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Control of Cellular Content of Chicken Egg White Protein Specific RNA during Estrogen Administration and Withdrawal†

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ABSTRACT: The mRNAs for the egg white proteins ovalbumin, ovomucoid, and lysozyme have been isolated [Groner, B., Hynes, N. E., Sippel, A. E., Jeep, S., Nguyen-Huu, M. C., & Schütz, G. (1977) *J. Biol. Chem. 252*, 6666]. In this communication we describe the isolation of conalbumin-specific mRNA. DNA complementary to these mRNAs served as hybridization probes to determine the content of egg white protein mRNA sequences in total RNA of oviducts from chicks after estradiol administration and withdrawal. Chicks that had been hormone stimulated and subsequently rapidly withdrawn from the hormone show a rapid and preferential loss of egg white protein mRNAs. Within 1 day of hormone withdrawal ovalbumin mRNA content had decreased 50-fold and conalbumin mRNA content 17-fold, while the concentration of non-egg-white protein mRNAs did not change significantly. When chicks were restimulated with estrogens following acute hormone withdrawal, ovalbumin mRNA, ovomucoid mRNA, and lysozyme mRNA accumulate with a constant rate only after a lag of approximately 3 h while conalbumin mRNA accumulates with no lag. The method of hormone administration and withdrawal used in these studies does not lead to changes in the proportion of tubular gland cells in the oviduct. Therefore, the accumulation pattern observed cannot arise from complex effects due to cellular differentiation. Transferrin which is synthesized in the liver has a protein moiety identical with conalbumin. Using stringent hybridization and assay conditions we have found that conalbumin cDNA hybridizes to liver RNA. We have determined that 500–600 molecules per cell of conalbumin mRNA are present in livers isolated from immature chicks and from hens. This result shows that estrogen can affect regulation of the same gene product in two different target tissues in a different manner.

The chicken oviduct is an organ which is sensitive to the action of the steroid hormones estradiol, progesterone, and testosterone. In the tubular gland cells of the hen oviduct approximately 75% of the proteins synthesized are the egg white proteins ovalbumin, conalbumin, ovomucoid, and lysozyme (Palmiter, 1972), while in the immature or hormone-withdrawn organ their synthesis is not detectable (Oka & Schimke, 1969; Palmiter et al., 1970). It has been shown that the hormonal induction of egg white proteins is a consequence of accumulation of their mRNAs (Chan et al., 1973; Rhoads et al., 1973; Palmiter & Smith, 1973), and investigations are aimed at determining the mechanism by which steroid hormones exert control of mRNA content.

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Fifteen to thirty minutes following administration of estrogen to hormone-withdrawn animals the binding of the estrogen receptor complex to oviduct chromatin reaches a saturating level (Palmiter et al., 1976; Kalimi et al., 1976). Yet it has been demonstrated that there is a lag in the appearance of $mRNA_{oa}$[1] activity (Palmiter, 1973) and more recently in the appearance of $mRNA_{oa}$ sequences (Cox et al., 1974; McKnight et al., 1975; Harris et al., 1975; Palmiter et al., 1976; Schütz et al., 1978). Cell-free translation experiments have shown no lag in the appearance of $mRNA_{con}$ activity (Palmiter et al., 1976), a result which suggests that although these two proteins are controlled by the same hormone in the same target cell, their mode of regulation is different.

Using cDNA hybridization techniques we have measured the concentration and kinetics of accumulation following hormone stimulation of the mRNAs coding for the four major egg white proteins ovalbumin, conalbumin, ovomucoid, and lysozyme. We have also studied the effects of hormone withdrawal upon these specific mRNAs. To avoid difficulties arising from changes in the cellular composition of the oviduct we have developed a new method of estrogen administration and withdrawal. The acute hormone withdrawal has allowed us to measure specific mRNA content in oviducts containing the same proportion of differentiated tubular gland cells. Our results indicate that, in addition to $mRNA_{oa}$, the mRNA sequences coding for ovomucoid and lysozyme only begin to accumulate with a constant rate approximately 3 h following hormone administration while $mRNA_{con}$ accumulates with no detectable lag period.

Measurements of $mRNA_{oa}$ synthesis in isolated oviduct nuclei (Schütz et al., 1978; Nguyen-Huu et al., 1978a,b) have revealed that its accumulation following hormonal induction is primarily due to transcriptional activation of the ovalbumin gene. Additionally, estradiol might directly influence the stability of $mRNA_{oa}$ since it has been shown that following acute hormone withdrawal ovalbumin mRNA activity is lost more rapidly than expected (Palmiter & Carey, 1974) from the $t_{1/2}$ of 24 h measured in the presence of estrogens (Palmiter, 1973). We report herein that following acute hormone withdrawal there is rapid and specific decay of egg white protein mRNA sequences. Recently, similar findings have been obtained for the decay of $mRNA_{oa}$ sequences upon hormone withdrawal (Cox, 1977). The effect of estradiol upon mRNA synthesis as well as upon mRNA stability illustrates the complexity of its mode of action.

The polypeptide chain of conalbumin which is an iron-binding protein appears to be identical with that of transferrin, synthesized in the liver (Ogden et al., 1962; Williams, 1962; Schimke et al., 1975). Using stringent hybridization and assay conditions we have determined that $cDNA_{con}$ hybridizes to liver RNA. We have also determined that, in contrast to the striking increase in the amount of $mRNA_{con}$ present in the oviduct following hormone administration, the $mRNA_{con}$ level in the liver is rather independent of the presence of estrogen. These results suggest that although both tissues are target organs for estrogens and both possess cytoplasmic and nuclear receptors, estrogen has a profound effect on the expression of the conalbumin gene in the oviduct, whereas the synthesis of the same gene product in the liver is independent of estrogen.

Experimental Procedures

Hormone Treatment. Silicon tubing (1.5-mm inner diameter) was cut into 25-mm pieces and filled with 25–30 mg of DES. Three tubings were implanted subcutaneously into the leg of 3–4 week old White Leghorn chicks (primary stimulation). Ten days following implantation the tubings were removed, and 3 days later the chicks were injected with 2 mg of DES daily in sesame oil (secondary stimulation). At various times following secondary stimulation the animals were sacrificed, and the oviducts and livers were removed, weighed, and quick frozen in liquid nitrogen. When oviducts were removed from mature hens the mucosal lining of the magnum portion was removed, stored frozen at −70 °C, and used for the isolation of nucleic acids.

