Ovalbumin Messenger Ribonucleic Acid Accumulation in the Chick Oviduct during Secondary Stimulation: Influence of Combinations of Steroid Hormones and Circannual Rhythms†

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ABSTRACT: We have analyzed by hybridization the accumulation of ovalbumin mRNA after the administration of estrogen, progesterone, these two hormones together, or each hormone with testosterone to "withdrawn" chicks (chicks previously stimulated with estrogen but then withdrawn from the hormone). We were interested in determining if the time lag between hormone treatment and a significant increase in ovalbumin mRNA levels could be shortened. After every hormonal treatment we found that there was a 1.5–2.5-h lag period before a significant increase in ovalbumin mRNA levels. This lag period was not simply due to the slow delivery of the hormone to the oviduct. Nuclear estrogen receptor levels had plateaued by 1 h. Rather unexpectedly, the rate of ovalbumin mRNA accumulation after this lag period seemed to depend on the time of the year. Ovalbumin mRNA accumulated faster during the summer and early fall than during the winter and early spring. These seasonal differences were also observed in ovalbumin mRNA levels 24 h after hormone treatment. Such a circannual rhythm may help explain reported differences in the rate of ovalbumin mRNA accumulation during secondary stimulation.

The chick oviduct is an excellent system in which to study the hormonal regulation of gene expression (Oka & Schimke, 1969; Kohler et al., 1969; O'Malley et al., 1969; Palmiter, 1975). The administration of estrogen to immature chicks (primary stimulation) causes the cytodifferentiation of the oviduct and the production of the egg white proteins of which ovalbumin is the most plentiful. Upon cessation of estrogen treatments the oviduct weight decreases and egg white proteins and mRNAs are destroyed. Readministration of hormone to these "withdrawn" chicks causes a rapid increase in oviduct weight and egg white proteins. While only estrogen stimulates the immature chick oviduct, both estrogen and progesterone stimulate the withdrawn chick oviduct. Testosterone alone does not stimulate the immature or withdrawn chick oviduct but does enhance the effects of the other hormones. In fact, it is only following the combined administration of all three hormones that egg white protein synthesis in the chick closely resembles that in the laying hen (Palmiter, 1972).

Numerous investigators have shown that for many of the egg white proteins the amount of that protein in the oviduct is determined by the levels of its mRNA; there is at no time sequested mRNA (McKnight et al., 1975; Lee et al., 1978; Tsai et al., 1978; Hynes et al., 1979). However, there is often a lag period between hormone treatment and an increase in egg white protein mRNA levels [such as ovalbumin mRNA (Hynes et al., 1979; McKnight et al., 1975; Palmiter et al., 1976; Spelsberg & Cox, 1976; Roop et al., 1978), lysozyme mRNA (Hynes et al., 1979), and ovomucoid mRNA (Tsai et al., 1978; Hynes et al., 1979)]. This lag period is not caused by the slow delivery of hormone and receptor to the nucleus; within 30 min after estrogen administration, nuclear levels of estrogen receptor have reached a plateau value (Palmiter et al., 1976). Furthermore, at least one egg white protein mRNA, conalbumin mRNA, starts to accumulate within 30 min after estrogen treatment (Palmiter et al., 1976). The cause of this time lag between hormone treatment and an increase in mRNA levels is unknown.

The accumulation of a particular mRNA depends not only on its rate of synthesis but also on its half-life. Both the synthesis and the half-life of the mRNA reflect the rate of several processes, many of which are interrelated. We reasoned that not every hormone may stimulate all of these processes to the same extent, nor may every mRNA be so dependent on a further (hormonally controlled) stimulation of every process. For instance, it has been reported that for ovalbumin mRNA the lag period was shorter after progesterone treatment (1.5–2 h) than after estrogen treatment (3–4 h) (McKnight et al., 1975; Palmiter et al., 1976). If this were the case, combined hormone administration might affect the early accumulation of mRNA, perhaps even eliminating this lag period before ovalbumin mRNA levels increase. To test these ideas, we decided to measure the effects of the combined administration of estrogen and progesterone or either hormone with testosterone on ovalbumin mRNA accumulation. We found that the early accumulation of ovalbumin mRNA including this initial lag period between hormone treatment and a significant increase in ovalbumin mRNA levels was similar after the administration of estrogen, progesterone, these two hormones together, or each hormone with testosterone. However, ovalbumin accumulation was greatly influenced by the time of year of the experiment. During the winter months the rate of ovalbumin mRNA accumulation after the initial lag period was reduced when compared to that observed during the summer months. This sometimes made it more difficult to define a precise lag period during the winter months because ovalbumin mRNA started to accumulate gradually and with a constantly increasing rate.

