were neutralized and counted in Aquasol. A parallel gradient containing sheared \( E. \text{coli} \) DNA, 3.8 \( \text{sex}^{{11}} \) and 5.08 \( \text{sex}^{{11}} \), as well as the open (16.1 \( \text{sex}^{{11}} \)) and closed circular form (18.4 \( \text{sex}^{{11}} \) of \( \Phi \) X174 DNA were used as markers. The \( \text{sex}^{{11}} \) values of the \( E. \text{coli} \) DNA were obtained by analytical sedimentation measurements in a Spinoce model E Ultracentrifuge using the alkaline buffer described above without sucrose. The \( \text{sex}^{{11}} \) values of the open and closed forms of \( \Phi \) X174 DNA in alkaline have been previously reported by Studier (26).

Synthesis of \( \text{[H]} \) Poly(dT)—To obtain an accurate estimate of the amount of poly(A)-RNA in the nucleic acid material bound to the oligo(dT)-cellulose column and to determine the molecular weight of some of poly(A)-RNA, the sample of poly(A)-RNA obtained from different groups of oviducts was subjected to paper chromatography. It was necessary to prepare \( \text{[H]} \) Poly(dT) of high specific activity (>10 cpm/μg). The high specific activity allows convenient analysis of small amounts of poly(A).

\( \text{[H]} \) Poly(dT) was made using AMV reverse transcriptase (described below) to transcribe poly(A) using an oligo(dT) primer. Two milligrams of \( \text{[H]} \) dTTP (adjusted to 50 Ci/mmol or 5 Ci/mmol) were divided into small fractions with a hammer and immediately homogenized in a Waring Blender with 5 volumes of 0.01 M NaOAc buffer (pH 5.0) containing 0.5% sodium saccharide and 5 volumes of phenol m-cresol mixture at room temperature (21). Homogenates were extracted for 5 min at 37°, rapidly cooled to 0°, and then centrifuged at 12,000 rpm for 10 min. The aqueous and interphase were removed and re-extracted as described above. The RNA was precipitated from the final aqueous phase with 2 volumes of ethanol, 0.1 volume of 2 M NaOAc (pH 5.5), and maintained at -20° overnight. The pellet was dissolved in 0.01 M Tris-Cl (pH 8.0)/0.5% sodium dodecyl sulfate/20 μg/ml EDTA/50 μg/ml RNase A and incubated at 37° for 30 min. The sample was then again treated with phenol, the aqueous phase removed, and the RNA precipitated as described above.

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1\text{sex}^{{11}} \) were prepared by shearing twice in a French press as described above except 0.5% sodium dodecyl sulfate was also present in the shearing buffer.
A sample was placed on a Sephadex G-50 column equilibrated with 5 mM ethanol. Stock solutions of \(^{3}H\)poly(dT) were synthesized having specific activities of about 1 x 10^6 and 1 x 10^7 cpm/µg.

Quantitation of Poly(A) in (dT)-Cellulose-bound RNA — The amount of poly(A) present in the nucleic acid bound to oligo(dT)-cellulose in high salt was estimated by hybridization with \(^{3}H\)poly(dT). The \(^{3}H\)poly(dT) was hybridized to poly(A) standards and various dilutions of oligo(dT)-cellulose-bound RNA. After digestion with S1 nuclease, the amount of poly(A) hybridized to \(^{3}H\)poly(dT) could be determined as described by Kaufman and Gross (27).

Estimation of Number Average Molecular Weight of Poly(A)-RNA — The number average molecular weight distribution of each total cellular poly(A)-RNA preparation was determined by formamide gradient centrifugation as described above. A 10-µl aliquot of each gradient fraction in a polypropylene test tube was added to 10 µl of a buffer containing 3 mM NaCl, 0.06 M Tris-HCl (pH 7.0), 0.006 M EDTA, 20 µl of H₂O, and 10 µl of \(^{3}H\)poly(dT) containing 50,000 cpn with a specific activity of 8 x 10⁶ cpm/µg. The solution containing 0.4 µg NaOAc (pH 4.5), 0.8 µg NaCl, 5 mM ZnOAc, and 0.15 µl of S1 nuclease (6) were added to each tube. The vials were incubated for 2 hours at 37°C. Hybrids resistant to S1 nuclease were precipitated with cold 20% trichloroacetic acid, collected on Millipore filters, and dried. The dried filters were then dissolved in 3 ml of Cellulase (exynhydrol gumonunamethyl ether) by shaking for 2 hours at room temperature and counted in 0 ml of Aquasol. The distribution of hybridizable \(^{3}H\)poly(dT) across the formamide gradient gives the molecular weight distribution of the total cellular poly(A)-RNA. From a cumulative plot of the fraction of poly(A)-RNA molecules against their length calculated as described previously by Sarrin (28), it was, therefore, possible to estimate the number average molecular weight of the poly(A)-RNA. No attempt was made to correct for any slight changes in the size distribution of the poly(A)-RNA present in the poly(A)-containing RNA across the gradients.