Isolation of Total Oviduct RNA. Total RNA was isolated from oviduct and liver by a method similar to that described by McKnight & Schmike (1974). Tissue (0.5 g) was homogenized for 90 s with a Polytron at setting 4.5 in 10 mL of 25 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 1 mg/mL of heparin, and 5% sucrose. Five-tenth milliliter was immediately removed and used to determine the DNA and RNA content. Ten milliliters of 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1% $NaDodSO_4$ was added to the homogenization mixture, followed by 20 mL of phenol equilibrated with 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, and 5 mM EDTA. The mixture was shaken for 10 min at room temperature and 20 mL of chloroform–isoamyl alcohol (24:1) was added. The mixture was shaken for 10 min and the phases were separated by centrifugation. The aqueous phase was reextracted once more with phenol and chloroform–isoamyl alcohol, followed by two extractions with two volumes of chloroform–isoamyl alcohol. The nucleic acids were precipitated at −20 °C by the addition of two volumes of ethanol and were collected by centrifugation. To degrade DNA the pellet was dissolved in 2 mL of DNase buffer (5 mM magnesium acetate, 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5), 80 μg of iodoacetate-treated DNase I (Zimmerman & Sandeen, 1966) was added, and the mixture was incubated 1 h at 26 °C. The RNA was extracted with phenol and chloroform and the aqueous phase was chromatographed on a Sephadex G-50 column (1.3 × 40 cm) in water. The excluded volume was adjusted to 0.2 M sodium acetate, pH 6.0, and the RNA precipitated at −20 °C by the addition of two volumes of ethanol. The RNA was collected by centrifugation and resuspended in water. Gel electrophoresis (Loening, 1969) of the RNA samples and spectrophotometric scanning of the gels revealed that this method of preparation yielded undegraded RNA. The RNA preparation was also assayed by the diphenylamine test (Burton, 1968) and was found to be free of DNA.

Nucleic Acid Determinations. The tissue homogenates were assayed for their DNA and RNA content by a method described by Bhargava & Halvorson (1971). Ten volumes of 70% ethanol were added to 0.5 mL of the homogenization mixture and the pelleted material was washed with 10 volumes of 0.015 N $HClO_4$ in 70% ethanol. The pellet was extracted twice with 10 vol of 3:1 alcohol–ether at 65 °C for 3 min, and the remaining sediment was collected and washed with 1.0 N $HClO_4$. The pellet was resuspended in 0.1 N NaOH and incubated at 100 °C for 10 min. The mixture was chilled on ice, $HClO_4$ to 0.5 N was added, and the pellet collected by centrifugation. The supernatant was used to determine the RNA content of the homogenization mixture by the orcinol assay (Schneider, 1957). The pellet was extracted twice at

[1] Abbreviations used: $mRNA_{oa}$, ovalbumin mRNA; $mRNA_{con}$, conalbumin mRNA; $mRNA_{omu}$, ovomucoid mRNA; $mRNA_{ly}$, lysozyme mRNA; $t_{1/2}$, half-life; cDNA, complementary DNA; $cDNA_{oa}$, $cDNA_{con}$, $cDNA_{omu}$, $cDNA_{ly}$, cDNA complementary to $mRNA_{oa}$, $mRNA_{con}$, $mRNA_{omu}$, $mRNA_{ly}$; EDTA, ethylenediaminetetraacetic acid; DES, diethylstilbestrol; $NaDodSO_4$, sodium dodecyl sulfate.

70 °C with 1.0 N $HClO_4$, and the supernatant was collected and analyzed for its DNA content by the diphenylamine assay (Burton, 1968).

[^{125}I]Anticonalbumin Binding to Polysomes. Conalbumin (Sigma type I) was chromatographed on DEAE-cellulose (Mandeles, 1960) and subsequently found to be electrophoretically pure. Anticonalbumin was raised in rabbits and purified by antigen Sepharose chromatography as described (Schütz et al., 1977; Groner et al., 1977). Anticonalbumin was iodinated with the Bolton and Hunter reagent to a specific activity of 40000 cpm/μg as described earlier (Groner et al., 1977). [^{125}I]Anticonalbumin (25 μg) was incubated with 5 A_{260} units of oviduct polysomes in 25 mM NaCl, 5 mM $MgCl_2$, 10 mM Hepes, pH 7.5, and 100 μg/mL of heparin (polysomal buffer) for 1 h at 2 °C and separated by sucrose gradient centrifugation as described (Groner et al., 1977). The radioactivity in the gradient fractions was determined in a γ counter.

Purification of $mRNA_{con}$. Conalbumin synthesizing polysomes were isolated as described previously (Schütz et al., 1977; Groner et al., 1977). Chicken oviduct polysomes (2500 A_{260} units; 15 A_{260} unit/mL) were incubated with 12.5 mg (5 $\mu g/A_{260}$ unit of polysomes) of anticonalbumin in polysomal buffer for 1 h at 2 °C. The unreacted anticonalbumin was separated from the polysomes by centrifugation through 1 M sucrose in polysomal buffer. The banded polysomes were diluted to 10 A_{260} units/mL with polysomal buffer, and conalbumin-synthesizing polysomes were immunoadsorbed to sheep antirabbit antibodies coupled to cellulose. Immunoadsorbant (200 mg) was added in 200 mL of polysomal buffer and incubated for 1 h at 2 °C. The cellulose was thoroughly washed, the bound polysomes were released with 20 mM EDTA, 10 mM Hepes, pH 7.5, and 100 μg/mL of heparin, and 155 A_{260} units of polysomes were recovered. Poly(A)-containing RNA was directly isolated from the polysomes by disruption with 1% $NaDodSO_4$ and 1% sarkosyl, followed by poly(U)-Sepharose chromatography. RNA (60 μg) was eluted from the poly(U)-Sepharose column and further purified by preparative gel electrophoresis as previously described (Groner et al., 1977).

In Vitro Translation of $mRNA_{con}$. Oviduct polysomal poly(A)-containing RNA and purified $mRNA_{con}$ were translated in vitro using the rabbit reticulocyte lysate system (Pelham & Jackson, 1976). One hundred and one hundred and sixty nanograms, respectively, were added to 25-μL reactions. [^{35}S]Methionine (2.3×10^6 and 1.6×10^6 cpm, respectively) was incorporated into acid insoluble material. Labeled protein containing about 10^5 cpm from each assay was analyzed by $NaDodSO_4$ polyacrylamide gel electrophoresis (Laemmli, 1970). Five micrograms of each in vitro protein synthesis assay was diluted into 500 μL of NaCl-*P* containing 1% Triton X-100 and 5 μg each of ovalbumin and conalbumin. Antiovalbumin and anticonalbumin (120 μg each) were added, and the resulting immunoprecipitates were washed, dissolved, and analyzed simultaneously with the poly(A)-containing RNA and $mRNA_{con}$ directed radioactive protein samples by $NaDodSO_4$ gel electrophoresis. The gel was fluorographed (Bonner & Laskey, 1974) and the film scanned with a densitometer. The two lanes of the gel containing the immunoprecipitates were subsequently cut into 2-mm slices and the radioactivity profile was determined.