Experimental Section

Methods

Hormone Treatments. Day-old White Leghorn chicks (strain W36) were injected subcutaneously in the neck with 12 mg of diethylstilbesterol pellets (Pfizer Stimplants, New York, NY). After 12 days the pellets were removed surgically and the chicks were left 21 days before further treatment.

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Chicks were then injected with hormone dissolved in sesame oil into the leg muscle. When two hormones were being injected, hormone solutions were mixed before injection. Chicks were sacrificed at the appropriate time by decapitation. Oviduct magna were removed, immediately frozen on dry ice, and stored in liquid nitrogen. Estradiol valerate was purchased from Mutual Pharmaceutics of Nashville, TN. All other hormones were purchased from Sigma.

RNA Isolation. Total cellular RNA used to quantify ovalbumin mRNA levels was isolated according to the method of Rosen et al. (1975) as modified by us (Tokarz et al., 1979). The RNA was active in a cell-free translational system (Schimke et al., 1974).

Ovalbumin mRNA Isolation. Polysomes from laying hen oviduct were isolated by magnesium precipitation (Palmiter, 1974). Ovalbumin mRNA containing polysomes were specifically immunoprecipitated according to the method of Shapiro et al. (1974) using 150 μg of sheep antiovalbumin and 1.5 mg of rabbit antisheep IgG antibodies per 20 optical density units of polysomes. This RNA directed the synthesis of 1.7–1.9 times as much ovalbumin per microgram of RNA as did total polysomal RNA in a cell-free rabbit reticulocyte system (Schimke et al., 1974). This is the purification expected from oviducts of laying hens in which ovalbumin mRNA constitutes 50–60% of all mRNA.

Both antibodies used for the immunoprecipitation were purified from a 35% ammonium sulfate precipitation fraction of serum by passage over an ovalbumin–Sepharose or a sheep IgG–Sepharose column, respectively. The antiserum was loaded on the appropriate column in PBS[1] (0.15 NaCl and 10 mM potassium phosphate, pH 7.4) and rinsed with PBS until eluted optical density was negligible. Pure antibody was eluted in 0.2 M acetic acid, neutralized with 0.5 equiv of ammonium hydroxide, and dialyzed against PBS. Such antisera were RNase-free as determined by RNA or polysome profiles on sucrose gradients (results not shown).

The ovalbumin (ICN) used to immunize the sheep and make the affinity column was purified by DEAE-cellulose chromatography (Mandeles, 1960). The sheep IgG was purified from the 35% ammonium sulfate fraction of serum by passage through DEAE-cellulose in 10 mM sodium phosphate, pH 7.5, and 15 mM NaCl. Both proteins were linked to CNBr-activated Sepharose 6B according to the method of Arndt-Jovin et al. (1975) (20 mL of Sepharose 6B was activated by 5 g of CNBr dissolved in 10 mL of cold dimethylformamide).

Ovalbumin cDNA. Tritiated ovalbumin cDNA was made from ovalbumin polysomal mRNA (600 μg/mL) by using the technique of Friedman & Rosbach (1977), except that the concentration of actimomycin D was 200 μg/mL, the concentration of [^{3}H]dCTP (ICN), 25 Ci/mmol, was 40 μM, and that of RNA-dependent DNA polymerase was 507 units/mL. The avian myeloblastosis virus RNA-dependent DNA polymerase (reverse transcriptase) was a gift of Dr. Joseph Beard and the Office of Program Resources and Logistics, Virus Cancer Program, National Cancer Institute. After the synthesis the RNA was destroyed by 0.3 N NaOH at 68 °C for 15 min. The cDNA was purified by chromatography on a 0.7 × 60 cm G-50 medium column in 10 mM Tris, pH 7.9, 1 mM EDTA, and 0.5% *tert*-butyl alcohol. It had a specific activity of (1–2) × 10^7 cpm/μg.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; RNase, ribonuclease; CNBr, cyanogen bromide; PBS, phosphate-buffered saline.

