Estrogen Withdrawal in Chick Oviduct. Selective Loss of High Abundance Classes of Polyadenylated Messenger RNA†

Ronald F. Cox

ABSTRACT: It has been demonstrated both theoretically [Harris, S. E., Rosen, J. M., Means, A. R., and O'Malley, B. W. (1975), *Biochemistry 14*, 2072] and experimentally [Palmiter, R. D. (1973), *J. Biol. Chem. 248*, 8260] that in estrogen-stimulated chick oviduct, ovalbumin mRNA (Ov mRNA) is extremely stable, having a half-life ($t_{1/2}$) of at least 24 h. To investigate the role of estrogen in maintaining mRNA stability, the effect of removing a subcutaneously implanted estrogen-containing pellet (withdrawal) on cellular levels of Ov mRNA and total polyadenylated mRNA [poly(A$^+$) mRNA] classes was examined, using in vitro translation and hybridization techniques. Withdrawal caused a rapid loss of Ov mRNA activity and concomitant degradation of Ov mRNA sequences (both to less than 10% of nonwithdrawn levels) within 24 h. A similar rate of decay was seen in isolated nuclei, and in total cellular RNA the level of Ov mRNA sequences declined with a $t_{1/2}$ of 4–5 h. Several lines of evidence indicate that the total mRNA population is more refractory to withdrawal. Total translatable mRNA in polysomal RNA is only reduced to 50% of nonwithdrawn levels after 4-days withdrawal. Back-hybridization of complementary DNA (cDNA) copies of polysomal poly(A$^+$) mRNA revealed that prior to withdrawal, three mRNA abundance classes were present, containing approximately 1, 200, and 10 000 gene equivalents per class. After withdrawal, this high abundance class was undetectable, but total mRNA complexity was unchanged. Furthermore, withdrawal for 24 h decreased the concentration of poly(A$^+$) mRNA in total RNA by a factor of 6, compared with the 100-fold decrease in Ov mRNA sequences. The results suggest that Ov mRNA stability is estrogen dependent, and not simply an inherent property of the molecule. The data are consistent with the hypothesis that maintenance of high levels of Ov mRNA in chick oviduct is mediated in part by a selective stabilization of these molecules within the total mRNA population.

Primary administration of estrogen to immature female chicks stimulates cell division, cytodifferentiation of oviduct epithelial cells to tubular gland cells, and induction of egg white proteins (Oka and Schimke, 1969; O'Malley and Means, 1974). When chicks are withdrawn from steroid treatment for several days, then given a secondary stimulation, egg white proteins are again induced but the response is more rapid (Palmiter, 1972a), since fully differentiated tubular gland cells are already present. At the molecular level, estrogen triggers the accumulation of mRNA[1] species coding for egg white proteins as measured by translation (Palmiter and Smith, 1973; Chan et al., 1973) and of Ov mRNA in particular, as measured by hybridization (Cox et al., 1974; Harris et al., 1975). With respect to the mechanism of estradiol action, two principal hypotheses emerge from these studies: (a) the steroid selectively enhances the rate of transcription of ovalbumin genes and (b) it confers selective stability on the gene transcript.

Evidence suggesting that estrogens stabilize ovalbumin gene copies is provided to date by a single report (Palmiter and Carey, 1974) which showed that the rate of disappearance of translatable Ov mRNA activity in polysomal RNA, after acute withdrawal of a secondary stimulation of estrogen, was considerably more rapid than would be predicted from the known $t_{1/2}$ of Ov mRNA in the presence of estrogen. The present work analyzes this phenomenon in more detail. The levels of specific (Ov mRNA) and total mRNA populations in chick oviduct were measured in total, nuclear, and polysomal RNA in response to withdrawal of a primary stimulation with estrogen, using both translation and hybridization assays. Also, a detailed analysis of mRNA abundance classes was made by synthesizing cDNA copies of polysomal poly(A$^+$) mRNA using both oligo(dT) and oligodeoxynucleotides as primers for reverse transcriptase, and performing cross-hybridization experiments. The data indicate that the concentration of mRNA species which constitute the high abundance, low complexity mRNA classes, including Ov mRNA, is selectively and rapidly reduced in polysomes after withdrawal, whereas the complexity of low abundance mRNA classes remains essentially unchanged.

Materials and Methods

Implantation of Steroid. Pellets containing 15 mg of hexestrol (Evans Medical Ltd., Liverpool, England) were implanted subcutaneously into the necks of 1–2 weeks old White Leghorn pullets. Withdrawal was achieved by removing this intact pellet 15–35 days after implantation.

Wheat Germ Protein Synthesizing System. Wheat germ was obtained from The Pillsbury Company (Minneapolis, Minn.) and the S-30 fraction was prepared (Roberts and Paterson, 1973), except that preincubation was omitted. The fraction excluded on Sephadex G-25 was stored in liquid nitrogen. Each assay contained (in a final volume of 250 μL) 20 mM Hepes (pH 7.4), 2.5 mM $Mg(C_2H_3O_2)_2$, 60 mM KCl, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 100 μg/mL creatine phosphokinase, 10 μM ^{3}H-labeled Ile (2.6 Ci/mmol), 20 μM Cys, 100 μM Arg, Asp, Asn, Glu, Gln, Gly, His, Met, Phe, Pro, Trp, and Tyr, 150 μM Lys, Ser, and Thr, 200 μM Ala, Leu, and Val, 50 μL of S-30 fraction and RNA

† From the Department of Molecular Medicine, Mayo Clinic, Rochester, Minnesota 55901. *Received February 3, 1977.* This work was supported by National Institutes of Health Grant No. HD-9140-C.

[1] Abbreviations used are: mRNA, messenger RNA; Ov mRNA, ovalbumin mRNA; poly(A$^+$) mRNA, polyadenylated mRNA; cDNA, complementary DNA; $cDNA_{ov}$, a cDNA copy of ovalbumin mRNA; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Cl_3AcOH, trichloroacetic acid; A_{260}, absorbance at 260 nm; $\overline{MW}$, average molecular weight; $t_{1/2}$, half-life; EDTA, ethylenediaminetetraacetic acid.

(0–50 μg). Assays were incubated at 26 °C for 2 h, mixed with 50 μL of a solution containing 10 μg of ovalbumin, 10 μg of conalbumin, 10 μg of bovine serum albumin, 2.5% sodium deoxycholate, 2.5% Triton X-100, 50 mM Ile, 50 mM Leu, and 5 mM NaN_3, and centrifuged (9500*g*; 10 min). Aliquots of supernatant (200 μL) were mixed with 50 μL of rabbit antiserum (containing antiovalbumin), incubated at 21 °C for 30 min, and layered over 100 μL of a solution of 1 M sucrose, 1% Triton X-100, 10 mM EDTA, 10 mM sodium phosphate, and 150 mM NaCl in a Beckman microfuge tube. Tubes were centrifuged (10 000*g*; 2 min) and 25-μL aliquots of the upper supernatant were spotted onto Whatman No. 3MM papers, washed 5 times in 5% Cl_3AcOH, heated to 90 °C for 2 min in 5% Cl_3AcOH, and dried. Tubes containing antigen–antibody pellets were frozen at −70 °C and the tips were cut off. Radioactivity on filters (total acid-precipitable protein) and in tube tips (ovalbumin protein) was estimated after digestion in 0.5 mL of NCS (Amersham/Searle) at 80 °C for 30 min followed by addition of toluene-based scintillation fluid. Background values (no exogenous RNA) were subtracted. Total and ovalbumin protein synthesis increased linearly with RNA added (up to 50 μg), and the activity of a given sample was estimated from the slope of the line.

Ovalbumin (Sigma) was purified by chromatography on DE-52 cellulose (Whatman). Antiserum to ovalbumin was obtained from rabbits by multiple-site injection of a mixture of 1 mg of ovalbumin, 1 mL of Freund's adjuvant, 5 mg of tuberculin bacillus (Difco), and 1 mL of normal saline. To determine the specificity of the antibody for ovalbumin, wheat germ assays were set up containing nonwithdrawn polysomal RNA, and the antibody–antigen precipitates obtained were redissolved and subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis. At least 90% of the loaded radioactivity was found in a peak migrating slightly ahead of the ovalbumin marker. This peak was not seen using exogenous liver polysomal RNA.

To analyze protein chains synthesized in the wheat germ system, samples were adjusted to 100 mM isoleucine and centrifuged (100 000*g*; SW 50.1; 1 h; 4 °C) to sediment ribosomes. The supernatant was adjusted to 10 mM EDTA and 10 μg/mL RNase A and incubated at 37 °C for 15 min, and an equal volume of 10% Cl_3AcOH was added. Precipitates were collected by centrifugation, washed three times in 5% Cl_3AcOH containing 100 mM isoleucine, and then dissolved, heated, and subjected to electrophoresis on 10% polyacrylamide gels containing sodium dodecyl sulfate (Palmiter et al., 1971). The average molecular weight ($\overline{MW}$) of labeled proteins was determined as described (Cox, 1973).