Synthesis and Purification of $cDNA_{con}$. The $mRNA_{con}$ recovered from the preparative gel shown in Figure 2 was used as a template for reverse transcriptase. [^{3}H]cDNA was synthesized and isolated as described previously (Hynes et al., 1977). To ensure that this cDNA was specific for $mRNA_{con}$ it was incubated with an excess of purified $mRNA_{oa}$ to an R_0t of 1×10^{-2}, a value greater than 10 times the $R_0t_{1/2}$ of the hybridization of $mRNA_{oa}$ with $cDNA_{oa}$. The single-stranded cDNA was then separated from the hybrids by fractionation on hydroxyapatite, as described previously (Sippel et al., 1977). The hybridization mixture was diluted 50-fold with 0.03 M sodium phosphate buffer, pH 6.8, and applied to hydroxyapatite, followed by a wash with 4 mL of this same buffer heated to 60 °C. Single-stranded cDNA was eluted by washing the column with 0.12 M sodium phosphate, pH 6.8, at 60 °C. Double-stranded material was eluted by raising the buffer concentration to 0.6 M. The single-stranded cDNA was pooled, incubated for 16 h in 0.3 N KOH at 37 °C, neutralized, and passed through a column of Sephadex G-50. The excluded volume was pooled, and the cDNA was precipitated by the addition of two volumes of ethanol and collected by centrifugation.

cDNA Synthesis and Hybridization Analysis. mRNA specific for the egg white proteins ovalbumin, ovomucoid, and lysozyme was isolated by the immunoadsorption method (Schütz et al., 1977; Groner et al., 1977). cDNA specific for each mRNA was synthesized and was hybridized in excess with the various RNA populations to probe for their specific egg white protein mRNA sequence content as previously described (Hynes et al., 1977). cDNA complementary to the hormone-withdrawn oviduct mRNA population was synthesized and hybridized with an excess of various RNA populations, and the percent double-stranded cDNA in the various reaction mixtures was determined by S1 nuclease digestion as described previously (Hynes et al., 1977).

Results

Purification of $mRNA_{con}$ and Preparation of $cDNA_{con}$. In order to compare the effects of hormone withdrawal and secondary hormone administration of the cellular concentration of the egg white protein mRNAs coding for ovalbumin, conalbumin, ovomucoid, and lysozyme, we purified the mRNAs and synthesized the corresponding cDNA probes. The procedure for the isolation of specific mRNAs and the synthesis of cDNA probes for the ovalbumin, ovomucoid, and lysozyme specific nucleic acid sequences has been described previously (Groner et al., 1977; Hynes et al., 1977). The same approach was employed to purify the mRNA coding for conalbumin. This method is based on the specific recognition of nascent polypeptide chains by highly purified antibodies raised against conalbumin and the subsequent immunoadsorption of the polysome antibody complex by matrix bound anti-antibody. To test the binding of rabbit anticonalbumin to the polysome fraction synthesizing conalbumin the purified antibodies were iodinated and incubated with total oviduct polysomes. The polysomes were separated according to their size on a succrose gradient and the region containing the radiolabel was determined (Figure 1). [^{125}I]Anticonalbumin was associated with polysomes larger than the peak of ovalbumin polysomes and containing approximately 12–15 ribosomes as seen on the optical density profile. The ovalbumin and conalbumin synthesizing polysomes have been previously identified (Gonzales et al., 1974). The anticonalbumin polysome complexes were isolated by immunoadsorption to sheep antibodies coupled to cellulose. The polysomes were released with EDTA and the poly(A)-containing RNA from these polysomes was obtained by poly(U)-Sepharose chromatography. The RNA fraction was applied to a polyacrylamide gel and the optical density tracing of this gel is shown in Figure 2. Although residual 28S and 18S rRNA were present, a peak

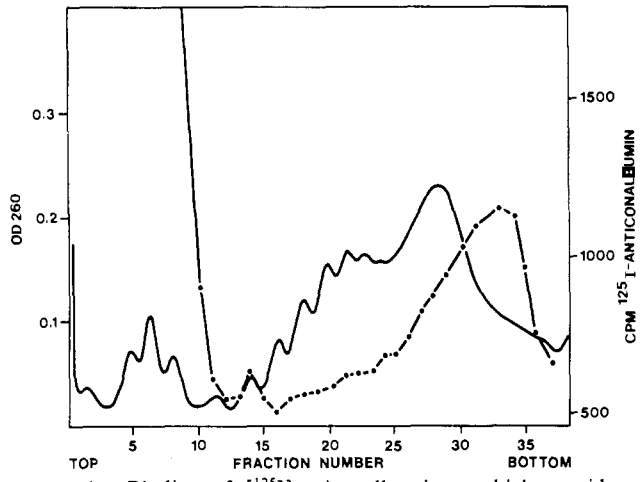

FIGURE 1: Binding of [^{125}I]anticonalbumin to chicken oviduct polysomes. Five A_{260} units of polysomes were incubated with 25 μg of ^{125}I-labeled anticonalbumin and sedimented for 90 min at 38 000 rpm through a 0.5–1.5 M sucrose gradient in a SW 41 Beckman rotor. The absorbance profile of the polysomes (—) was monitored with a Gilford gradient scanner and 5-drop fractions were collected and counted in a γ counter (●).

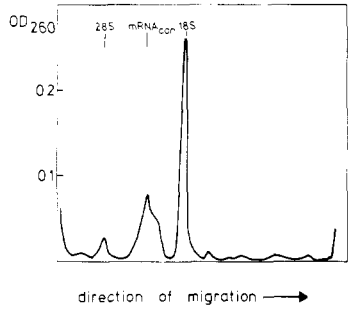

FIGURE 2: Gel electrophoresis of mRNA$_{con}$. Poly(A)-containing RNA (25 μg) obtained by poly(U)-Sepharose chromatography of RNA isolated from immunoadsorbed conalbumin synthesizing polysomes were electrophoresed for 4 h at 5 mA in a 2.5% polyacrylamide gel (Loening, 1969). The gel was scanned at 260 nm and the region containing the mRNA$_{con}$ was excised. The gel fragment was reinserted into a gel tube and overlayered with 0.25 g of oligo(dT)-cellulose. The mRNA$_{con}$ was recovered by electrophoresis for 5 h at 4 mA in 1 × electrophoresis buffer containing 0.1 M NaCl and was eluted from oligo(dT)-cellulose with water (Groner et al., 1977).