Hybridization Assay. Hybridizations were done at 68 °C in plastic tubes overlaid with mineral oil for 2 days as described previously (Tokarz et al., 1979). Ovalbumin mRNA concentration was determined by comparing the kinetics of the hybridization of each RNA sample with that of a standard (Tokarz et al., 1979). The data were plotted as 1/(fraction of single-stranded [^{3}H]cDNA) vs. the concentration of RNA times time (C_rt) (Bishop, 1972). This method of expressing the data should result in a straight line even when the hybridization is not carried to completion or done under "RNA-excess" conditions (see theory below). Straight-line fits of these data using a least-squares program gave correlation coefficients (r^2) between 0.95 and 0.999. [for examples, see Tokarz et al. (1979)]. Every RNA sample was subjected to at least three hybridizations to determine a range of values for the amount of ovalbumin mRNA present. The results of these experiments showed the error in our determination of ovalbumin mRNA levels to be within 10–15%. The error was not larger when several different ovalbumin mRNA standards and/or different preparations of ^{3}H-labeled ovalbumin cDNA were used.

Hybridization Theory. Bishop (1972) has shown that under RNA excess conditions, the rate of hybridization of single-stranded RNA to single- or double-stranded DNA can be expressed as

$$D_0/D = 1 + kR_0t \tag{1}$$

where D, R_0, D_0, k, and t are the concentration of single-stranded DNA, the initial concentrations of RNA or DNA, the rate constant, and time, respectively. A plot of D_0/D vs. R_0t will be linear until the later stages of hybridization. If the DNA cannot reanneal with itself, as in the case of cDNA, eq 1 is valid even when the RNA is not in excess. In fact it is possible to define the exact limits of hybridization over which eq 1 is valid.

Young & Paul (1973) have shown that when single-stranded RNA is hybridized to single-stranded DNA, the amount of hybrid, H, formed with time is given by eq 2 when $R_0 \neq D_0$

$$H = \frac{R_0D_0(1 - e^{(R_0-D_0)kt})}{D_0 - R_0e^{(R_0-D_0)kt}} \tag{2}$$

where

$$k = \frac{1}{(R_0 - D_0)t} \ln \frac{D_0(R_0 - H)}{R_0(D_0 - H)} \tag{3}$$

(When $R_0 = D_0$, eq 1 is valid throughout the hybridization.) If $|(R_0 - D_0)kt| \leq 1$, eq 2 can be expanded to

$$H = D_0 - D = \frac{R_0D_0[1 - [1 + (R_0 - D_0)kt + \ldots]]}{D_0 - R_0[1 + (R_0 - D_0)kt + \ldots]} = \frac{R_0D_0kt}{1 + R_0kt}$$

or

$$D_0/D = 1 + R_0kt$$

Again a plot of D_0/D vs. R_0t is linear if $|(R_0 - D_0)kt| \leq 1$. By use of eq 3, this is equivalent to

$$\ln \frac{D_0(R_0 - H)}{R_0(D_0 - H)} \leq 1 \tag{4}$$

or

$$H \leq \frac{1.73D_0R_0}{2.73(R_0 - D_0)} \tag{5}$$

For RNA excess reactions, eq 1 will be linear until the hybridization is over 60% finished. When $R_0 > D_0$ but not much

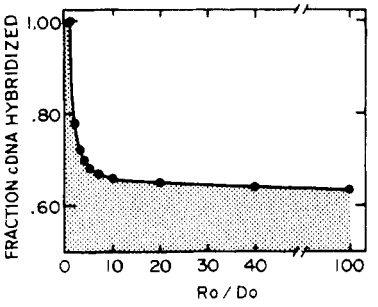

FIGURE 1: Graph of eq 5. A plot of the inverse of the fraction of single-stranded ovalbumin cDNA at the end of the hybridization vs. R_0t, the initial concentration of RNA times time, will be linear as long as the fraction of the cDNA hybridized is less than the value given by the curve (i.e., is in the shaded area).

greater than D_0, numerical calculation shows that eq 1 is linear for even higher levels of hybrid formation (Figure 1).