Preparation of RNA. Polysomal RNA was prepared by magnesium precipitation, followed by phenol/chloroform extraction and 3 M sodium acetate washes (Palmiter, 1974), except that precipitates were centrifuged through buffer containing 0.3 M sucrose. This preparation is referred to as polysomal RNA, but free ribosomes are also precipitated. Recovery of polysomal RNA was 30–70 A_{260} units/g of tissue wet wt.

Polysomal poly(A^+) mRNA was prepared by passing polysomal RNA twice through oligo(dT)-cellulose by a modification of the method of Aviv and Leder (1972). Prior to each passage, samples (3 mg or less in 20 mM Hepes, pH 7.5) were incubated at 65 °C for 10 min to disaggregate RNA, rapidly cooled in ice, diluted to 10 mL in binding buffer, and passed through Econo columns (Bio-Rad) containing 1 g of T3 oligo(dT)-cellulose (Collaborative Research Inc.). Poly(A) containing RNA was eluted with 20 mM Hepes (pH 7.5).

Total RNA was prepared using aqueous buffers adjusted to 0.05% diethyl pyrocarbonate and boiled (15–30 min) before use. Tissue (0.5 g) was minced, homogenized (Tekmar; 50 V; 60 s × 2) in 4 mL of 0.1 M sodium acetate (pH 6.0) containing 1 mg/mL heparin, 0.5% sodium dodecyl sulfate, and 10 mM EDTA, and then mixed with 4 mL of phenol saturated in 0.1 M sodium acetate (pH 6.4). After addition of 4 mL of chloroform, the mixture was shaken and centrifuged (25 000*g*; 10 min). The aqueous phase (and interface) was reextracted twice with phenol/chloroform (1:1) containing hydroxyquinoline (0.5%), twice with chloroform, and then precipitated with ethanol. The precipitate was collected, redissolved in 10 mM Tris (pH 7.5) containing 10 mM NaCl, and incubated at 37 °C for 15 min in the presence of 0.5 mg/mL deoxyribonuclease I (Worthington) and 10 mM $MgCl_2$. After the addition of sodium dodecyl sulfate to 0.5%, the mixture was extracted with phenol/chloroform and chloroform prior to ethanol precipitation. Treatment with deoxyribonuclease, phenol/chloroform extraction, and ethanol precipitation were repeated and RNA was dissolved in 20 mM Hepes (pH 7.5).

To prepare nuclear RNA, nuclei were isolated as described (Cox, 1976), except that high-speed centrifugation was omitted. The crude nuclear pellet was washed (2×) in 1 mM Tris (pH 8.0), 0.1 mM EDTA, 0.5 mM dithiothreitol, 12.5% glycerol, and RNA was isolated by the method of Holmes and Bonner (1973) with modifications. After resuspension of nuclei in the lysis mixture, phenol extraction, and ethanol precipitation, RNA was subjected to two cycles of treatment with deoxyribonuclease I, phenol/chloroform and ethanol precipitation (as for total RNA), and then dissolved in 20 mM Hepes (pH 7.5).

Purification of Ovalbumin mRNA. This was performed as described (Cox et al., 1974) except that after the oligo(dT)-cellulose step, and prior to final purification on sucrose gradients, poly(A^+) mRNA was subjected to preparative agarose gel electrophoresis using a modification of the method of Woo et al. (1974), to separate 18S rRNA from Ov mRNA.

Chemical Estimations. RNA content was estimated from its absorbance ($A_{260} - A_{320}$), assuming that 20 absorbance units were equivalent to 1 mg of RNA. DNA was estimated by the method of Burton (1956).

Synthesis and Isolation of Complementary DNA. Reverse transcriptase from avian myeloblastosis virus was the kind gift of Dr. J. W. Beard (Life Sciences, Inc., St. Petersburg, Fla.). To prepare oligodeoxynucleotide primer, calf-thymus DNA (Sigma; 5 mg/mL) was incubated at 37 °C for 2 h in 10 mM Tris (pH 7.4) containing 100 μg of deoxyribonuclease I (Worthington) and 10 mM $MgCl_2$. The mixture was then boiled for 10 min, to denature DNA and destroy enzyme activity, and stored at −20 °C.

Reactions (1 mL) for cDNA synthesis were incubated at 37 °C for 60 min and contained 2–10 μg of template RNA, 1.25 μg of oligo(dT_{18-20}), 25 mM Tris (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 mM dATP and TTP, 10 μM ^{3}H-labeled dGTP and dCTP (20–30 Ci/mmol each), 200 μg/mL actinomycin D, 500 μg/mL bovine serum albumin, 6% glycerol, and 40 units of reverse transcriptase in 0.2 M potassium phosphate (pH 7.2) containing 2 mM dithiothreitol, 0.2% Triton X-100, and 50% glycerol (diluted 20× in the reaction). In some cases, calf-thymus oligodeoxynucleotides (250 μg/mL) were used as primer in place of oligo(dT). After incubation, cDNA was isolated and centrifuged on alkaline sucrose gradients (Cox et al., 1974). Marker DNA fragments, obtained by digestion of ^{3}H-labeled SV40 DNA with *Hae* III restriction endonuclease (Yang et al., 1976), were kindly supplied by Dr.

Sue Astrin. Selected gradient fractions were pooled, neutralized, lyophilized, resuspended in H_2O (0.5 mL), and chromatographed on a Sephadex G-50 column (0.9 × 15 cm) containing a pad of Chelex-100 resin (Bio-Rad) at its base. Excluded radioactivity was pooled, lyophilized, and resuspended in 20 mM Hepes (pH 7.5).

Hybridization. Buffers were passed over Chelex-100 resin, filtered through a Millipore filter (0.45 μm), treated with diethyl pyrocarbonate (see above), and boiled. Formamide (Fluka, Switzerland) was pretreated with AG 501-X8 mixed bed resin (Bio-Rad). Lyophilized mixtures of cDNA were resuspended in hybridization buffer (25 mM Hepes (pH 6.8), 0.1% sodium dodecyl sulfate, 0.5 M NaCl, 0.5 mM EDTA, 50% formamide), and aliquots (5 or 10 μL) were sealed in glass capillaries (Dade; siliconized; treated with diethyl pyrocarbonate), heated to 90 °C for 3 min, and incubated at 43 °C. Hybridization was arrested by cooling capillaries to −10 °C; the contents were expelled into 485 or 490 μL of digestion buffer (Cox et al., 1974) and stored at −20 °C.

To determine the extent of hybridization, mixtures were incubated at 45 °C for 90 min after addition of 10 units (5 μL) of S_1 nuclease (Vogt, 1973). To estimate total input cDNA (D_0), 100 μL was transferred to a scintillation vial. Nuclease-resistant hybrid was then precipitated with 50 μL of a solution containing 2 mg of bovine serum albumin and 200 μg of calf-thymus DNA per mL, and 50 μL of 6 N perchloric acid. Mixtures were cooled in ice (20 min) and centrifuged (7000*g*; 10 min), and the amount of single-stranded (nuclease sensitive) cDNA (D) was estimated in 200 μL of the supernatant. Radioactivity in aqueous samples was estimated after addition of H_2O (to 0.5 mL) and 5 mL of scintillation fluid containing Triton X-100 (Gilmour et al., 1974). The fraction of cDNA remaining single stranded was obtained from the ratio D/D_0, and plotted as a function of R_0t or D_0t (R_0, D_0 = initial concentration of RNA or DNA in moles per liter; t = time of hybridization in seconds). For each set of reactions, control capillaries, containing the same cDNA and an equivalent amount of *Escherichia coli* tRNA, were assayed in parallel. These background levels (0–5% of total cDNA input) were subtracted.

Fractionation of High Abundance Class cDNA. To isolate early cDNA copied from nonwithdrawn polysomal poly(A$^+$) (see Figure 7), standard reactions were scaled up 40-fold and hybridized to an R_0t of 4.94×10^{-1} mol s L^{-1}. After digestion with S_1 nuclease (using 400 units of enzyme in 10 mL of digestion buffer), the mixture was adjusted to 0.5% sodium dodecyl sulfate and 10 mM EDTA, extracted once with phenol/chloroform/hydroxyquinoline and twice with chloroform, and precipitated overnight (−20 °C) with ethanol in the presence of 0.2 M NaCl and 100 μg of *E. coli* tRNA. The precipitate was collected, resuspended in 0.5 mL of H_2O, and passed through a Sephadex G-50/Chelex-100 column (see above). Excluded fractions were pooled, lyophilized, resuspended in 0.2 N NaOH, 10 mM NaCl, 10 mM EDTA, and incubated at 37 °C for 6 h to hydrolyze RNA. The sample was then neutralized and passed through Sephadex/Chelex, and the excluded cDNA was pooled, lyophilized, and resuspended in H_2O. Approximately 7% of the original cDNA was recovered.