of mRNA$_{con}$ can be seen in the 21S region of the gel. This region was excised and poly(A)-containing RNA was recovered by electrophoresis onto oligo(dT)-cellulose. In order to verify that the isolated RNA was mRNA$_{con}$ it was translated in a reticulocyte lysate system. Figures 3A,B show the polypeptide patterns obtained when, respectively, total oviduct poly(A)-containing RNA and purified mRNA$_{con}$ were offered as exogenous templates. The nature of the main peaks observed in Figures 3A,B was determined by immunoprecipitation of ovalbumin and conalbumin from the in vitro synthesized proteins. The radioactive polypeptides were immunoprecipitated simultaneously from the in vitro protein synthesis assays and the immunoprecipitated material was electrophoresed on polyacrylamide gels. For a more accurate quantitation of the amounts of conalbumin and ovalbumin present, the lanes of the gel were sliced and the radioactivity in each fraction was determined (Figures 3C,D). Ovalbumin and conalbumin comigrated with the major peaks in Figures 3A,B. The translation of purified mRNA$_{con}$ and the subsequent immunoprecipitation revealed a residual presence of mRNA$_{oa}$. This contamination is also indicated by the shoulder located between mRNA$_{con}$ and 18S rRNA apparent in the gel

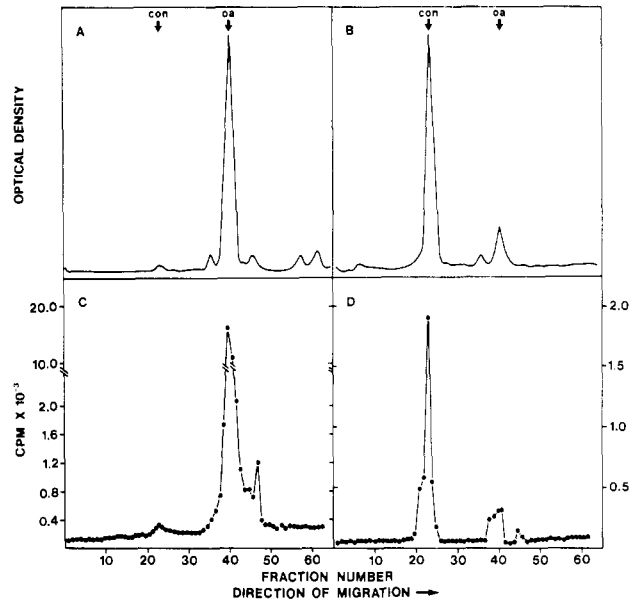

FIGURE 3: NaDodSO$_4$ polyacrylamide gel electrophoresis of in vitro synthesized protein products. In vitro protein synthesis in a rabbit reticulocyte lysate dependent upon exogeneous mRNA was directed by (A) 100 ng of oviduct polysomal poly(A)-containing RNA and (B) 160 ng of purified mRNA$_{con}$ in a 25-μL assay. Approximately 10^5 cpm of [^{35}S]methionine-labeled protein was loaded on lanes (A) and (B). After electrophoresis the gel was fluorographed and the film scanned with a densitometer. Ovalbumin and conalbumin were immunoprecipitated simultaneously from 5 μL of both protein synthesis assays. Immunoprecipitated ovalbumin and conalbumin from the protein synthesis assay were directed by (C) total poly(A)-containing RNA and (D) purified mRNA$_{con}$. Lanes (C) and (D) were sliced into 2-mm fractions after electrophoresis, Aquasol was added, and the fractions were counted in a scintillation counter.

profile of the immunoprecipitated RNA (Figure 2).

This sized mRNA$_{con}$ was used as a template for reverse transcriptase. The final cDNA product, a mixture of sequences specific for mRNA$_{oa}$ and mRNA$_{con}$, was further purified by hybridization with an excess of purified mRNA$_{oa}$. The hybridization reaction was incubated to a R_0t of 1.0×10^{-2}, a value greater than 10 times the $R_0t_{1/2}$ of mRNA$_{oa}$ with cDNA$_{oa}$ (Hynes et al., 1977). The single-stranded cDNA was separated from the hybrids by fractionation on hydroxyapatite as described under Experimental Procedures. The purity of the final cDNA$_{con}$ was analyzed by hybridization with purified mRNA$_{oa}$. Increasing amounts of cDNA$_{con}$ were hybridized to completion with excess mRNA$_{oa}$. In all cases the percent double-stranded cDNA remained at the background level of 7%. The size of the cDNA$_{con}$ was determined by electrophoresis on alkaline agarose gels (McDonell et al., 1977). The cDNA$_{con}$ was comprised of a heterogeneous polulation containing molecules with an average size of approximately 800 nucleotides. The cDNA contained molecules electrophoresing in the area of the gel expected for full-length transcripts, but these molecules represented a smaller proportion of the total product than is seen for cDNA$_{oa}$, cDNA$_{omu}$, and cDNA$_{ly}$. The observation that the reverse transcription of mRNA$_{con}$ leads to a low proportion of full-length transcripts has recently been described by others (Buell et al., 1978; Lee et al., 1978). In the presence of an excess of hen oviduct polysomal poly(A)-containing RNA, purified cDNA$_{con}$ hybridized to a maximum value of 65%. This lowering from the theoretical 100% value is due in part to the hydroxyapatite purification step in which any nonhybridizable cDNA fragments are isolated with the single-stranded cDNA$_{con}$ fraction. The cDNA$_{con}$ was hybridized with the various RNA preparations described below and the mRNA$_{con}$ content was determined

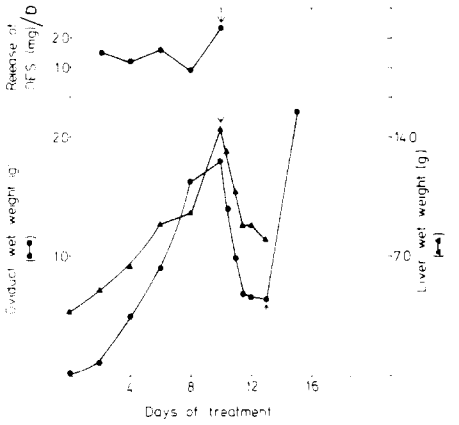

FIGURE 4: Wet weight of oviduct and liver following estrogen administration and withdrawal. Three silicon tubings each containing 25–30 mg of DES were implanted subcutaneously in the legs of immature chicks. At day 10 the tubings were removed and at day 13 the chicks received daily intramuscular injections of 2 mg of DES. At various times the oviducts (●) and livers (▲) from three animals were removed and weighed. The upper panel shows the amount of DES released per day as determined by weighing the tubings before and after implantation.