Occasionally, hybridizations containing low levels of ovalbumin mRNA are actually in slight DNA excess. Equation 1 is still valid depending on the degree of saturation (hybrid formation) and the ratio R_0/D_0. It can be shown numerically that if the hybridization is allowed to go to 63% completion, then R_0 may be 10 times less than D_0 and eq 1 is still valid. As the hybridization goes to further completion, R_0 must be a larger fraction of D_0. At 70% completion (or saturation) R_0 must be $>0.26D_0$, at 80% completion, $>0.58D_0$, and at 90% completion, $>0.81D_0$. In practice it is not difficult to satisfy these conditions even when low amounts of a specific RNA are present in the sample.

Estrogen Receptor Assay. Cytoplasmic and nuclear estrogen receptor concentrations were quantified by using the method of Sutherland & Baulieu (1976). The number of high-affinity estrogen binding sites was determined by a Scatchard (1949) plot analysis. Only "soluble" or 0.5 KCl-extractable nuclear receptor levels were measured since there is some controversy as to the meaning of the "insoluble" or non-salt-extractable receptor sites (Barrack et al., 1977).

Results

Effects of Estrogen or Progesterone Alone on Ovalbumin mRNA. Before examining the effects of combinations of hormones on ovalbumin mRNA accumulation during secondary administration, we examined the effects of estrogen or progesterone alone on ovalbumin mRNA accumulation. Day-old chicks were primed with diethylstilbestrol pellets for 12 days and then left for 21 days so that all traces of hormone would be eliminated. These withdrawn chicks were stimulated with 2 mg of estrogen or progesterone. Figures 2 and 3 (filled squares) show the accumulation of ovalbumin mRNA after the administration of estrogen or progesterone, respectively. Both groups of chicks were born and raised during the summer. Total oviduct RNA was isolated, and ovalbumin mRNA levels were determined by hybridization and expressed as the percentage of total RNA. In both cases there was no increase in ovalbumin mRNA levels above those found in withdrawn chicks until after ~2 h. In fact, ovalbumin mRNA levels in the progesterone-treated chicks increased at rates nearly identical with those found in the estrogen-treated chicks for the first 4–6 h of secondary stimulation.

These results were in agreement with those of Spelsberg & Cox (1976). However, they differed from those reported by Palmiter et al. (1976) and McKnight et al. (1975). These groups reported a faster increase in ovalbumin mRNA levels and at an earlier time after progesterone administration than after estrogen administration. Our preliminary experiments

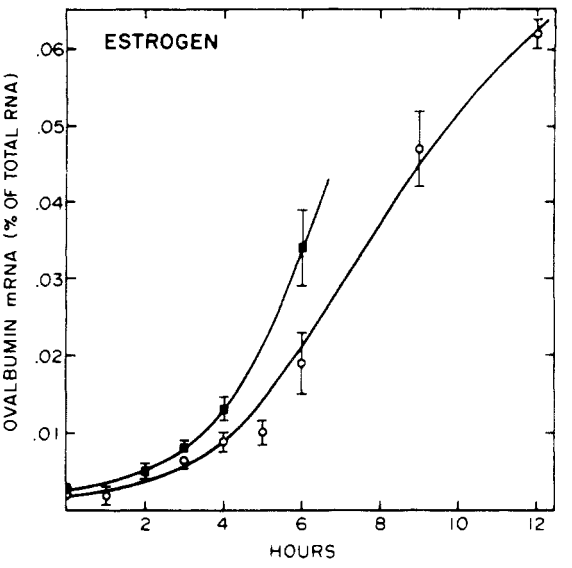

FIGURE 2: Ovalbumin mRNA accumulation after the administration of estrogen. 2 mg of estradiol valerate in sesame oil was injected into the leg muscle of chicks which had been primed with diethylstilbesterol pellets for 12 days and then withdrawn for 21 days. Total oviduct RNA was extracted and hybridized to ovalbumin cDNA according to the procedure outlined under Methods. Ovalbumin mRNA levels were determined as described in Tokarz et al. (1979). Ovalbumin mRNA levels were expressed as the percentage of total RNA. Each point represents the results of at least three separate hybridizations. The experiments were conducted in late July (■) or at the end of March (○).