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- \(d\) = \(\frac{\sum_{i=1}^{n} P_i [1 - \exp((-0.69R_{i,t}/R_{i,t})^{a+1})]}{D_0}\) (1)
- \(d = \frac{\sum_{i=1}^{n} P_i [1 - (1 + (Ct/Ct_{i,t}))^{-a+1}]^{a+1}}{D_0}\) (2)
evaluated. Each parameter was then increased or decreased by an incremental amount and the mean sum of squares for all these adjacent models evaluated. The best new value became the new starting model and the process was continued with a further increment in the same direction as the previous one until there was no significant improvement in the degree of curve fitting. The program then investigated all neighboring increments looking for a new search vector. When no improvement was possible, the final \( C^2 \) or \( R^2 \) values and \( P \) values were printed out and the curve was drawn by computer. For each set of hybridization data, the F-test of equality of variance of a 1, 2, or 3 component hybridization curve (i.e. for \( n = 1, 2, \) or 3) was compared with an \( (n - 1) \) component curve. All curves shown gave best fits of \( n \) within 99% confidence levels over a value of \( (n - 1) \).

RESULTS

Characterization of RNA—Total cellular poly(A)-RNA was isolated from oviduct tissues by affinity chromatography using an oligo(dT)-cellulose column (23, 34). Most of the cellular DNA, rRNA, and tRNA failed to bind to the column. However, because a small amount of contaminating RNA, primarily ribosomal RNA, bound to the column, it was first necessary to quantitate the amount of poly(A)-containing RNA in each preparation of (dT)-cellulose-bound material. In order to make this estimate, it was necessary to determine the percentage of poly(A) in each preparation, the number average length of these poly(A) sequences, and the number average length of the total poly(A)-RNA.

The percentage of poly(A) in total poly(A)-RNA of oviducts of hen and estrogen-stimulated and withdrawn chicks was determined by hybridizing 1 ng of RNA with an excess of \( [\text{3H}]\text{poly(dT)} \) as described under “Methods.” As shown in Fig. 1 and Table I, the preparations contained between 0.52% and 1.6% of the total poly(A)-RNA as poly(A) sequences. The reason for the larger percentage of rRNA (and thus, lower percentage total A sequences) in poly(A)-RNA from estrogen-stimulated chick oviduct tissue is unclear.

The number average length of the poly(A) sequences present in each poly(A)-RNA preparation was obtained by labeling the 3'-terminal end of each RNA with \( [\text{3H}]\text{NaBH}_4 \), hydrolyzing the non-poly(A) sequences with RNase, and analyzing the remaining \( [\text{3H}] \)-labeled poly(A)s by polyacrylamide gel electrophoresis. The profile of \( [\text{3H}] \)-labeled poly(A), \( (\text{A}_{25}) \), and \( (\text{A}_{45}) \) standards run on the electrophoretic system is shown in Fig. 2A. As shown in Fig. 2B, there was a linear relationship between the log of the length of the poly(A) chain versus the distance migrated in the gel. The polyacrylamide gel profiles of the poly(A) sequences from the three tissues are shown in Fig. 3. The calculated number average sequence lengths of poly(A) sequence in total poly(A)-RNA obtained from hormone-withdrawn, estrogen-stimulated chicks and hen were found to be similar, approximately 70 nucleotides (Table I).