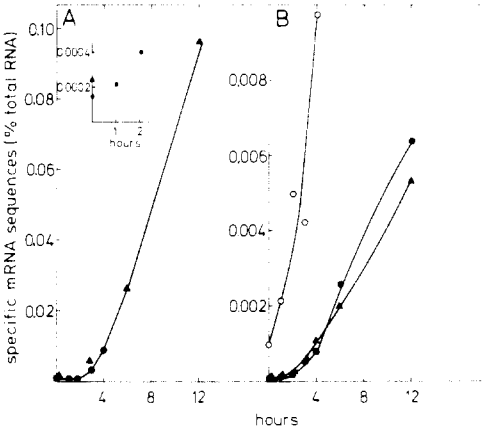

FIGURE 5: Accumulation of ovalbumin, conalbumin, ovomucoid, and lysozyme mRNA sequences following secondary hormone stimulation. Total RNA isolated from oviducts secondarily stimulated with hormone for the indicated times was assayed for panel A, $mRNA_{oa}$ content; panel B, $mRNA_{omu}$ (●), $mRNA_{ly}$ (▲—▲), and $mRNA_{con}$ (○) content. Increasing amounts of the various RNA preparations were incubated with an excess of cDNA specific for each egg white protein mRNA for a length of time sufficient to ensure completion of each hybridization reaction. Percentage of hybridization was determined as described under Experimental Procedures.

by comparison with the standard curve shown in Figure 8A.

Induction of Egg White Protein mRNAs Following Secondary Hormone Stimulation. To characterize the effect of estrogens on the regulation of different gene products we have isolated the mRNAs coding for the major egg white proteins ovalbumin, conalbumin, ovomucoid, and lysozyme (Groner et al., 1977) and have studied the kinetics of their induction following secondary hormone stimulation. A novel regime of hormone administration and withdrawal allowed us to perform this study with oviducts containing a constant proportion of tubular gland cells throughout the period of induction and deinduction. This method involves primary administration of hormone to immature chicks by implantation of silicon tubings containing DES. The tubings which act as a continuous source of hormone are removed after 10 days, and 3 days later the chicks are secondarily stimulated with hormone for various time periods. This method of hormone withdrawal is suitable because, with respect to the egg white protein mRNA sequence content, acutely withdrawn oviducts are comparable to chronically withdrawn organs. Morphologically, however, 80% of the cell mass of the acutely withdrawn organ, as estimated by histological criteria (not shown), is comprised of tubular gland cells. This is in contrast to the chronically withdrawn oviducts which contain only 10–20% tubular gland cells (Palmiter, 1973).

Figure 4 shows that within the 10 days of hormone implantation the oviduct increases steadily in weight from 20 mg to 1.8 g. Removal of the DES-containing tubings after 10 days is followed by a rapid weight loss, and, after 3 days of withdrawal, secondary hormonal stimulation is accompanied by a rapid weight gain. The hormone-dependent fluctuations have also been observed in the weight of chicken liver and are represented in Figure 4. The ratio of RNA content to DNA content fluctuates with the hormonal state of the oviduct and allows calculations of the RNA content per cell. Following hormone removal there is a rapid loss in total RNA (Figure 6), and the RNA/DNA ratio drops from a value of 3.7 to 1.6 after 3 days of acute hormone withdrawal. A ratio of 1.5 has previously been reported for chicks chronically withdrawn from hormone for a 12-day period (Oka & Schimke, 1969). The number of cells present in the oviduct tissue, however, does not change appreciably as evidenced by the slight change in DNA content following hormone withdrawal (Oka and Schimke, 1969, unpublished observations). Therefore, the change in the RNA/DNA ratio reflects a loss of RNA within the tubular gland cells. To further characterize the withdrawn state of the organ, 3 days after removal of the hormone source the concentration of $mRNA_{oa}$ was determined and compared to its concentration in oviducts chronically withdrawn from hormone. $mRNA_{oa}$ was found to represent 2.8×10^{-4} and 2.5×10^{-4}% of the total RNA of, respectively, acutely and chronically hormone-withdrawn oviducts. These values agree well with published values for the percent of total RNA of chronic hormone-withdrawn oviducts represented by $mRNA_{oa}$ (Palmiter et al., 1976). Taken together, these results suggest that 3 days after removal of the DES implant (acute withdrawal) the oviduct can be considered withdrawn and is appropriate to use in studies of secondary hormone stimulation.

Chicks from which the DES-containing tubes had been removed for a 3-day period were given hormone injections for various periods of time. The total RNA from the oviduct was isolated and specific mRNA content was determined by hybridization to the egg white protein mRNA specific cDNAs. Figure 5A shows the induction kinetics for $mRNA_{oa}$ sequences, measured as a percent of total RNA, in response to secondary hormone stimulation. The inset shows that even at very early times there is a slight increase in $mRNA_{oa}$ sequence concentration. The constant rate of accumulation, however, does not begin until after a lag of approximately 3 h. Figure 5B shows the secondary induction kinetics of $mRNA_{con}$, $mRNA_{omu}$, and $mRNA_{ly}$. $mRNA_{omu}$ and $mRNA_{ly}$ also display a lag of approximately 3-h duration before beginning to accumulate with a maximal rate while $mRNA_{con}$ appears to accumulate linearly with little or no lag phase.

These results suggest that since the mRNAs coding for ovalbumin, ovomucoid, and lysozyme have similar kinetics of induction, that is, they accumulate following a lag phase, they may be controlled by estrogen in a similar manner. This control mechanism appears to be different for conalbumin, since the mRNA coding for it accumulates with little or no lag. These results suggest that the lag phase seen here and in other reports (Palmiter, 1973; Cox et al., 1974; McKnight et al., 1975; Palmiter et al., 1976) is not related to effects of the hormone upon cellular differentiation but rather upon the rate of mRNA synthesis or upon its stabilization since the

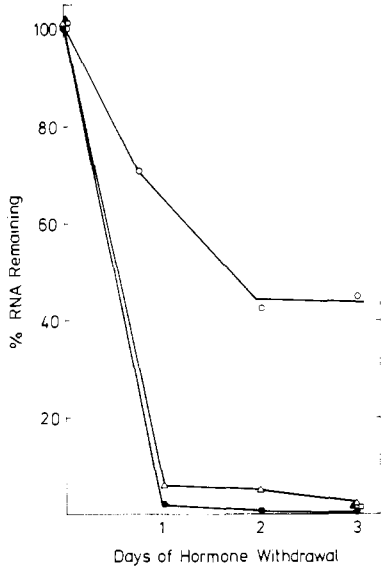

FIGURE 6: Loss of total oviduct RNA and egg white protein specific mRNA following acute hormone withdrawal. Total RNA was isolated from oviducts removed from chickens which had been primary stimulated for 10 days and subsequently withdrawn from the hormone. The concentrations of $mRNA_{oa}$ (●), $mRNA_{con}$ (△), $mRNA_{omu}$ (□), and $mRNA_{ly}$ (▲) sequences were measured by hybridizing total RNA with an excess of the specific cDNA. The data are plotted as the percent of the mRNA concentration found after 10 days of primary induction. The loss in total RNA (○) was determined from the RNA/DNA ratios.

oviducts used in our studies had the same proportion of tubular gland cells in the presence or absence of the hormone.