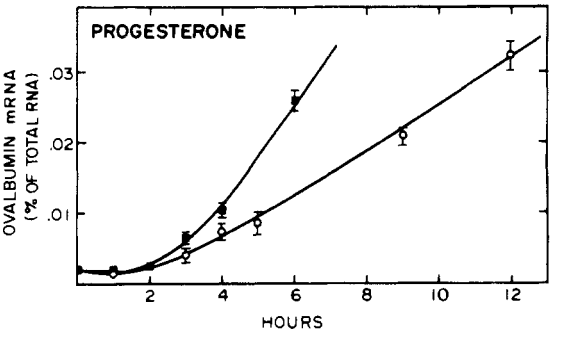

FIGURE 3: Ovalbumin mRNA accumulation after the administration of progesterone. 2 mg of progesterone in sesame oil was administered to estrogen-withdrawn chicks, and ovalbumin mRNA levels were determined as described in Figure 2. The experiments were conducted in the middle of August (■) or at the end of March (○).

indicated that these differences in the length of the lag period and the rate of accumulation of mRNA are probably not due to the method of RNA preparation or ovalbumin mRNA quantitation. We found similar yields of RNA and ovalbumin mRNA levels from laying hen oviduct by using the phenol–chloroform method of McKnight & Schimke (1974) to prepare the RNA and cell-free translation or saturation hybridization (Palmiter et al., 1976; Robins & Schimke, 1978) for quantitating ovalbumin mRNA (data not shown). These differences may be due to the strains of chicks used. Both Spelsberg (personnel communication) and we used White Leghorn Hyline W36 (see also Discussion).

Our results have been verified with a second group of chicks which were born at the same time and raised together (during the winter). During the first 5 h of secondary stimulation both estrogen and progesterone induced ovalbumin mRNA accumulation to similar degrees (Figures 2 and 3, open circles). After that time ovalbumin mRNA accumulated faster in estrogen-treated chicks than progesterone-treated chicks. Rather

Table I: Ovalbumin mRNA Levels 24 h after Secondary Stimulation

hormone administered[a]	ovalbumin mRNA[b] expt A[c]	expt B[d]
estrogen	0.23 ± 0.01	0.063 + 0.005
estrogen + progesterone	0.28 ± 0.047	0.12 ± 0.01
estrogen + testosterone	0.21 ± 0.04	0.11 ± 0.01
progesterone	0.077 ± 0.015	0.060 ± 0.003
progesterone + testosterone	0.11 ± 0.01	

[a] 2 mg of each hormone was administered. [b] As a percentage of total cellular RNA. [c] Experiment done at the end of November. [d] Experiment done at the end of March except for estrogen + progesterone which is from the beginning of March of the same year.

unexpectedly, ovalbumin mRNA levels in chicks born and raised during the winter months seemed to be less responsive to the administration of either estrogen or progesterone. This is especially apparent in chicks treated with progesterone. It is also true for estrogen-treated chicks and is even more apparent 24 h after treatment (Table I). Such seasonal (circannual) rhythms have been reported by Spelsberg et al. (1979) and Boyd & Spelsberg (1979) and affect both progesterone and estrogen nuclear receptor levels (see Discussion). In general, we (Tokarz et al., 1979) and Mulvihill & Palmiter (1977) have found that ovalbumin mRNA levels and the rate of accumulation are closely correlated with the level of the receptor in the nucleus. Thus, if less estrogen and progesterone receptor is translocated to the nucleus in the winter and spring than in the summer and fall, this should lead to decreased ovalbumin mRNA levels.

As with the data obtained during the summer months, no significant increase in ovalbumin mRNA levels was observed until after 2 h. However, with this set of chicks born and raised during the winter, it was much harder to define the lag period precisely. The rate of ovalbumin mRNA accumulation increased gradually after hormonal treatment; there was no clear break in the rate (for contrast, see Figures 5 and 6). This is especially apparent in the estrogen-treated chicks (Figure 2, open circles).