The number average polynucleotide chain length of each poly(A)-RNA preparation was determined by tannamide gradient centrifugation followed by hybridization of each gradient fraction with \( [\text{3H}]\text{poly(dT)} \) as described under “Methods.” Fig. 4 contains sedimentation profiles of oligo(dT)-cellulose poly(A)-RNAs obtained from oviduct tissues of hen (Fig. 4A), estrogen-stimulated chick (14 days diethylstilbestrol (Fig. 4B), and chicks withdrawn from all hormone for 12 days (Fig. 4C). From each \( [\text{3H}]\text{poly(dT)} \) hybridization profile, it was clear that the poly(A)-RNA sedimented with a broad range of S values.
ranging from 5 S to 30 S with a peak in each case slightly behind the 18 S rRNA marker. It was also apparent from the absorbance profiles that there was a considerable amount of 18 S and 28 S rRNA in each poly(A)-RNA preparation, particularly in the case of estrogen-stimulated and withdrawn chick tissues. Using the $s_{20,W}$ values of 4 S, 18 S, and 28 S as standards, it was possible to draw a curve from which the nucleotide lengths of a RNA species in each fraction of the gradient could be calculated using the relationship:

$$\text{No. of nucleotides} = 4.697 \times (s_{20,W})^{1.1}$$

As described by Spirin (28). Cumulative plots of the fraction of the total poly(A)-RNA against the nucleotide chain length of the RNA from hen and estrogen-stimulated and withdrawn chicks are shown in Fig. 5 A, B, and C, respectively. The number average nucleotide chain length calculated for the poly(A)-RNA from each tissue is summarized in Table I and was between 1925 and 2000 nucleotides for all three RNA preparations.

Hybridization of Poly(A)-RNA cDNAs to Chick DNA—In order to determine if the poly(A)-RNA sequences isolated from oviduct tissue were transcribed from unique or repetitive DNA sequences, the cDNAs synthesized using poly(A)-RNAs from hen and estrogen-stimulated and hormone-withdrawn chicks were each hybridized with a vast excess of chick DNA. The hybridization data were analyzed by computer and the resulting curves are shown in Fig. 6, A, B, and C. The data were statistically best fit by two component curves. Also included in Fig. 6 is a curve (Fig. 6D) for $^{32}$P-labeled unique sequence chick DNA annealed to a vast excess of chick DNA. This DNA has been characterized elsewhere (21, 35). Using the computer program described above to fit the data to Equation 2, a $C_{ot}$ for the unique DNA value was calculated to be $\sim 725$ mol s$^{-1}$, when corrected for the fact that approximately 22% of the total cDNA consisted of repeated DNA sequences. These sequences would, on the average, be repeated $\sim 35$ times/gene. A somewhat similar amount of total polysomal mRNA sequences transcribed from repeated DNA sequences has been observed by other investigators (14-19).

Hybridization of $[^{32}P]cDNA_{poly(A)}$ to Total Poly(A)-RNA—Under conditions of a large excess of poly(A)-RNA, the rate of hybridization of cDNA$_{poly(A)}$ is inversely proportional to the base sequences complexity of the poly(A)-RNA population itself (32). The sequence complexity of an unknown RNA population can be determined by a comparison of its $R_{c}^{A}$ value with that of a kinetic standard of known sequence complexity. The kinetic standard chosen for this work was the back hybrid between pure ovalbumin mRNA and its DNA complement ($[^{32}P]cDNA_{ov}$). Ovalbumin mRNA has been purified to ho-
mogeneity and shown to contain a sequence complexity of approximately 1900 nucleotides (22). This was very close to the number average nucleotide length of our poly(A)-RNA preparations, so no correction for the effect of length of the RNA upon the rate of hybridization was required. However, there is evidence that the kinetics of hybridization between RNA and cDNA is affected by a variation in the size of cDNA (36). In order to minimize deviations from ideal reaction kinetics, all cDNApoly(A) preparations were fractionated on alkaline sucrose gradients. Only those fractions corresponding to a nucleotide size between 280 and 900 (\(\sim 5.0 \) to 8.0 \(s_{0.18}^H\)) were used in the hybridization studies described below. A second advantage in sizing the cDNA preparation before hybridization was the achievement of a final extent of hybridization reaction exceeding 90%. We have consistently observed that if the cDNApoly(A) was not fractionated as described above, the back hybridization with total poly(A)-RNA seldom reached a final extent of reaction greater than 60%. It is possible that the short cDNApoly(A) hybrids are not completely stable to S_{1} nuclease under the conditions used for assay procedures. Using a cDNApoly(A) preparation of 280 to 900 nucleotides in length, >95% hybridization was achieved and a \(R_{f_{RNA}}\) value of \(6.58 \times 10^{-3}\) mol s\(^{-1}\) was obtained (Fig. 7) with purified ovalbumin mRNA.