Analysis of Hybridization Data. Pseudo-first-order reactions between RNA and cDNA, in which the initial RNA concentration (R_0) is in excess of the initial DNA concentration (D_0), can be described by the equation $D/D_0 = e^{-KR_0t}$, where D is the concentration of cDNA remaining single stranded in moles per liter, K is the rate constant of the reaction, and t is the time of hybridization in seconds (Bishop, 1972). To analyze hybridization kinetics in mixtures containing components with different, overlapping, reaction rates, a computer program, based on that of Britten et al. (1974), was employed to fit the data to a number of pseudo-first-order reactions, using the least sum of squares as a criterion for goodness of fit. For each analysis, the number of reaction components (1–3) is specified, together with "guessed" values for the rate constant and the fraction of cDNA contributing to each. A computer-generated best fit plot is obtained, together with a root-mean-square error value (rms), an estimate of the sum of the squares of the deviation of the data points from the mean. Since rms depends on more than one parameter, an objective choice between two solutions is not possible if rms values differ by less than 1.0% (M. J. Getz, personal communication), and other approaches must be used (see Results).

The sequence complexity of mRNA classes in poly(A$^+$) mRNA was determined by comparison with a standard reaction (Ov mRNA and its cDNA), assuming that reaction rates are directly proportional to sequence complexity (Birnstiel et al., 1972). Differences in hybridization rate due to variation in cDNA fragment length were minimized, since all cDNAs used were between 300 and 1000 nucleotides long. No correction was made for differences in the base composition of cDNA fractions, since this parameter has little effect on reaction rate (Wetmur, 1976) when differences are small (<5%). The concentration of Ov mRNA sequences in RNA fractions was determined as described (Cox et al., 1974).

Results

Characteristics of Estrogen Withdrawal. Implantation of hexestrol pellets into female chicks stimulates rapid oviduct growth; tissue wet weight (magnum region) increases from less than 30 mg (unstimulated) to approximately 600 mg (10 days after implantation). Removal of the pellet (withdrawal) initiates a rapid decline in tissue weight such that after 4 days withdrawal, magnum wet weight is reduced to approximately 200 mg. There is also a decline in DNA and protein content per oviduct, indicative of cell loss, and in the protein/DNA ratio, suggesting a decrease in cell protein content. The recovery of RNA also decreases; polysomal RNA levels are shown in Table I, and total RNA declines linearly from 2 to 1 mg of RNA per mg of DNA before and after 4 days of withdrawal, respectively. These changes are characteristic of the general involution of tubular gland tissue. Examination of oviduct tissue sections (control and 24 h withdrawn) revealed that 80–90% of the tissue consisted of tubular gland cells. Hence, studies described below comparing these tissues are unlikely to be strongly influenced by changes in the proportion of different cell types.

Translation of mRNA in Polysomal RNA. To analyze changes in mRNA activity in response to withdrawal, polysomal RNA was assayed in a wheat germ protein synthesizing system (Figure 1), and total and ovalbumin protein synthesis was monitored. After 4-days withdrawal, total mRNA activity declines to 50% of nonwithdrawn levels, although the maximum rate of decrease is observed at early times (0–1 day). In contrast, Ov mRNA activity is only 5% of nonwithdrawn values after 1 day of withdrawal, and by 4 days is almost undetectable. Clearly, total and Ov mRNA activities per unit of polysomal RNA are diminished by withdrawal, but the rate of inactivation of Ov mRNA is considerably greater; as indicated in Figure 1, in nonwithdrawn oviduct, 9% of total protein synthesized by polysomal RNA is immunoprecipitated by

TABLE I: Quantitation of Ovalbumin mRNA Sequences in Oviduct Polysomes during Estrogen Withdrawal.

Duration of withdrawal (days)	Concentration of ovalbumin mRNA in polysomal RNA				
	$R_0t_{1/2}$ (mol s L^{-1})	mRNA sequences[a] (%)	RNA content (μg/mg DNA)	Ovalbumin mRNA per unit of DNA[b] (ng/mg DNA)	Molecules of ovalbumin mRNA per tubular gland cell[c]
0	1.36	0.58	850	4930	13 330
0.5	1.57	0.50	880	4400	11 900
1	9.58	0.083	545	452	1 200
2	4.0×10^2	0.0020	515	10.3	30
4	1.0×10^3	0.0008	365	2.9	8

[a] Calculated as described (Cox et al., 1974), assuming that the Ov mRNA standard is 100% pure and reacts with homologous cDNA with a $R_0t_{1/2}$ value of 8.0×10^{-3} (see Figure 3). [b] Values are the product of columns 3 and 4. [c] Calculated from values in column 5, assuming that the molecular weight of Ov mRNA is 7×10^5 (Shapiro and Shimke, 1975), each tubular gland cell contains 2.5 pg of DNA per cell (Sober, 1968), and that 80% of oviduct cells are tubular gland cells (see Results).

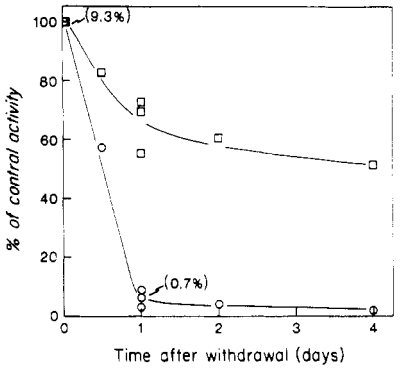

FIGURE 1: Effect of withdrawal on translatable mRNA activity in polysomal RNA from chick oviduct (magnum region). Aliquots (0-10 μg) of polysomal RNA isolated at various stages of withdrawal were assayed in vitro in a wheat germ protein-synthesizing system. Total (□) and ovalbumin protein synthesis (○) were measured and expressed as a percentage of nonwithdrawn levels. Values in parentheses indicate the percent contribution of ovalbumin protein synthesis to total synthesis in nonwithdrawn and 1-day withdrawn tissue. Three replicates of analysis (using separate animals) are shown for 1-day withdrawn RNA.

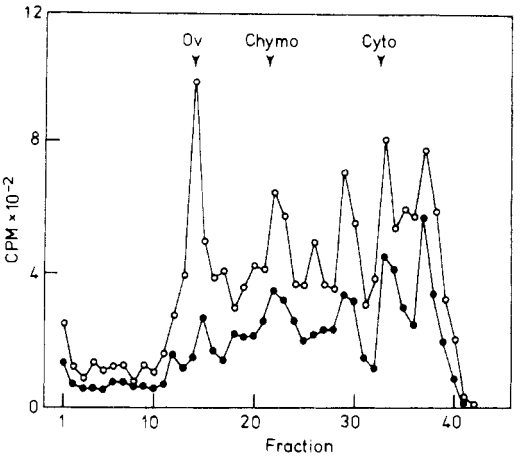

FIGURE 2: Effect of withdrawal on the size distribution of peptides synthesized in the wheat germ system in response to exogenous polysomal RNA. Aliquots (20 μg) of polysomal RNA were incubated in a wheat germ system. Acid-precipitable radioactivity was recovered and subjected to electrophoresis on 10% polyacrylamide gels, and the distribution of radioactivity in the gels was estimated (see Materials and Methods). Patterns obtained using polysomal RNA from nonwithdrawn (○) or 1-day withdrawn (●) oviduct are shown, after the endogenous background (no RNA added; less than 20% of the total acid-precipitable radioactivity) had been subtracted. The positions to which ovalbumin (42 000 daltons), α-chymotrypsinogen (22 500), and cytochrome c (12 400) migrated under identical conditions are indicated.

antiovalbumin, but this value drops to 0.7% after 1 day of withdrawal. This apparent inactivation of Ov mRNA in oviduct confirms similar observations made previously (Palmiter and Carey, 1974).

The size distribution of protein chains synthesized in the wheat germ system in response to exogenous polysomal RNA is shown in Figure 2. Approximately 12% of the total label migrates with ovalbumin marker when nonwithdrawn RNA acts as template, but no distinct peak of this size is seen using withdrawn RNA. Both RNA preparations direct the synthesis of a number of peptides which co-migrate, particularly in the lower molecular weight region. Similar experiments were performed using polysomal RNA from 2- and 4-day withdrawn tissue (not shown); very little radioactivity co-migrated with ovalbumin marker and the radioactive peaks coincided poorly with those obtained using nonwithdrawn RNA, suggesting that a major change in the pattern of synthesis of predominant proteins had occurred.

The average molecular weights ($\overline{MW}$) of labeled peptides synthesized by nonwithdrawn and 1-, 2-, and 4-day withdrawn polysomal RNAs were found to be 18 400, 17 700, 18 400, and 18 600, respectively, and, in all cases, no peptides smaller than 10 500 daltons were detected on the gels. These results argue against the likelihood that differential degradation of mRNA occurs during the isolation of polysomes from each of these tissues, and indicate that measurement of total mRNA activity (by acid precipitation) is not influenced by recovery of small peptide fragments. However, some degree of mRNA degradation cannot be entirely ruled out. Limited scission of mRNA molecules during isolation may be responsible for the apparent discrepancy between $\overline{MW}$ values for oviduct proteins synthesized in vitro and those synthesized in organ culture (33 000-38 000) as determined by Palmiter (1972b), but other factors, such as translation efficiency and differential translation of mRNAs in the wheat germ system, may also contribute.