Effects of Combined Administration of Estrogen and Progesterone on Ovalbumin mRNA Accumulation. Despite the findings reported in the previous section, it was still possible that the combined administration of estrogen and progesterone might shorten or eliminate the lag period between hormone treatment and a significant increase in ovalbumin mRNA levels. Alternatively, the hormones might be antagonistic and might decrease the rate of ovalbumin mRNA accumulation and/or lengthen this lag period. Such antagonistic behavior has been reported in immature chicks but not withdrawn chicks. The effects of such a combined hormone treatment are an important result needed for the construction and/or testing of any hypothesis about the regulation of ovalbumin mRNA accumulation.

Because secondary stimulation is routinely initiated by administering 2 mg of a single hormone, withdrawn chicks were treated with 2 mg each of estrogen and progesterone (i.e., a total of 4 mg of hormones) or 1 mg each of estrogen and progesterone (i.e., a total of 2 mg of hormones) (Figure 4, open or closed symbols, respectively). The hormones were mixed together prior to injection. In both cases there was about a 1–2-h lag between hormone administration and an increase in ovalbumin mRNA levels. In fact ovalbumin mRNA accumulation after 2 mg of each hormone (Figure 4, open symbols) was not significantly different than that observed after estrogen was administered alone during the same period

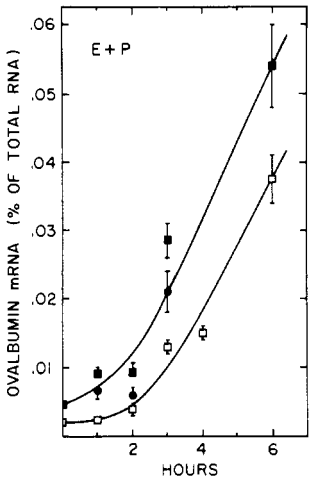

FIGURE 4: Ovalbumin mRNA accumulation after the combined administration of estrogen and progesterone. Ovalbumin mRNA levels were determined as described in Figure 2. Estrogen-withdrawn chicks were treated with 2 mg each of estrogen and progesterone at the end of October (□) or 1 mg each of estrogen and progesterone at the beginning of October (■). Another group of chicks was also treated with 1 mg each of progesterone and estrogen at the beginning of November (●) of the following year.

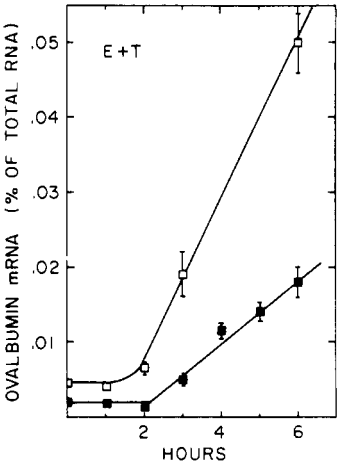

FIGURE 5: Ovalbumin mRNA accumulation after the combined administration of estrogen and testosterone. Estrogen-withdrawn chicks were treated with 2 mg each of estrogen and testosterone, and ovalbumin mRNA levels were determined as described in Figure 2. The experiments were conducted at the beginning of December (■) or the beginning of November (□).

of the year (Figure 2, closed squares). Note that similar results were obtained in two groups of chicks which were treated with hormone during the fall but in different years (Figure 4, closed symbols). From our data it seemed that after administration of 1 mg of each hormone, ovalbumin mRNA accumulation was somewhat faster than after 2 mg of each hormone. This is consistent with the data of Spelsberg & Cox (1976). Large doses of hormone tend to suppress the activation of and even inhibit RNA polymerase II, the polymerase responsible for mRNA transcription. However, even though ovalbumin mRNA levels increased more rapidly when only 1 mg each of estrogen and progesterone was injected, the lag periods before ovalbumin mRNA levels increased significantly were the same after either dose.

Effects of Testosterone on Ovalbumin mRNA Accumulation. Withdrawn chicks were treated with 2 mg each of estrogen and testosterone (Figure 5) or 2 mg of progesterone and testosterone (Figure 6). In every case the two hormones were mixed together before injection. After the combined administration of estrogen and testosterone there was a period