Preferential Decay of Egg White Protein mRNAs Following Estradiol Withdrawal. The results in the previous section show that estradiol has an effect upon the egg white protein mRNA production. Evidence arising from measurements of egg white protein mRNA synthesis in isolated oviduct nuclei (Schütz et al., 1978; Nguyen-Huu et al., 1978a,b) shows that the accumulation of these mRNAs is primarily due to transcriptional activation of their respective genes. Steroid-dependent changes in the stability of these mRNAs may, however, also be of importance in achieving the high accumulation rates observed. Following acute hormone withdrawal oviduct translatable $mRNA_{oa}$ activity decreases more rapidly than would be predicted (Palmiter & Carey, 1974) from the $mRNA_{oa}$ $t_{1/2}$ of 24 h determined in the hormonally stimulated organ (Palmiter, 1973). To distinguish between inactivation and degradation of mRNA sequences we followed the deinduction of the mRNAs by hybridization with cDNA.

Figure 6 shows the loss of oviduct egg white protein specific mRNA sequences following acute hormone withdrawal. After 1 day of hormone withdrawal, the amount of $mRNA_{oa}$ in the total oviduct RNA drops by a factor of 50 and by 3 days of withdrawal by a factor of several thousand. The level of $mRNA_{con}$ drops 50-fold while the levels of $mRNA_{omu}$ and $mRNA_{ly}$ are several hundred fold lower following 3 days of hormone withdrawal. Figure 6 also shows the loss of total oviduct RNA. Following 1 day of hormone withdrawal, the total RNA decreases by a factor of 1.4 and at 3 days by a factor of 2.2. These results show that, while total RNA also decreases upon hormone withdrawal, there is a preferential loss of mRNAs coding for egg white proteins.

The specificity of the decay of egg white protein mRNAs is further revealed by an analysis of total mRNA content of the withdrawn organ. cDNA was prepared using polysomal poly(A)-containing RNA isolated from chronically hormone-withdrawn oviducts. This cDNA has been shown to

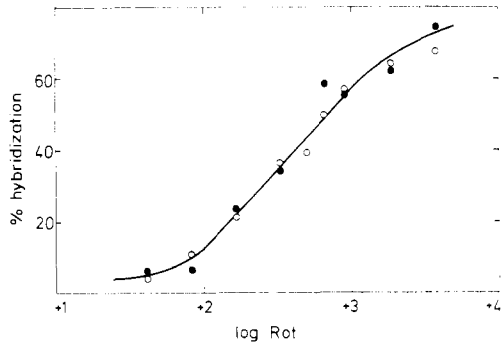

FIGURE 7: Analysis of total mRNA content following hormone withdrawal. DNA complementary to the polysomal poly(A)-containing oviduct mRNA of hormone-withdrawn animals was annealed with 15 mg/mL of oviduct RNA obtained from chicks which had been treated with DES for 10 days (●) and subsequently withdrawn from the hormone for 1 day (○). Aliquots were removed and assayed as described under Experimental Procedures. The percentage of cDNA hybridized is plotted against log R_0t, the product of RNA concentration (mol of nucleotides/L) × time (s).

represent all the different mRNA species present in the hen oviduct (Hynes et al., 1977) and thus can be used to probe the total RNA isolated from 10-day hormone-stimulated chick oviducts and from 1-day hormone-withdrawn chick oviducts for total mRNA sequences. Figure 7 shows the kinetics of hybridization of this cDNA in the presence of total RNA isolated from both oviduct types. This cDNA can be hybridized to greater than 90% with an excess of oviduct mRNA (see Figure 3 of Hynes et al., 1977), but in order to hybridize it to completion in the presence of total oviduct RNA extremely high R_0t values must be reached. Figure 7 shows that at the highest R_0t values attained (4×10^3) both preparations of RNA drove approximately 70% of the cDNA into hybrid molecules. Additionally, the figure reveals that the kinetics of hybridization of the cDNA to the two different oviduct RNA populations are essentially identical with a $R_0t_{1/2}$ values of $(3–4) \times 10^2$ in both cases. If the concentration of total mRNA in the total oviduct RNA decreased as the content of $mRNA_{oa}$ or $mRNA_{con}$, that is, by a factor of 17–50 in 1 day, then the kinetics of hybridization of 1-day withdrawn RNA would be shifted to 17–50-fold higher $R_0t_{1/2}$ values. The fact that the kinetics of hybridization of both RNA populations are essentially identical suggests that the 30% loss in total RNA following 1 day of hormone withdrawal reflects a parallel loss of 30% in both non-egg-white mRNA and rRNA species.

Conalbumin mRNA Content in Chick Liver Is Not Enhanced by Estradiol. Genetic (Ogden et al., 1962) and biochemical (Williams, 1962; Williams, 1968) evidence strongly suggests that the egg white protein conalbumin is identical with the serum iron-binding protein transferrin which is synthesized in chicken liver. These proteins seem to differ only in their carbohydrate moiety. It was therefore of interest to determine whether liver RNA hybridizes with $cDNA_{con}$ and to determine the influence of estrogen on the concentration of $mRNA_{con}$ in the liver of immature chick and hen. When we used total RNA in excess of conalbumin cDNA from either hen liver or hen oviduct, no difference in the kinetics and in the extent of hybridization was observed. To determine the concentration of conalbumin mRNA total RNA was isolated from immature chick liver and from hen liver, and increasing amounts were hybridized to completion with an excess of $cDNA_{con}$ as previously described (Hynes et al., 1977). The results, shown in Figure 8, reveal that $cDNA_{con}$ is able to hybridize to the liver RNA populations under stringent hybridization and assay conditions. By use of the same conditions

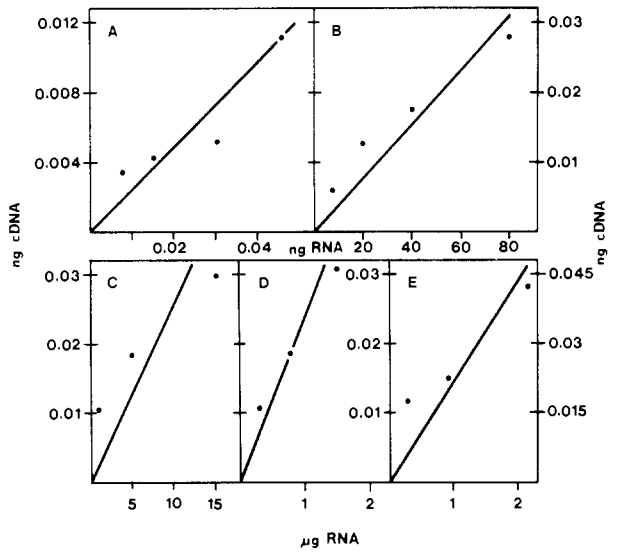

FIGURE 8: Saturation hybridization of various RNA populations with $cDNA_{con}$. $cDNA_{con}$ (0.06–0.10 ng) was incubated with increasing amounts of various RNA populations for a length of time sufficient to ensure completion of each hybridization reaction. Percentage of hybridization was determined as described under Experimental Procedures. A background value of 7% has been subtracted. The amount of RNA in each reaction is plotted vs. the amount of cDNA hybridized. The panels represent (A) immunoprecipitated and sized $mRNA_{con}$, (B) total hen oviduct RNA, (C) total acute hormone-withdrawn chick oviduct RNA, (D) total hen liver RNA, and (E) total acute hormone-withdrawn chick liver RNA.