The possibility that Ov mRNA was being selectively degraded within the total mRNA population in withdrawn tissue homogenates during isolation was checked by adding constant amounts of a preparation of hen oviduct polysomes to homogenates and then preparing polysomal RNA as usual. In all cases, at least 80% of the additional Ov mRNA activity (measured by translation) was recovered, compared to the

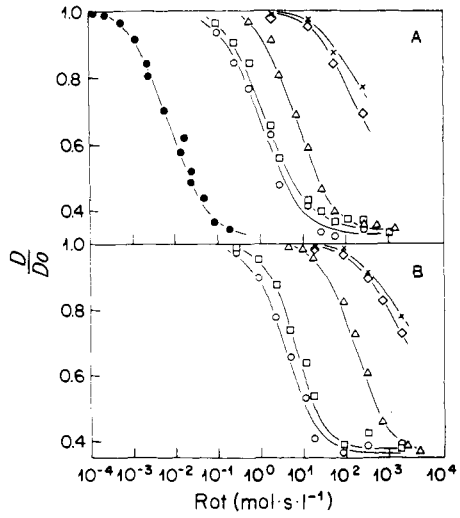

FIGURE 3: Hybridization kinetics of reactions between $cDNA_{ov}$ and polysomal and nuclear RNAs isolated during withdrawal. cDNA (0.02 ng; 1000 cpm) was hybridized back to 5–60 ng of purified Ov mRNA (●), or to 0.5–160 μg of polysomal RNA (panel A) or 2–100 μg of nuclear RNA (panel B) isolated at various times during withdrawal: nonwithdrawn (○); 12-h withdrawn (□); 1-day withdrawn (△); 2-days withdrawn (◇); 4-days withdrawn (×). The best fit single component curve for each set of data (0–1 day withdrawn samples only) was obtained by computer analysis. Polysomal RNA preparations were the same as those used in the wheat germ assay (Figure 1).

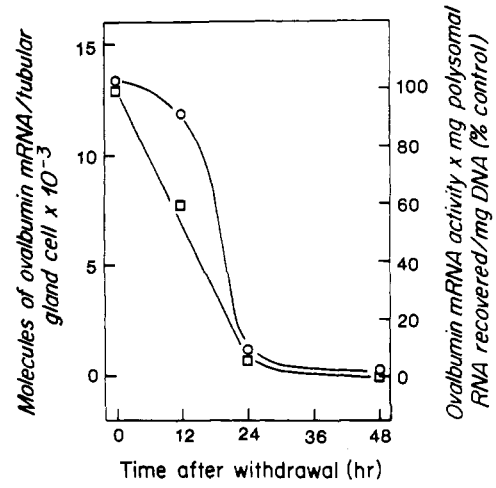

FIGURE 4: Effect of withdrawal on the kinetics of disappearance of Ov mRNA in polysomal RNA as measured by translation and by hybridization. Estimates of the number of molecules of Ov mRNA in polysomal RNA per tubular gland cell (○), as measured by hybridization, are from Table I. The total level of translatable Ov mRNA in the polysome fraction (□) was calculated from the product of the specific activity of polysomal RNA in the wheat germ assay (counts per minute incorporated into ovalbumin protein per microgram of RNA, derived from Figure 1) and the total yield (micrograms) of RNA (see Table I), and expressed as a percentage of nonwithdrawn levels.

recovery in controls (hen polysomes minus homogenate).

Quantitation of Ovalbumin mRNA Sequences in Polysomal RNA. To distinguish between mechanisms which could account for a rapid decrease in translatable Ov mRNA activity, the concentration of Ov mRNA sequences was measured by hybridization of polysomal RNA to labeled $cDNA_{ov}$. Back-hybridization of $cDNA_{ov}$ to Ov mRNA is shown in Figure 3A. The reaction exhibits a $R_0t_{1/2}$ (the R_0t value at which the reaction is 50% complete) of 8×10^{-3} mol s L^{-1}, and the data points fit a computer-generated curve which delineates the kinetics of a reaction between a homogeneous RNA component and its cDNA. Using identical conditions of hybridization, Getz et al. (1976) demonstrated that globin mRNA (4×10^5 daltons) back-hybridizes with a $R_0t_{1/2}$ of 5×10^{-3} mol s L^{-1}. Since the analytical complexity of Ov mRNA is 7×10^5 daltons (Shapiro and Schimke, 1975), ovalbumin mRNA is expected to back-hybridize to its cDNA at a $R_0t_{1/2}$ of $(5 \times 10^{-3}) \times (7 \times 10^5)/(4 \times 10^5) = 8.75 \times 10^{-3}$. Such considerations strongly suggest that the purified Ov mRNA, and hence the cDNA copy, are homogeneous.

Hybridization of $cDNA_{ov}$ to the polysomal RNA fractions assayed in Figure 1 is shown in Figure 3A. Little change in the Ov mRNA concentration is observed after 12 h of withdrawal but a marked decrease is seen after 1 day, and after 2 and 4 days the concentration is extremely low. Based on these data, an estimate of the number of Ov mRNA molecules per tubular gland cell in response to withdrawal was made (Table I). The results indicate that 10 000–15 000 Ov mRNA copies are present in each cell when estrogen is present in the circulation, but less than 10 are present 4 days after withdrawal. This rapid depletion of Ov mRNA sequences also occurred in nuclear RNA as well, as shown in Figure 3B. The rate of loss may in fact be greater than that observed in polysomes, there being at least a 40-fold decrease in Ov mRNA sequence concentration after 1 day of withdrawal. Analysis of total oviduct RNA (see below) gave comparable results, strengthening the conclusion that the onset of degradation of Ov mRNA induced by withdrawal occurs in all major compartments of the tubular gland cell.

Direct comparison of the kinetics of loss of translatable Ov mRNA activity (per cell) and of hybridizable Ov mRNA sequences is made in Figure 4. As might be predicted, translatable activity degrades more rapidly than hybridizable sequences, since a single nick in Ov mRNA will affect the former, but not the latter, assay. No significant lag time between withdrawal and the observed decrease in translational activity is seen, whereas the maximum rate of removal of Ov mRNA sequences is not observed until 12–24 h after withdrawal. The data confirm that the decreased contribution to total protein synthesis of immunoprecipitable ovalbumin (9 to 0.7%) observed in polysomal RNA after 24 h of withdrawal is mediated by a rapid decay of Ov mRNA molecules.

Quantitation of mRNA Sequences in Polysomal Poly(A^+) mRNA. Polysomal poly(A^+) mRNA was isolated by affinity chromatography using oligo(dT)-cellulose. The recovery of these fractions is summarized in Table II. Approximately 1–2% of polysomal RNA was recovered in the poly(A^+) fraction, and the yield of poly(A^+) mRNA after withdrawal was approximately 60% of that obtained from nonwithdrawn polysomal RNA. Reduced yields of poly(A^+) mRNA probably reflect a general decrease in the level of mRNA relative to ribosomal RNA after withdrawal. Major alterations in the length of poly(A) tracts (which could modify RNA yields on oligo(dT)-cellulose) are unlikely, since Monahan et al. (1976) showed that these tracts are about 70 nucleotides in length in both estrogen-stimulated and withdrawn oviduct. However, withdrawal may alter the proportion of total mRNA which is polyadenylated. Estimates of the amount of poly(A^+) mRNA recovered in polysomes per tubular gland cell are also shown in Table II.

The synthesis of cDNA copies of poly(A^+) mRNA was studied using two different primer molecules, namely oligo(dT) and a random mixture of oligodeoxynucleotides prepared by digesting calf-thymus DNA with deoxyribonuclease I (Ross et al., 1972; Taylor et al., 1976). The RNA and primer de-

TABLE II: Effect of Withdrawal on Yields of Oviduct Polysomal Poly(A$^+$) mRNA during Estrogen Withdrawal.

Duration of withdrawal (days)	Poly(A$^+$) mRNA recovered[a] (μg)	% of polysomal RNA	Poly(A$^+$) mRNA per unit of DNA[b] (μg/μg of DNA)	Poly(A$^+$) mRNA content[c] (pg/cell)
0	63.3 ± 6.9 (7)	2.10	0.018	0.045
1	37.9 ± 7.9 (11)	1.26	0.008	0.020

[a] Values represent poly(A$^+$) mRNA recovered after passage of 3 mg of polysomal RNA through oligo(dT)-cellulose, and are the average of the number of estimations shown in parentheses, ± standard deviation. [b] Obtained from the product of values in column 3 and the yield of polysomal RNA per unit DNA (see Table I). [c] Assuming 2.5 pg of DNA per cell (Sober, 1968).

TABLE III: Efficiency of Oligodeoxynucleotides and Oligo(dT) as Primers for Reverse Transcriptase.

Additions (μg per reaction)		Poly(A$^+$)[a] mRNA	[^{3}H]dGTP and [^{3}H]dCTP incorpd into DNA[b] (cpm)
Oligodeoxynucleotides	Oligo(dT$_{18\text{-}20}$)		
		0.98	2 900
25			800
	0.125		700
25		0.98	53 400
50		0.98	64 300
	0.125	0.98	95 200

[a] From nonwithdrawn oviduct. [b] Assay mixtures were as described under Materials and Methods, but scaled down × 10. Reactions were incubated at 37 °C for 60 min, and acid-precipitable radioactivity was determined as described (Cox et al., 1974).