The data obtained in a hybridization experiment between cDNApoly(A), and hen total poly(A)-RNA are plotted in Fig. 8. Inspection of the range of the reaction (\(\sim 5\) log units) revealed that different classes existed which could be separated on the basis of sequence abundance. An ideal pseudo-first order reaction for bimolecular reaction with one of the reactants being in a large excess will have a range of reaction of only \(1/2\). We have found that the cDNApoly(A) hybridization curve containing three different RNA classes provided a statistical best fit for the data. The \(R_{f_{RNA}}\) values and the fraction of the total RNA that each component represented are summarized in Table III. The \(R_{f_{RNA}}\) values were then corrected for two factors. First, a correction was made for the fact that only 42.3% of the RNA was poly(A)-RNA (Table III, third column). Second, the \(R_{f_{RNA}}\) values of the three components were corrected for the fact that each component represented only a fraction of the total RNA (\(R_{f_{RNA}}\) x fraction of total; see Table III, fifth column.) Based on a \(R_{f_{RNA}}\) value of 0.00658 mol s\(^{-1}\) for the back hybridization of ovalbumin mRNA to cDNApoly(A), the numbers of different 1900 long nucleotide sequences in the three different abundance classes were approximately 3, 90, and 24,500.

It was also possible to calculate, based upon our previous estimates, that the hen oviduct contains 12.7 pg of RNA/cell (6), and that 3.7% of the total cellular RNA was bound to the (dT)-cellulose column (and, of this, 42.3% was poly(A)-RNA). Thus, there were \(\sim 0.2\) pg of poly(A)-RNA/cell. Using this value, we calculated that there were, on an average, 25,400 molecules/cell of length 1900 nucleotides for each of the three different sequences in the first component. In the same way, the second and third hybridization components were calculated to contain 450 and 2.8 molecules of each sequence per cell, respectively. It has been previously established that the messenger RNAs for the egg white proteins are in high concentrations in the hen oviduct tubular gland cell (6). It is likely that the first hybridization component represented these messenger RNAs.

Since there are approximately \(8.69 \times 10^8\) of unique sequence nucleotide pairs of DNA per haploid chick genome by simple calculation, we could demonstrate that the first, second, and third poly(A)-RNA abundance classes represented an expression of \(0.00067\), \(0.019\), and \(5\)%, respectively, of the unique DNA sequences present in the hen (Table III).

Similar studies were carried out with [H]cDNApoly(A), synthesized from poly(A)-RNA prepared from chicks stimulated with diethylstilbestrol for 14 days. Again, a three component curve provided a best fit for the data and indicated \(R_{f_{RNA}}\) values of 0.80, 33.10, and 4000 mol s\(^{-1}\), respectively, for the three classes (Fig. 9). Again correcting these data for the actual percentage of poly(A)-RNA in the preparation and the fraction of the total which each component represents, the three abundance classes correspond to 5, 330, and 20,300 different nucleotide sequences, respectively. The RNA content of an estrogen-stimulated oviduct cell was calculated to be 9.23 pg/cell (6). Using similar calculations to those described above.
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The kinetics for the back hybridization of \(^{3}H\) poly(A)-RNA prepared from hormone-withdrawn chicks was in marked contrast to the hybridization of hen and estrogen-stimulated chick poly(A)-RNAs. A two component curve provided a best fit for these data and indicated \(R_{0.5}\) values of 4.546 and 300, respectively (Fig. 10). Calculations similar to those described above reveal that 121 different sequences were present in the first abundance class and 10,200 separate components in the second. Based on a RNA content of 3.9 pg of RNA/cell for hormone withdrawal (12 days) tissue (6), there were an estimated 220 molecules of each of the first abundance class sequences and 3.4 of each of the second. The first and second abundance classes of poly(A)-RNA represented 0.026 and 2.18% of the unique sequences in the chick genome, respectively (Table III).