the cDNA representing another egg white protein mRNA, $cDNA_{oa}$, was not able to hybridize to liver RNA above background levels (data not shown). As a comparison, the hybridization of total RNA isolated from hen oviduct and from 3-day acute hormone-withdrawn chick oviduct with $cDNA_{con}$ is also shown in Figure 8. By comparing the hybridization of $cDNA_{con}$ to purified $mRNA_{con}$ with its hybridization to each of the other RNA populations, it is possible to calculate the $mRNA_{con}$ content in each of the four organs. The standard hybridization curve reveals that approximately 4 pg of $mRNA_{con}$ is needed to hybridize 1 pg of $cDNA_{con}$. This divergence from the expected ratio of 1:1 is most likely due to the fact that the average cDNA molecule is 800 nucleotides, a length approximately four times shorter than $mRNA_{con}$ (Buell et al., 1978; Lee et al., 1978). Table I presents the percentage of $mRNA_{con}$ in the RNA populations of hen and hormone-withdrawn chick oviduct and its percentage in the RNA of hen and hormone-withdrawn chick liver. The number of $mRNA_{con}$ molecules per cell was calculated based upon the RNA/DNA ratio of each organ type and upon the molecular weight of $mRNA_{con}$. The results reveal the dramatic effect which estrogen has upon the $mRNA_{con}$ content in the tubular gland cells of the oviduct. There is approximately a 400-fold increase in the number of $mRNA_{con}$ molecules per oviduct cell when the hormone-withdrawn organ is compared to the mature organ. In the liver cells, however, there is only a minor increase in the number of $mRNA_{con}$ molecules per cell during development of the chicken to a laying hen, a result which indicates a differential mechanism of control of $mRNA_{con}$ sequences in the two organs.

Discussion

This and our previous study (Hynes et al., 1977) have been performed to obtain insight into the events of oviduct mRNA production during hormone stimulation and withdrawal. By analysis of the total mRNA population we have shown that

Table I: Concentration of $mRNA_{con}$ Sequences in the Total RNA of Chick Liver and Oviduct

tissue	$mRNA_{con}$ sequences in total RNA (%)[a]	RNA/DNA[b]	molecules of $mRNA_{con}$ per cell[c]
oviduct			
immature chick[d]	0.00104	1.6	25[e]
hen	0.156	3.7	9099
liver			
immature chick[d]	0.0088	4.15	576
hen	0.0098	4.25	657

[a] Values were taken from experiments in Figure 8 and calculated as explained in the Results section according to the formula % $mRNA_{con}$ in total RNA = [(pg of $cDNA_{con}$ hybridized × 100)/(pg of total RNA input)] × 4. [b] Determined for each organ as described under Experimental Procedures. [c] Molecules per cell = fraction $mRNA_{con}$ × (RNA/DNA) × [(1 μg of DNA)/(4×10^5 cells)] × [(6×10^{23} molecules)/(9.5×10^{11} μg of $mRNA_{con}$)]. [d] Value from 3-day acutely hormone-withdrawn chick oviducts. [e] The method employed for acute hormone withdrawal did not lead to any noticeable loss of tubular gland cells as determined histologically and, therefore, no correction for the fraction of cells synthesizing $mRNA_{con}$ was made.

the number and type of structural genes expressed are very similar in the presence and absence of the hormone, implying that estrogens lead to quantitative rather than qualitative changes in the mRNA population. In this communication we present data on the detailed time course of the accumulation of the mRNAs coding for the egg white proteins following hormone stimulation as well as evidence that in addition to changes in the rate of transcription of the egg white protein genes (Schütz et al., 1978; Nguyen-Huu, 1978b) the stability of these specific mRNAs is controlled by estrogens.

It has previously been observed that upon withdrawal of estrogen-stimulated chicks from hormone the ovalbumin-synthesizing mRNA activity decreases more rapidly than expected (Palmiter & Carey, 1974). Our results and those of Cox (Cox, 1977) indicate that not only the translational activity disappears rapidly but also that the RNA sequences are lost more rapidly after estrogen withdrawal than predicted from the $t_{1/2}$ of 24 h determined for $mRNA_{oa}$ (Palmiter, 1973). Following 1 day of hormone withdrawal, $mRNA_{oa}$ has decreased a factor of 50 and $mRNA_{con}$ a factor of 17 and total RNA a factor of 1.4. The results show furthermore that this rapid decay is specific for the egg white protein mRNAs, since the concentration of the non-egg-white-protein mRNAs in total RNA does not change significantly during estrogen withdrawal. There are not enough measurements of mRNA content in the early times of hormone withdrawal to accurately determine a $t_{1/2}$ for the mRNAs, but the fact that $mRNA_{oa}$ has dropped a factor of 50 and $mRNA_{con}$ a factor of 17 in 24 h suggests that both mRNAs will have a $t_{1/2}$ in the order of 2–5 h in the absence of the hormone. This finding indicates that estrogens selectively stabilize the egg white mRNA sequences in the tubular gland cell. This effect most likely occurs in the cytoplasm and it will be of great interest to see whether it is a direct or indirect result of the action of the hormone receptor complex.

An interesting point which has arisen from these experiments is the fact that the level of $mRNA_{con}$ in the 3-day acute hormone-withdrawn oviduct never reaches the low level seen for the other egg white protein mRNAs. Approximately (1.0×10^{-3})% of the 3-day hormone-withdrawn oviduct RNA represents $mRNA_{con}$ while ovalbumin, ovomucoid, and lysozyme mRNAs represent respectively (2.8×10^{-4})%, (1.0×10^{-4})%, and (6.0×10^{-5})% of the RNA. These percentages

represent 7–9 molecules per cell of $mRNA_{oa}$, $mRNA_{omu}$, and $mRNA_{ly}$, while the mRNA coding for conalbumin is present at approximately 25 copies per cell. This is probably due to the action of endogenous estrogens since the dose for half-maximal induction of conalbumin is 2.5 times lower than for ovalbumin (Mulvihill & Palmiter, 1977).