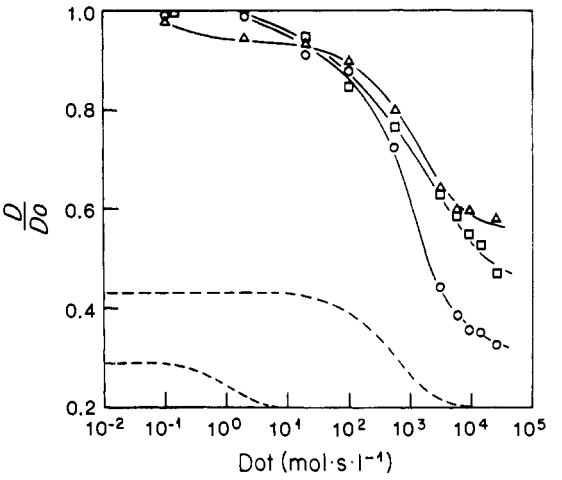

FIGURE 5: Hybridization kinetics of total chick DNA and cDNAs synthesized from polysomal poly(A$^+$) mRNA fractions. Chick oviduct DNA was prepared from oviduct chromatin (Marmur, 1961), sonicated to reduce fragment length to approximately 400 nucleotides, and passed over Chelex-100 before use. Aliquots of DNA (25 μg) were hybridized with cDNA (1000 cpm) synthesized from nonwithdrawn RNA using either oligo(dT) (□) or oligodeoxynucleotide (△) primer, or from 1-day withdrawn RNA using oligo(dT) (○). The reason for the variation in the extent of hybridization between different cDNAs is not clear. Curves approximating the rates of renaturation of unique (upper) and repetitive (lower) chick DNA fractions (see Mizuno et al., 1977) are indicated by dashed lines. The relative height of these curves on the ordinate is arbitrary.

pendence of the reaction is shown in Table III. As indicated, enzyme activity is dependent on the presence of both poly(A$^+$) mRNA and primer, and high concentrations of calf-thymus primer (at least 50 μg per reaction) were needed to match the priming efficiency of small amounts (0.12 μg per reaction) of oligo(dT).

The size of cDNA copies synthesized from nonwithdrawn poly(A$^+$) mRNA, and using either oligo(dT) or oligodeoxynucleotides as primer, was estimated on alkaline sucrose gradients by comparison with restriction endonuclease digests of SV40 DNA (not shown). Oligodeoxynucleotide-primed cDNA copies were, on the average, about 300 nucleotides in length. When oligo(dT) was used, cDNA copies were much longer (600 nucleotides average), and 10–20% of the chains were in excess of 1000 nucleotides. This difference in priming ability probably relates to the fact that oligodeoxynucleotides hybridize randomly along the entire length of the template; consequently (a) on the average, each enzyme/primer complex will only copy 50% of the length of a given template and (b) primed enzymes may be unable to traverse regions where other primer molecules are interposed between them and the 5′ end of the template. The size of oligo(dT)-primed cDNA copies of 1-day withdrawn poly(A$^+$) mRNA was marginally smaller (500 nucleotides average) than when using nonwithdrawn RNA.

To establish whether poly(A$^+$) mRNA sequences in oviduct are transcribed from unique or repetitive DNA sequence, aliquots of cDNA were renatured with total chick DNA. Figure 5 shows that when oligo(dT) primer is used, the major fractions of cDNA copied from either nonwithdrawn or 1-day withdrawn poly(A$^+$) mRNA renatured between 10^1 and 10^4 mol s L^{-1}, the region in which unique chick DNA copies renature (Mizuno et al., 1977). Judging by the degree of renaturation which takes place before 10^1 mol s L^{-1}, 10–15% of both poly(A$^+$) mRNA fractions contain moderately repetitive sequences, in agreement with similar studies performed in chick oviduct and other eukaryotic tissues (Monahan et al., 1976; Ryffel and McCarthy, 1975).

Since oligodeoxynucleotides will prime nonpolyadenylated RNA species, this property was used to estimate the contamination of poly(A$^+$) mRNA fractions with ribosomal RNA. When oligodeoxynucleotide-primed cDNA copied from nonwithdrawn RNA was renatured with chick DNA (Figure 5), the kinetics were similar to those obtained using oligo(dT)-primed cDNA, except for the presence of a repetitive cDNA fraction renaturing between 10^{-1} and 10^1 mol s L^{-1}. Similar kinetics were observed using withdrawn RNA templates. Birnstiel et al. (1971) estimate that ribosomal cistrons in the chick genome are 400-fold repetitive, suggesting that the rapidly renaturing cDNA fraction represents copies of ribosomal RNA. The data allow a maximum limit to be put on the level of ribosomal RNA contamination in poly(A$^+$) mRNA fractions, that is, about 10–15%.

The total complexity of an mRNA population can be estimated by back-hybridization of a homologous cDNA copy

TABLE IV: Base Sequence Complexity Analysis of Poly(A$^+$) mRNA.

Duration of withdrawal (days)	cDNA primer used	Component	Fraction of hybridizable cDNA	$R_0t_{1/2}$ (mol s L^{-1}) Obsd	$R_0t_{1/2}$ (mol s L^{-1}) Corrected[a]	Sequence complexity[b] (daltons)	Gene equiv[c]	Genome expressed[d] (%)	Abundance[e] (molecules per gene per cell)
0	Olido(dT)	1	0.250	0.035	0.008	7.0×10^5	1.0	0.00009	9640
		2	0.319	3.58	1.142	9.9×10^7	201	0.013	87
		3	0.429	133.9	57.4	5.0×10^9	10 190	0.65	2.3
0	Oligo-deoxy-nucleotides	1	0.235	0.031	0.0073	6.3×10^5	0.91		
		2	0.357	0.859	0.306	2.7×10^7	55		
		3	0.406	97.7	39.6	3.5×10^9	7 130		
1	Oligo(dT)	1							
		2	0.220	1.27	0.279	2.4×10^7	49	0.003	110
		3	0.779	85.1	66.2	5.8×10^9	11 820	0.76	1.6

[a] The products of columns 4 and 5. [b] Sequence complexity = $R_0t_{1/2}$ corrected × molecular weight of Ov mRNA/$R_0t_{1/2}$ of the Ov mRNA·cDNA reaction. Values used for the latter two parameters are given in the legend to Table I. [c] For component 1, obtained by dividing sequence complexity values by the molecular weight of Ov mRNA; for components 2 and 3, by dividing by the molecular weight of the average oviduct mRNA (assumed to be 1500 nucleotides, or 4.905×10^5 daltons). [d] Percent genome expressed = sequence complexity × 100/molecular weight of the haploid chick genome. The latter figure is taken as 7.6×10^{11} (Mizuno et al., 1977). [e] Abundance = amount of poly(A$^+$) mRNA per cell (in grams) × fraction of hybridizable cDNA × (6×10^{23})/sequence complexity of the component. Values for the amount of poly(A$^+$) mRNA per cell are taken from Table II.

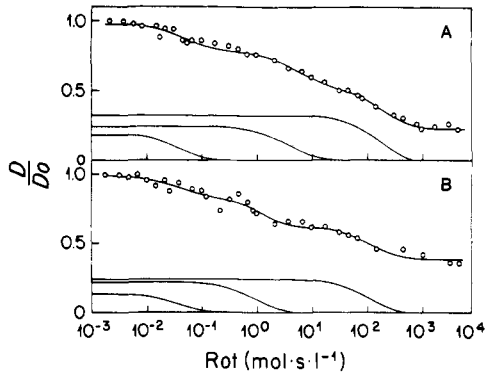

FIGURE 6: Hybridization kinetics of reactions between nonwithdrawn polysomal poly(A$^+$) mRNA and homologous cDNAs synthesized using two different primers. (A) RNA (0.0005-3 μg) was hybridized with cDNA (1000 cpm) synthesized using oligo(dT) as primer. (B) RNA hybridized with cDNA synthesized using oligodeoxynucleotide primer. In each case, the line through the data points represents the best least-squares solution to the data. Curves without data points represent computer-generated first-order rate components; the fraction of total input cDNA contributing to each component is obtained on the ordinate.

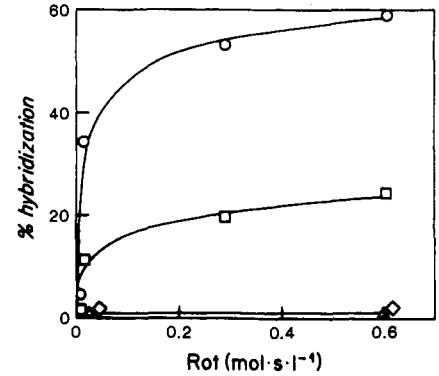

FIGURE 7: Hybridization of Ov mRNA with total and "early" cDNA fractions synthesized from nonwithdrawn polysomal poly(A$^+$) mRNA. After synthesis of cDNA using oligo(dT) as primer, early or first transition cDNA was isolated (see Materials and Methods). Purified Ov mRNA (33 ng) and aliquots (500 cpm) of either unfractionated (□) or early (○) cDNAs were then hybridized to R_0t values sufficient to saturate all DNA copies complementary to Ov mRNA. Control reactions contained 33 ng of *E. coli* tRNA and unfractionated (◇) or early (△) cDNAs.