**DISCUSSION**

There is now considerable evidence to suggest that the production of mRNA in eukaryotic cells involves a series of nuclear post-transcriptional events (19, 37-43), one of which is the post-transcriptional addition of poly(A) to the 3'-terminal of many mRNA sequences (19, 37). Although many of the details remain unclear, it is very possible that total cellular poly(A)-RNA represents much of the protein-specifying sequences of the cell (19). By carefully analyzing the kinetics of the back hybridization of total cellular poly(A)-RNA to cDNAs synthesized from the same poly(A)-RNA fraction, we hoped to gain some additional insight into the extent of differential gene expression occurring in oviduct tissue during hormone treatment. From the data discussed above, it is apparent that although there were large differences in the complexity of RNA in hen or diethylstilbestrol-stimulated chick oviduct tissues as compared to hormone-withdrawn oviducts, there was little change in the overall number average size of the total cellular poly(A)-RNA. Furthermore, little change was also found in the number average size of poly(A) sequences attached to the 3'-terminal end of each poly(A) RNA. Thus, hormone treatment did not appear to affect the overall size distribution of the poly(A)-RNA or the length of the poly(A) sequence itself.

Most mRNAs in eukaryotic cells are transcribed from nonrepeated DNA sequences. As expected, 70 to 80% of the cDNA,ow c z, / hybridized with an excess of chick DNA at a high \(C_{t}\) value which was consistent with the interpretation that the poly(A)-RNA sequences of chick are transcribed from the unique sequences of the genome. However, it was also clear from the data that approximately 20 to 25% of cDNA,,,,, hybridized with chick DNA at \(C_{t}\) values which were lower than that seen with unique sequence transcripts. Such poly(A)-RNA sequences would be represented an average \(-35\) times/genome. Recent studies indicate that in HeLa cells (17), L-cells (16), rat myoblast cells (14), and a Drosophila melanogaster cell line (15), approximately 20 to 30% of poly(A)-RNA is transcribed from repeated DNA sequences. The function of such repeated DNA sequence transcripts is unclear. Campo and Bishop (14) have provided evidence that these RNAs are transcribed in their entirety from such repeated DNA sequences. In other words, short repeated sequence transcripts are not present in a number of otherwise unique DNA transcripts. Ryffel and McCarthy (16), using density-labeled cDNA,,,, from mouse L-cells, showed that many repeated cDNA,,,, sequences hybridized preferentially to...
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TABLE II

| Tissue                           | Observed C<sub>0.6</sub> (1) | Isolated C<sub>0.6</sub> (1) | % of total cDNA<sub>poly(A)</sub> (2) |
|----------------------------------|-----------------------------|----------------------------|--------------------------------------|
| Hen                              | 89.4                        | 20.7                       | 23.2                                 |
| Diethylstilbestrol-stimulated    | 98.3                        | 23.6                       | 24.1                                 |
| Diethylstilbestrol-withdrawn     | 73.9                        | 15.0                       | 20.3                                 |
| Unique sequence chick DNA        | 724                         |                            | 79                                    |

Fig. 7. Hybridization of cDNA<sub>poly(A)</sub> with purified ovalbumin mRNA. Varying amounts of ovalbumin mRNA were incubated with 0.1 ng of cDNA<sub>poly(A)</sub> at 68° in 50 μl of 0.6 M NaCl/0.01 M Tris-HCl/1 mM Hepes (pH 7.0)/2 mM EDTA to the indicated R<sub>f</sub> values. Hybrid was assayed with S<sub>1</sub> nuclease. Hybridization curves were determined and drawn by computer.

Before discussing our interpretation of the cDNA<sub>poly(A)</sub> hybridization experiments with total cell poly(A)-RNA, it may be appropriate to consider the validity of the approach. Using conditions of vast poly(A)-RNA excess, the rate of hybridization of cDNA<sub>poly(A)</sub> to RNA is inversely proportional only to the base sequence complexity of the RNA (32). For cytoplasmic poly(A)-RNA, probably the first 280 to 900 nucleotides from the 3'-terminal end of each poly(A)-RNA were transcribed into cDNA (36). Since this was also the case for the cDNA<sub>poly(A)</sub> used as a base sequence complexity standard (36), and because the number average size of ovalbumin mRNA approximately equaled that obtained for the number average size of our poly(A)-RNA preparations, no correction for differences in lengths of the RNAs was required. However, the poly(A)-RNA complexity could be underestimated if a repetitive sequence element were present in a region close to the poly(A)-containing region of the poly(A)-RNA and thereby was transcribed into cDNA<sub>poly(A)</sub>. Such a repetitive sequence element would displace the curve to a lower R<sub>f</sub> value causing an underestimation of the base sequence complexity. Indeed, a short sequence at the poly(A) end of several different mRNAs has been recently reported to have a number of bases in common (44). However, these sequences are quite short, and would not form a stable hybrid with cDNA<sub>poly(A)</sub> under our S<sub>1</sub> assay conditions. If, on the other hand, a repetitive sequence element was located in a region toward the 5'-terminal end of the poly(A)-RNA and was, therefore, not transcribed into cDNA<sub>poly(A)</sub>, then the R<sub>f</sub> value would be shifted to a higher value leading to an overestimation of the base sequence complexity. Dina et al. (45) and Firtel and Lodish (46) have suggested the presence of such a 5'-terminal repeated sequence segment in Xenopus and Dictyostelium mRNAs, respectively. However, Campo and Bishop (14) have been unable to detect such "mixed molecules" (i.e. composed partly of repeated sequence transcripts and partly of nonrepeated transcripts) in mRNA preparations from rat myoblast cells. Instead, it appears that mRNA sequences that hybridize to repeated DNA sequences represent a distinct molecular entity containing repeated DNA sequence in their entirety.