In order to link entry of the hormone receptor complex into the nucleus to changes in mRNA production, the time courses of accumulation of the mRNAs for ovalbumin, conalbumin, ovomucoid, and lysozyme have been studied. The patterns of accumulation for these mRNA species which are synthesized in the same target cells are not the same. Following hormone treatment, there is a lag phase of approximately 3 h during which the mRNAs for ovalbumin, ovomucoid, and lysozyme change only slightly in concentration. After 3 h, the rate of accumulation changes sharply and is at least an order of magnitude greater than during the lag phase. In contrast, conalbumin-specific mRNA accumulates without a lag. This suggests that three of the egg white protein mRNAs are coordinately controlled and that some as yet undefined step occurs between hormone receptor–protein complex migration into the cell nucleus and their accumulation. This lag period is obviously of great importance for our understanding of steroid action. Experiments from our laboratory indicate that the lag is also present in the transcriptional activation of these egg white protein genes (Nguyen-Huu et al., 1978b).

In this work we have ruled out the possibility that the lag phase arises from an artefact due to tubular gland cell differentiation since we have used a regime of hormone treatment in which the hormone-withdrawn oviduct retains almost all of its tubular gland cells. In previously reported studies, withdrawn oviducts have apparently involuted into organs retaining only 10–20% tubular gland cells (Cox et al., 1974; Harris et al., 1975; McKnight et al., 1975; Palmiter et al., 1976).

A third series of experiments which we have performed reveal that estrogen has different effects upon the accumulation of the same gene product in different target tissues. The chicken liver is also responsive to the action of estrogens since it is the site of synthesis of the egg yolk proteins (for review see Tata, 1976). Transferrin which is synthesized in chicken liver has a protein moiety identical with that of conalbumin (Williams, 1962; Williams, 1968). Employing stringent hybridization and assay conditions we have determined that $cDNA_{con}$ is able to hybridize to liver RNA isolated from immature chicks and hens and that steroid hormones cause no or only a slight increase in the number of $mRNA_{con}$ molecules in liver cells of hormonally stimulated, mature chicken. This is in contrast to the striking effect which estrogen has upon the $mRNA_{con}$ content of oviduct cells. The hormonally stimulated, mature oviduct cells contain a 400-fold increased level of $mRNA_{con}$ when compared to the hormone-withdrawn chick oviduct cells. During the preparation of this manuscript Lee et al. (1978) reported similar results. These authors also find that the effect of steroids upon the liver $mRNA_{con}$ content is much less pronounced than the effect upon the oviduct $mRNA_{con}$ levels.

Two interpretations of the results are possible. One is that each chicken cell contains two genes coding for conalbumin. One gene is estrogen sensitive and active in hormone-mature oviduct cells, while the other is insensitive to the action of estrogen and is constitutively active in liver cells. This possibility can be tested in part by determining the conalbumin-specific gene number with cDNA excess hybridizations as first described by Bishop & Freeman (1973) and by identification of restriction enzyme-digested DNA fragments containing the conalbumin gene and its adjacent DNA sequences (Southern, 1975). Lee et al. (1978) show by hybridization analysis that there are no more than two genes per haploid chicken cell specific for conalbumin so this possibility still remains open. The second possibility is that only one gene specific for conalbumin exists in all chicken cells and that various factors allow estrogen to exert an effect upon its transcription in oviduct cells but not in liver cells. These differential effects might be based upon differences in the cellular components thought to be involved in the mediation of estrogen action. The steroid receptors existing in the oviduct and in the liver may be dissimilar, thus resulting upon hormone stimulation in the activation of a different set of genes in the two organs. Alternatively, the hormone receptor–acceptor sites may not be identical. These sites may have different non-histone chromosomal proteins or different chromosomal structural features which lead to activation of the conalbumin gene in the oviduct but do not affect its transcription in the liver.

The results emphasize the pleiotropic nature of estrogen action in the chick liver and oviduct. First, the high accumulation rates of the egg white protein mRNAs after hormonal induction are due to (a) activation of the transcription of the egg white protein genes (Schütz et al., 1978; Nguyen-Huu et al., 1978b) as well as to (b) selective stabilization of the egg white protein mRNAs. Second, estrogen has various modes of action in the same target cell; this is illustrated by the lag observed in the accumulation of the ovalbumin, ovomucoid, and lysozyme mRNA, whereas conalbumin mRNA accumulates without a lag. Third, the same gene product, the conalbumin polypeptide chain, is under different types of control in liver and oviduct, even though both organs are targets for estrogens and possess cytoplasmic as well as nuclear estrogen receptors.

Acknowledgments

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Stimulation of Protein Synthesis and Met-tRNA$_f$ Binding by Phosphorylated Sugars: Studies on Their Mechanism of Action†

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ABSTRACT: It has been previously reported by J. R. Lenz et al. [(1978) *Biochemistry 17*, 80–87] that certain phosphorylated sugars stimulate protein synthesis in extracts of mammalian cells. This effect was found to be due to a stimulation of Met-tRNA$_f$ binding to 40S ribosomal subunits, both in whole extracts and with isolated ribosomes. However, formation of a ternary complex of Met-tRNA$_f$, initiation factor eIF-2, and GTP was not stimulated. It was also shown that the stimulation is not due solely to metabolism of the sugars. The present communication further characterizes the stimulatory effect of the sugars. They were found to prevent the inactivation of ribosomes that occurs during protein synthesis incubations. The sugars were also found to inhibit cAMP-dependent protein kinases noncompetitively. However, they stimulate Met-tRNA$_f$ binding to 40S ribosomal subunits even under conditions in which an inhibition of protein kinase has no effect. Although it has not been possible to demonstrate a direct association of the sugars with the 40S initiation complex, the evidence suggests that their effect is mediated by an interaction with one of the components involved in the formation of this complex.

The initiation of protein synthesis in mammalian cells and their extracts has been extensively studied as the main site of translational regulation (Lodish, 1976). One of the compounds known to exert an effect at this level is hemin (Zucker & Shulman, 1968; Weber et al., 1975), which acts by preventing the formation of an inhibitor of translation, known as the hemin-controlled repressor or HCR[1] (Maxwell et al., 1971; Gross & Rabinovitz, 1972). HCR is a cAMP-independent protein kinase that phosphorylates eIF-2, the initiation factor responsible for the binding of Met-tRNA$_f$ to native 40S (40S^N) ribosomal subunits (Levin et al., 1975; Kramer et al., 1976; Lenz & Baglioni, 1977; Farrel et al., 1977). Phosphorylated eIF-2 is much less active in initiation because it cannot respond

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[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DEAE, diethylaminoethyl; HCR, hemin-controlled repressor; G6P, glucose 6-phosphate; dG6P, 2-deoxyglucose 6-phosphate; FDP, fructose 1,6-bis(phosphate); 2AP, 2-aminopurine; eIF, eukaryotic initiation factor (nomenclature adopted at the International Symposium on Protein Synthesis, Bethesda, MD, October 18–20, 1976; see *FEBS Lett. 76*, 1–10 (1977); cAMP, cyclic adenosine monophosphate; 40S^N, native 40S ribosomal subunits; IF, initiation factors; Met-tRNA$_f$, initiator methionyl-tRNA which can be formylated with *E. coli* transformylase.

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