(Bishop et al., 1974). In Figure 6A, the reaction between nonwithdrawn poly(A$^+$) mRNA and oligo(dT)-primed cDNA is shown, together with the computer-generated plot of the kinetics of reaction of each component when three components (of undetermined rate constant) are specified. Three were specified because: (a) the reaction occurs over more than 2 log units of R_0t, indicating that at least two components are present (Young et al., 1974); (b) when the data are plotted on a scale of linear R_0t, three major transitions in the kinetic curve were evident (results not shown), which tend to be obscured when using a logarithmic R_0t scale (Bishop et al., 1974); and (c) the root-mean-square error (see Materials and Methods) is considerably greater (by more than 1.0%) when two components are specified. The kinetics of hybridization of oligodeoxynucleotide-primed cDNA with its template was also measured (Figure 6B) and analyzed in terms of three components. The data obtained using both primers are summarized in Table IV. The fraction of hybridizable cDNA and $R_0t_{1/2}$ values for each component are shown (columns 4 and 5), and $R_0t_{1/2}$ values were then corrected, taking into consideration the real concentration of RNA contributing to each transition. The sequence complexity of RNA was then calculated by comparison with the $R_0t_{1/2}$ of the reaction between Ov mRNA and its cDNA.

In the reaction with oligo(dT)-primed cDNA, the first transition has a corrected $R_0t_{1/2}$ similar to that of Ov mRNA and its cDNA (that is, 8×10^{-3} mol s L^{-1}) suggesting that this transition is due to Ov mRNA. Direct proof of this possibility was obtained by isolating cDNA which hybridized in the first transition ("early" cDNA), and reannealing it back to Ov mRNA. At least 60% of the "early" cDNA hybridizes to Ov mRNA (Figure 7). Considering that a small fraction of this cDNA may be complementary to second transition mRNAs (due to overlap of these components) and that S_1 nuclease treatment used to isolate "early" cDNA may reduce cDNA size and hence its ability to hybridize, the significant enrichment of sequences complementary to Ov mRNA strongly

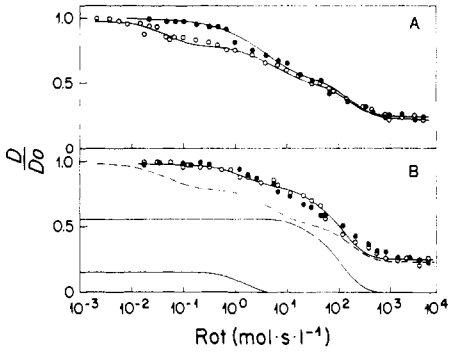

FIGURE 8: Kinetics of cross-hybridization reactions between nonwithdrawn and 1-day withdrawn polysomal poly(A$^+$) mRNA fractions and their respective cDNAs. RNA (0.0005-3 μg) was hybridized with cDNA (1000 cpm) synthesized using oligo(dT) as primer. (A) Heterologous reaction between 1-day withdrawn RNA and cDNA synthesized from nonwithdrawn RNA (●); data points for the homologous reaction between nonwithdrawn RNA and its cDNA (taken from Figure 6A) are superimposed (○). (B) Heterologous reaction between nonwithdrawn RNA and cDNA synthesized from 1-day withdrawn RNA (●); homologous reaction between 1-day withdrawn RNA and its cDNA (○). Lines drawn through data points represent the best least-squares solution to the data. In panel B, the line is the best fit for the homologous reaction, and continuous curves without data points represent individual first-order components for this reaction. The dotted line represents the kinetics of the homologous reaction between nonwithdrawn RNA and its cDNA (see panel A) and is included for comparison.

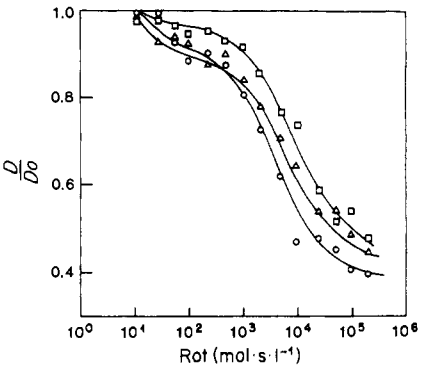

FIGURE 9: Effect of withdrawal on the kinetics of hybridization of cDNA, synthesized using 1-day withdrawn polysomal poly(A$^+$) mRNA as template, with total RNA. Aliquots of cDNA (1000 cpm) were hybridized with total RNA (100-150 μg) isolated at various times after withdrawal: nonwithdrawn (○); 12-h withdrawn (△); 1-day withdrawn (□).

suggests that Ov mRNA is the first major component of this reaction. Also, when unfractionated cDNA is hybridized to Ov mRNA (Figure 7), approximately 25% hybridizes, in agreement with the demonstration that 25% of the total cDNA copy of nonwithdrawn poly(A$^+$) hybridizes in the first transition (Table IV).

Assuming that the average mRNA species in oviduct is 1500 nucleotides long (Cox, 1976), Table IV shows that the second and third (more complex) components contain approximately 200 and 10 000 different mRNA species, respectively (column 8). The total poly(A$^+$) mRNA fraction represents the expression of less than 1% of the DNA sequence per cell (column 9). Using the data in Table II, the abundance of mRNA species in each class can be estimated (column 10). For the first transition (Ov mRNA), 9000-10 000 copies are present in the poly(A$^+$) fraction per cell, a figure close to that (13 000) calculated for the number of Ov mRNA copies in polysomes (Table I), suggesting that most Ov mRNA molecules contain poly(A$^+$) tracts. Species of mRNA which contribute to the second and third transitions are present at approximately 90 and 2 copies per cell, respectively.

The similarity between the three-component fit when poly(A$^+$) mRNA is hybridized back to either oligo(dT)-primed or oligodeoxynucleotide-primed cDNA is apparent (Figure 6 and Table IV). The $R_0t_{1/2}$ for the second transition, using oligodeoxynucleotide-primed cDNA, is somewhat lower, but may be related to a scatter of the data points between $R_0t = 10^{-1}$ and 10. These results have important implications. When oligo(dT) is used as primer, random copying of all poly(A$^+$) mRNA species is assumed, although the possibility that a significant fraction of mRNA contains sequences close to the 3′ end which reverse transcriptase fails to copy is not eliminated. The finding that oligo(dT)-primed cDNA is at least as complex as that synthesized using a random primer argues against this likelihood.

Quantitation of Poly(A$^+$) mRNA Sequences after Withdrawal. In Figure 8A, kinetics of hybridization of the homologous nonwithdrawn reaction are compared with those obtained using the same cDNA and 1-day withdrawn poly(A$^+$) mRNA. Withdrawn RNA is unable to react with first transition cDNA, indicating a marked depletion (lower abundance) of Ov mRNA sequences in withdrawn poly(A$^+$) mRNA, and confirming results obtained for polysomal RNA (Figures 1 and 3). However, all hybridizable cDNA is driven into hybrid by withdrawn poly(A$^+$) mRNA, and saturation is reached at the same R_0t (10^3). One can conclude that virtually all nonwithdrawn mRNA species are present after 1 day of withdrawal.

To determine the change in abundance levels of mRNA species after withdrawal, a homologous reaction between withdrawn poly(A$^+$) mRNA and its cDNA copy was analyzed (Figure 8B). Two components (of undefined rate constant) were specified, based on the number of major transitions observed when the data were plotted against linear R_0t. Comparison with the nonwithdrawn homologous reaction (dashed line) reveals that the entire first transition is absent and a significant fraction of the second transition is missing, indicating that Ov mRNA is no longer sufficiently abundant to contribute a separate rapidly hybridizing mRNA class, and that many mRNAs in the second transition prior to withdrawal are now considerably less abundant. The results are quantitated in Table IV. After 1 day of withdrawal, only 50 different mRNA species, at a concentration of 100 copies per cell, contribute to the second transition, and 11 000-12 000 mRNA species, at a level of 1-2 copies per cell, contribute to the third transition. The fraction of cDNA complementary to the low abundance mRNA class increases considerably during withdrawal (43-78%), although the total complexity of poly(A$^+$) mRNA is not greatly changed (10 400 and 12 000 gene equivalents before and after withdrawal, respectively).

Superimposed upon the withdrawn homologous reaction (Figure 8B) are data obtained when withdrawn cDNA is hybridized to nonwithdrawn poly(A$^+$) mRNA. The pattern of hybridization is almost identical with that of the homologous reaction, although minor differences in the rate of hybridization are seen because of the differing relative proportions of second and third transition mRNAs per unit mass of nonwithdrawn, as compared to withdrawn, mRNA fractions. However, since practically all mRNAs present after withdrawal are also present prior to withdrawal, very few if any new poly(A$^+$) mRNA species are present in polysomes when estrogen is removed from the circulation.

Levels of mRNA in Total RNA. Changes in mRNA levels in polysomal and poly(A$^+$) mRNA fractions during with-

TABLE V: Effect of Withdrawal on the Concentration of Ovalbumin mRNA and High Complexity mRNA Classes in Total RNA.