Some mRNAs (e.g. histone mRNA) lack a poly(A) tail (47). Such species would not be transcribed into cDNAs and would not, therefore, take part in the above hybridization reactions. They will also lead to a slight overestimation of the base sequence complexity.

The heterogeneity of the RNA populations, i.e. not all of the RNA sequences being present in equal proportions, presents a second complication in interpreting hybridization with
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TABLE III

Hybridization of cDNA<sub>poly(A)</sub> to total poly(A)-RNA

| Tissue                        | Observed $R_d$<sub>0</sub> | Corrected $R_d$<sub>0</sub> | Fraction of total | $R_d$<sub>0</sub> isolated | No. of different sequences | No. of molecules of each per cell | % of unique DNA |
|-------------------------------|----------------------------|----------------------------|-------------------|-----------------------------|---------------------------|-----------------------------------|----------------|
| Hen                           | 0.120                      | 0.051                      | 0.41              | 0.021                       | 3                         | 25,400                             | 0.00067        |
| Diethylstilbestrol-stimulated | 0.805                      | 0.120                      | 0.25              | 0.030                       | 5                         | 5,800                              | 0.00110        |
| Diethylstilbestrol-withdrawn  | 4.546                      | 2.000                      | 0.40              | 0.798                       | 120                       | 220                                | 0.026          |

Fig. 9. Hybridization of 0.1 ng of diethylstilbestrol-stimulated oviduct tissue cDNA<sub>poly(A)</sub> with an excess of diethylstilbestrol-stimulated oviduct poly(A)-RNA. The hybridization was carried out at 68° in 50 μl of 0.6 M NaCl/0.01 M Tris-HCl/1 mM Hepes (pH 7.0)/2 mM EDTA to the indicated $R_d$ values. Hybrids were assayed with $S_1$ nuclease. Hybridization curves were determined and drawn by computer. $R_d$ values indicated have been corrected for the effect of salt on the rate of hybridization. The concentrations of poly(A)-RNA used were: A, 2226 μg/ml; B, 222.6 μg/ml; C, 22.26 μg/ml; D, 2.23 μg/ml.

cDNA<sub>poly(A)</sub>. Any one abundance class will react with a $R_d$<sub>0</sub> value proportional to its base sequence complexity, but the extent of hybridization is proportional to the fraction of the total RNA represented by this abundance class. For this reason, it is important to run the hybridization reaction until the least abundant component class has hybridized to completion. This class may constitute, if it is large, a major fraction of the poly(A)-RNA population. Fortunately, we have obtained greater than 90% hybridization in our hybridization reactions. Any remaining unhybridized poly(A)-RNA sequences would, by definition, have to be complementary to a large segment of the single copy DNA and would be present in the tissue at less than 1 molecule/100 cells. The excellent correlation between our kinetic data and previous DNA saturation experiments (21) suggests that we are not grossly underestimating the sequence complexity of the total poly(A)-RNA.

When comparing the number of different poly(A)-RNA sequences present in oviduct tissue with that of RNA from tissue culture cells (14-19), it should also be remembered that the oviduct tissue contains a number of distinct cell types (6, 48). The large number of different poly(A)-RNA sequences we observed in oviduct tissue may not be present in all cells of the tissue. Many of the sequences may be restricted to different cell types. Lastly, it should be remembered that not all of the oviduct mRNA and HnRNAs contain 3'-terminal poly(A)s (14, 23, 37). The base sequence complexity, therefore, of total cellular poly(A)-RNA isolated as we described clearly represents a minimum value for the base sequence complexity of total oviduct cellular mRNA and HnRNAs.