Duration of withdrawal (days)	cDNA used for hybridization			
	cDNA to ovalbumin mRNA		cDNA to 1-day withdrawn polysomal poly(A$^+$) mRNA	
	$R_0t_{1/2}$ (mol s L^{-1})	Sequence concn[a] (%)	$R_0t_{1/2}$ corrected[b] (mol s L^{-1})	Sequence concn[a] (%)
0	1.45	100	2.56×10^3	100
0.5	1.00×10	14.5	6.26×10^3	40.8
1	2.10×10^2	0.69	1.65×10^4	15.5

[a] Calculated by assuming that the sequence concentration is inversely proportional to the $R_0t_{1/2}$ of the reaction, and is equivalent to 100% in nonwithdrawn total RNA. [b] Data shown in Figure 9 were analyzed using a computer program (see Materials and Methods). Two components (of undetermined rate constant) were specified and the corrected $R_0t_{1/2}$ value obtained for the most complex component (component 2) is shown.

drawal may not reflect the situation in all cell compartments. To address this question, the relative rates of loss of Ov mRNA and high complexity mRNA classes were measured in total oviduct RNA. To monitor high complexity mRNA species, oligo(dT)-primed cDNA synthesized from 1-day withdrawn poly(A$^+$) mRNA was hybridized back to total RNA prepared at various times during withdrawal (Figure 9). Some difficulty was experienced in hybridizing cDNA to completion due to the low mRNA concentration in total RNA, but an approximate estimate of the rate of hybridization of the high complexity component was obtained by computer analysis (Table V). The results obtained when $cDNA_{ov}$ was hybridized to total RNA are also shown.

The data indicate that Ov mRNA sequences are lost at a greater rate than the high complexity mRNA class in total RNA. If values for sequence concentration (Table V) are plotted on a semi-log scale (not shown), estimates of the rates of loss can be obtained. High complexity mRNA levels decrease with a $t_{1/2}$ of about 9 h, whereas Ov mRNA is removed at a considerably faster rate ($t_{1/2}$ = 4–5 h). Why the high complexity mRNA class appears to decay more rapidly as compared to translatable mRNA activity in polysomes (Figure 1) is not entirely clear. It may indicate that mRNAs are more stable in polysomes, but the fact that total RNA also contains nuclear pre-mRNA, that only poly(A$^+$) mRNA species are being monitored in total RNA, and that different ratios of non-mRNA species may be recovered in total and polysomal RNA at different stages of withdrawal (affecting estimates of mRNA concentration) precludes a simple explanation.

Discussion

The validity of the approach to measure mRNA complexity has been outlined by Bishop et al. (1974) and is discussed elsewhere (Levy and McCarthy, 1975). It depends on the assumption that mRNA molecules are randomly copied by reverse transcriptase, and evidence for this has recently been obtained (Hastie and Bishop, 1976). In the present report, different template primers were employed, random transcription being defined by different criteria in each case. Using oligo(dT), transcription begins on poly(A$^+$) tails, irrespective of the length of the attached mRNA coding sequence; for oligodeoxynucleotides, transcription is governed by the mass of each contributing mRNA species. Considering these differences, the overall complexity values obtained for polysomal poly(A$^+$) mRNA from estrogen-primed oviduct are reasonably close (5.0 and 3.5×10^9 daltons), as is the distribution pattern of mRNA classes. The use of random and poly(A$^+$)-specific primers also allows a limit to be set on ribosomal RNA contamination, an important factor since contamination by the latter elevates R_0t values and hence complexity estimates. Furthermore, an internal standard (Ov mRNA) was identified within the total mRNA/cDNA reaction, thus strengthening the validity of the estimate of 7000–10 000 different polysomal poly(A$^+$) mRNA species per oviduct cell. These figures are comparable to other reported values. In mouse and *Drosophila* cells, 6000–10 000 different poly(A$^+$) mRNA species exist in polysomes or cytoplasm (Ryffel and McCarthy, 1975; Getz et al., 1976; Levy and McCarthy, 1975) and Young et al. (1976) obtained complexity values of $6.8–7.2 \times 10^9$ daltons for similar RNA fractions in mouse embryo and liver. In birds, Axel et al. (1976) find 14 000 polysomal poly(A$^+$) mRNA species in hen oviduct, and in estrogen-treated oviduct, Monahan et al. (1976) indicate that there are 20 000 mRNA species in total poly(A$^+$) RNA. The latter figure cannot be directly compared to the present data, since nuclear RNA species probably contribute significantly to the total complexity.

The sequence of molecular events by which estrogens reprogram protein synthesis in chick oviduct remains unknown. Steroid-mediated changes in the expression of oviduct genes are probably achieved by controlling the cellular level of mRNAs coded by these genes, and not by selective initiation of translation on "preferred" mRNA species (Palmiter, 1975). Further evidence for this is the close correlation between Ov mRNA levels and ovalbumin synthesis during secondary stimulation by estrogen (Cox et al., 1974; Palmiter, 1973). At least two mechanisms could contribute to the accumulation of specific mRNA species. Estradiol may activate specific gene transcription, an idea strengthened by studies performed primarily in vitro (Harris et al., 1976; Tsai et al., 1976). A second, but not mutually exclusive, model is that by a series of processing or translation-related steps, certain gene products are considerably stabilized relative to other mRNAs, enabling these species to accumulate selectively in polysomes. This implies that the stability of Ov mRNA is not inherent, but is conferred only in the cellular milieu created by estrogen. It has been shown that Ov mRNA has a long half-life (at least 24 h) in the presence of estrogen (Palmiter, 1973; Harris et al., 1975). Consequently, if estradiol acts only as a positive effector on gene transcription, withdrawal should reduce ovalbumin gene transcription, and preexisting gene copies should degrade with $t_{1/2}$ of 24 h. If estradiol confers mRNA stability, withdrawal will result in rapid degradation of "protected" mRNAs.

The data described in this paper suggest that the latter mechanism is operative, since the rate of decay of Ov mRNA is significantly less than 24 h. Translatable Ov mRNA activity in polysomal RNA decays with an approximate $t_{1/2}$ of 12 h, and is mediated by complete physical degradation of the molecule. In total RNA fractions, the rate of removal of Ov

mRNA sequences was more rapid than this ($t_{1/2}$ = 4–5 h). Since both measurements were made under steady-state conditions, they represent the maximum limits for the actual decay rate of Ov mRNA. Similar results were seen in nuclear RNA and polysomal poly(A^+) mRNA, ruling out the possibility of relocation of Ov mRNA from polysomes to another cell compartment.

Several lines of evidence show that, relative to Ov mRNA, the majority of mRNA species are more refractory to estrogen withdrawal, although their cellular concentration is still reduced. Translatable Ov mRNA activity is virtually undetectable after 1 day of withdrawal, but total translatable mRNA levels in polysomes are reduced by less than half, even after 4 days. Secondly, when the concentration of low abundance poly(A^+) mRNA species was measured in total RNA, there was only a sixfold reduction in these levels after withdrawal compared to a 100-fold reduction in Ov mRNA. Thirdly, although the concentration of polysomal poly(A^+) mRNA per cell is reduced after withdrawal, cross-hybridization of cDNA copies of poly(A^+) mRNA fractions revealed that withdrawal caused selective loss of Ov mRNA. However, the total complexity was hardly altered, suggesting that within the limits of detection, no single mRNA species was totally destroyed.

These results provide no information about relative rates of degradation of mRNA classes, since mRNA levels are a function of rates of synthesis and degradation. Withdrawal diminishes the ability of form B RNA polymerases to synthesize RNA in isolated nuclei (Spelsberg and Cox, 1976; Mizuno et al., 1977). This effect would account, in part, for declining levels of total RNA, polysomal RNA, and mRNA, and the more rapid loss of Ov mRNA relative to total mRNA could then be explained by at least two mechanisms: (a) ovalbumin gene transcription is selectively reduced or shut down, but all mRNAs are degraded at equal rates after withdrawal; (b) rates of synthesis of both classes are reduced to a similar degree after withdrawal, but Ov mRNA is more susceptible to degradation. In the latter case, estrogen withdrawal might activate nucleases specific for Ov mRNA or, alternatively, Ov mRNA may be more susceptible than the average mRNA to the normal processes of mRNA degradation. Further work is needed to distinguish between these possibilities.

It is not clear from the present data whether estrogen stabilizes mRNA molecules in general in oviduct. However, it has been demonstrated on theoretical grounds that, since ovalbumin genes are not reiterated in the genome, Ov mRNA must be several-fold more stable than the average mRNA in order to maintain its high abundance (up to 60% of total mRNA) in the estrogen-primed oviduct (Kafatos and Gelinas, 1974). Taken together with the present results, and those of Palmiter and Carey (1974), it appears that some mechanism for *selectively stabilizing* this mRNA relative to total mRNA must be operative in the presence of estrogen. Control of specific gene expression by regulating mRNA stability may also be important in other systems, as, for example, in synchronized HeLa cells (Gallwitz, 1975; Perry and Kelley, 1973), where histone mRNA stability is coupled to DNA synthesis, and the $t_{1/2}$ varies from 13 min to 11 h. However, the mechanisms which regulate these events remain obscure.