With the above limitations in mind, it is possible, however, to reach some general conclusions concerning the base sequence and abundance complexities of poly(A)-RNAs in the whole cell of oviduct tissue. In hen oviduct tissue, three distinct transitions were seen in the hybridization curve (Fig. 8). The sequence complexity of the first transition was low (three different sequences of approximately 1900 nucleotides in length), and yet, this hybridization component represented some 41% of the total hybridizing cDNA<sub>poly(A)</sub>. It is thus clear that there must be a considerable number of molecules of these...
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We calculated an average of 25,448 of each. It has been previously established that the messenger RNAs for the egg white proteins are in high concentrations in the hen oviduct tubular gland cell (6). It seemed likely that the first component represented these messenger RNAs. In fact, when hen cDNA was hybridized to purified ovalbumin mRNA, 18% of the total cDNA was scored as hybrid (49). This would represent 44% of the total poly(A)-RNA in the first component or approximately 34,000 molecules/cell.

The sequence complexity of the third component of hen poly(A)-RNA was estimated to be very large—24,500 different sequences of approximately 1900 nucleotides in length. This is, with the exception of HeLa cell mRNAs (17), 2 or 3 times larger than that reported for cytoplasmic mRNAs of several cell types (14-16, 18, 19, 50). However, the sequence complexity of nuclear poly(A)-RNA appears to be more complex than cytoplasmic poly(A)-RNA when assayed by these (18, 50-52) and other (21) procedures. Since our extracts were prepared from whole cells, the nuclear poly(A) RNAs are almost certainly contributing to the total cellular poly(A)-RNA sequence complexity.

We should also point out that it is possible that some nuclear poly(A)s may be over-represented in the cDNA population in terms of their sequence complexity if internal short poly(A) sequences were present in HnRNA (53, 54). It is conceivable that these sequences could bind oligo(dT)12-18 primer and form a template for reverse transcriptase, resulting in a number of different cDNAs being transcribed from one HnRNA instead of just one at the 3'-terminal end. Further work is required to establish the relationship between nuclear and cytoplasmic poly(A) RNAs and to determine the mechanism of transcription by reverse transcriptase.

A somewhat similar sequence complexity was found for estrogen-stimulated oviduct tissue total poly(A)-RNAs. There were five sequences which were each present on an average of 5000 copies/cell. At this time, we feel that the small difference between the first component of hen and estrogen-stimulated chicks is not significant. The most complex abundance class contained 20,300 different sequences, each of which represented an average of 1.2 copies/cell. The total percentage of the genome transcribed into these sequences was 4.49%, quite similar to that found for the hen RNA (5%). It will be of interest in further experiments to determine the extent to which the two tissues have poly(A)-RNA sequences in common.

The hybridization kinetics of cDNA, with RNA prepared from oviducts of hormone-withdrawn chicks was distinctly different from the two cases discussed above. The data were statistically plotted best as a two component hybridization curve. One component represented 121 different sequences, each present as 221 copies/cell, and a more complex class containing 10,310 different sequences present as 3.4 molecules/cell on average. It was apparent that the analytical complexity of this tissue was approximately half that of hen or estrogen-stimulated oviduct tissue. The total percentage of unique sequence DNA represented was 2.18%. The few sequences present in great abundance in hen and hormone-stimulated chicks were absent in hormone-withdrawn chicks or alternatively were present in less than a few copies per cell. It is tempting to speculate that these RNAs were mRNAs coding for the egg white proteins, since they were not produced in hormone withdrawn tissue (1, 4, 6).

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Table IV

| Tissue                | No. of different sequences in poly(A)-RNA | No. of chromatin initiation sites per haploid genome* |
|-----------------------|------------------------------------------|------------------------------------------------------|
| Hen                   | 24,600                                   | 34,000                                               |
| Diethylstilbestrol-    | 90,600                                   | 12,000                                               |
| stimulated            |                                          |                                                      |
| Diethylstilbestrol-    | 10,300                                   | 14,000                                               |
| withdrawn             |                                          |                                                      |
| Unstimulated          | 14,000                                   |                                                      |

*Schwartz et al. (13).
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