Acknowledgments

The author wishes to thank Nancy Tallman for her expert technical assistance and Shigeki Mizuno and Michael Getz for their critical comments and helpful discussions.

References

Aviv, H., and Leder, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1408.
Axel, R., Feigelson, P., and Schultz, G. (1976), *Cell 7,* 247.
Birnstiel, M. L., Chipchase, M., and Speirs, J. (1971), *Prog. Nucleic Acid Res. Mol. Biol. 11,* 351.
Birnsteil, M. L., Sells, B. H., and Purdom, I. F. (1972), *J. Mol. Biol. 63,* 21.
Bishop, J. P. (1972), in Gene Transcription in Reproductive Tissue, Diczfalusy, E., Ed., Stockholm, Karolinska Institutet, p 247.
Bishop, J. O., Morton, J. G., Rosbash, M., and Richardson, M. (1974), *Nature (London) 250,* 199.
Britten, R. J., Graham, D. E., and Neufeld, B. R. (1974), *Methods Enzymol. 29E,* 363.
Burton, K. (1956), *Biochem. J. 62,* 315.
Chan, L., Means, A. R., and O'Malley, B. W. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1870.
Cox, R. F. (1973), *Eur. J. Biochem. 39,* 49.
Cox, R. F. (1976), *Cell 7,* 455.
Cox, R. F., Haines, M. E., and Emtage, J. E. (1974), *Eur. J. Biochem. 49,* 225.
Gallwitz, D. (1975), *Nature (London) 257,* 247.
Getz, J. J., Elder, P. K., Benz, E. W., Stephens, R. E., and Moses, H. L. (1976), *Cell 7,* 255.
Gilmour, R. S., Harrison, R. P., Windass, J. D., Affara, N. A., and Paul, J. (1974), *Cell Differ. 3,* 9.
Harris, S. E., Rosen, J. M., Means, A. R., and O'Malley, B. W. (1975), *Biochemistry 14,* 2072.
Harris, S. E., Schwartz, R. J., Tsai, M., O'Malley, B. W., and Roy, A. K. (1976), *J. Biol. Chem. 251,* 524.
Hastie, N. D., and Bishop, J. O. (1976), *Cell 9,* 761.
Holmes, D. S., and Bonner, J. (1973), *Biochemistry 12,* 2330.
Kafatos, F. C., and Gelinas, R. (1974), in Biochemistry of Cell Differentiation, Paul, J., Ed., London, Butterworths, p 223.
Levy, B. W., and McCarthy, B. J. (1975), *Biochemistry 14,* 2440.
Marmur, J. (1961), *J. Mol. Biol. 3,* 208.
Mizuno, S., Tallman, N. A., and Cox, R. F. (1977), *Biochim. Biophys. Acta* (in press).
Monahan, J. J., Harris, S. E., and O'Malley, B. W. (1976), *J. Biol. Chem. 251,* 3738.
Oka, T., and Schimke, R. T. (1969), *J. Cell. Biol. 43,* 123.
O'Malley, B. W., and Means, A. R. (1974), *Science 183,* 610.
Palmiter, R. D. (1972a), *J. Biol. Chem. 247,* 6450.
Palmiter, R. D. (1972b), *J. Biol. Chem. 247,* 6770.
Palmiter, R. D. (1973), *J. Biol. Chem. 248,* 8260.
Palmiter, R. D. (1974), *Biochemistry 13,* 3606.
Palmiter, R. D. (1975), *Cell 4,* 189.
Palmiter, R. D., and Carey, N. H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2357.
Palmiter, R. D., Oka, T., and Schimke, R. T. (1971), *J. Biol. Chem. 246,* 724.
Palmiter, R. D., and Smith, L. T. (1973), *Nature (London), New Biol. 246,* 74.
Perry, R. P., and Kelley, D. E. (1973), *J. Mol. Biol. 79,* 681.
Roberts, B. E., and Paterson, B. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2330.
Ross, J., Aviv, H., Scolnick, E., and Leder, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 264.

Ryffel, G. U., and McCarthy, B. J. (1975), *Biochemistry 14,* 1385.
Shapiro, D. J., and Schimke, R. T. (1975), *J. Biol. Chem. 254,* 1759.
Sober, H. A. (1968), in Handbook of Biochemistry, Cleveland, Ohio, Chemical Rubber Co., p 57.
Spelsberg, T. C., and Cox, R. F. (1976), *Biochim. Biophys. Acta 435,* 376.
Taylor, J. M., Illmensee, R., and Summers, J. (1976), *Biochim. Biophys. Acta 422,* 324.
Tsai, S. Y., Harris, S. E., Tsai, M., and O'Malley, B. W. (1976), *J. Biol. Chem. 251,* 4713.
Vogt, V. M. (1973), *Eur. J. Biochem. 33,* 192.
Wetmur, J. G. (1976), *Annu. Rev. Biophys. Bioeng. 5,* 337.
Woo, S. L. C., Harris, S. E., Rosen, J. M., Chan, L., Sperry, P. J., Means, A. R., and O'Malley, B. W. (1974), *Prep. Biochem. 4,* 555.
Yang, R. C., Van de Voorde, A., and Fiers, W. (1976), *Eur. J. Biochem. 61,* 101.
Young, B. D., Birnie, G. D., and Paul, J. (1976), *Biochemistry 15,* 2823.
Young, B. D., Harrison, P. R., Gilmour, R. S., Birnie, G. D., Hell, A., Humphries, S., and Paul, J. (1974), *J. Mol. Biol. 84,* 555.

Effects of Photoactivated Porphyrins at the Cell Surface of Leukemia L1210 Cells†

David Kessel

ABSTRACT: When murine leukemia L1210 cells are exposed to certain porphyrins, in the presence of light, a rapid loss of cell viability occurs. We have examined structure-activity relationships, using a series of porphyrins, and have studied early effects of these agents, to elucidate their mode of action. In the system employed here, only water-soluble porphyrins were cytotoxic. The first step in cytotoxicity involved binding of porphyrin to the cell surface. Porphyrins unable to bind were inactive. An important determinant of drug binding was the partition coefficient of a porphyrin between octanol and water. In the presence of light, presumably via a singlet oxygen intermediate, a variety of effects on the cell surface and cell membrane were then produced. These included inhibition of nucleoside and amino acid transport, perturbation of permeability barriers to actinomycin D uptake, enhanced binding of the fluorescent probe 8-anilino-1-naphthalenesulfonate, inhibition of activity of 5′-nucleotidase, an ectoenzyme, and altered cell-surface properties, measured with a two-phase aqueous polymer system. In the L1210 cell line, the most potent compound tested was deuteroporphyrin IX which produced the effects mentioned above at a 5×10^{-6} M level; this drug level also prevented subsequent cell division. A tenfold higher drug level caused inhibition of intracellular nucleoside kinase activity, along with inhibition of sugar transport and of the fluorogenic interaction between 8-anilino-1-naphthalenesulfonic acid and cell components. We conclude that the initial site of photoactivated porphyrin toxicity is at or near the cell surface.

Although the cytotoxic effects of photoactivated dyes have been known for many years, the system has been studied in detail only recently (Spikes, 1975). Treatment of experimental animal tumors with hematoporphyrin, followed by exposure to light, produced a large number of long-term cures (Dougherty et al., 1975). Cytotoxic effects of photoactivated hematoporphyrin were also detected in vitro (Dougherty et al., 1976). A singlet oxygen intermediate has been implicated as the proximate cytotoxic agent (Weishaupt et al., 1976).

In this study, we examined the properties of a series of porphyrins and their effects on the murine leukemia L1210 cell, to determine structure-activity relationships. Using the most potent agent, deuteroporphyrin IX, we measured the effects of photoactivation on cell viability and on other biological processes including transport of nucleosides, sugars, and a nonmetabolized amino acid, cell permeability barriers, uptake of a fluorogenic membrane probe, and behavior of cells in a two-phase polymer system. We identified three determinants of porphyrin toxicity: quantum yield of singlet oxygen, water solubility, and the octanol-water partition coefficient. Significant effects of photoactivated porphyrins on cell-surface phenomena were found at drug levels which markedly reduced cell viability, but which did not affect the action of intracellular nucleoside kinases, nor of incorporation of precursors into nucleic acid.

Rationale

The study of the mode of action of a cytotoxic agent involves the identification of early sites of drug action. Exposure of a cell line to a growth-inhibitory compound will eventually result in cessation of synthesis of nucleic acid and protein, resulting in marked alterations in all biophysical and biochemical parameters. Furthermore, procedures commonly employed in characterization of drug action can provide ambiguous data, e.g., an agent which inhibits transport of nucleosides will therefore inhibit incorporation of exogenous labeled nucleoside into nucleic acid, but the proximate effect is on a transport process. Procedures for evaluating drug-induced damage on cell-surface phenomena are still undergoing development.

† From the Departments of Pharmacology and Oncology, Wayne State University School of Medicine, Darling Memorial Center (Grace Hospital), Detroit, Michigan 48201. *Received January 11, 1977.* Supported by a contract (N-01 CM 53789) with the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health.