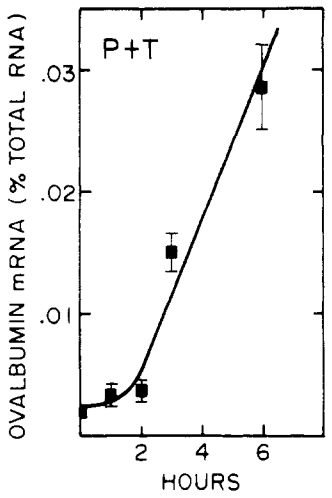

FIGURE 6: Ovalbumin mRNA accumulation after the combined administration of progesterone and testosterone. Estrogen-withdrawn chicks were treated with 2 mg of each progesterone and testosterone, and ovalbumin mRNA levels (■) were determined as outlined in Figure 2. The experiment was conducted at the end of November.

of 1.5–2 h before a rapid increase in ovalbumin mRNA levels took place (Figure 5). With this hormone combination the lag period was relatively well-defined unlike the results frequently obtained with the other hormone(s). Although the relative shapes of the two different curves in Figure 5 are similar, the rates of ovalbumin mRNA accumulation are very different. These data correlate very well with those of Boyd & Spelsberg (1979) which showed that for the years concerned chicks killed in early November had high levels of nuclear receptor while chicks killed in early December had low levels of nuclear receptor. [Ovalbumin mRNA accumulation has been shown to be strongly dependent on high levels of receptor in the nucleus (Mulvihill & Palmiter, 1977)].

When testosterone was administered with progesterone, there was again a 1.5–2-h lag period before ovalbumin mRNA levels had significantly increased over those observed in withdrawn chicks (Figure 6). In fact, the results obtained with progesterone and testosterone are not significantly different from those obtained with progesterone alone during the summer months (Figure 4, filled squares).

Delivery of Hormone and Receptor to the Nucleus. Since it had been reported that within 30 min after hormone administration the nuclear estrogen receptor levels had reached a plateau (Palmiter et al., 1976), it had been argued that the 1.5–2-h lag period was not due to the slow delivery of hormone to the oviduct. However, these studies did not state how the hormone was administered to the chicks. Since we injected the hormone(s) in sesame oil into the leg muscle, it might take longer than 15–30 min for the nuclear estrogen receptor levels to reach a plateau. Therefore, the rate of accumulation of estrogen receptor in the nucleus after estrogen plus testosterone treatment was compared with that measured after estrogen plus progesterone treatment. Of all the hormones or combinations of hormones, the best defined lag period before an increase in ovalbumin mRNA levels (2 h) occurred after estrogen plus testosterone treatment. The lag period was not as well-defined and perhaps the shortest after estrogen (1 mg) plus progesterone (1 mg) treatment. Thus, it was of interest to compare the rise in nuclear estrogen receptor levels after these two hormone treatments.

Chicks were treated with either 1 mg each of estrogen and progesterone or 2 mg each of estrogen and testosterone, and the levels of nuclear estrogen receptor were measured during

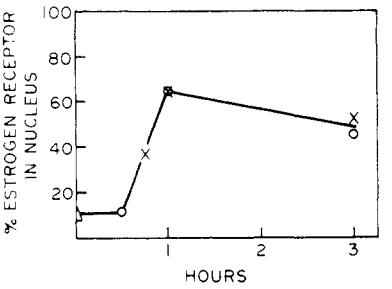

FIGURE 7: Time course of the appearance of the estrogen receptor in the nucleus after hormone treatment. Estrogen-withdrawn chicks were treated with 1 mg each of estrogen and progesterone (○) or 2 mg each of estrogen and testosterone (×), and the levels of soluble estrogen receptor were determined in the nucleus by using an exchange assay as outlined under Methods. The levels of estrogen receptor in the nucleus are expressed as the percentage of total cellular receptor. During the time course of the experiment the levels of estrogen receptor in the cell remained constant at ~2000 sites/cell. The experiment was conducted at the beginning of November.

the next 3 h (Figure 7). Nuclear estrogen receptor levels were expressed as the percentage of nuclear plus cytoplasmic receptor levels. During the first few hours following hormone treatment the total amount of estrogen receptor, corresponding to ~2000 sites/cell, did not change. Comparable values have been reported by Sutherland & Baulieu (1976). The results obtained for the two different hormone treatments were very similar. It took at least 0.5 h for estrogen to reach the oviduct nucleus after its injection in oil into the leg muscle. Nuclear estrogen receptor levels peaked ~1 h after hormone treatment. This was still ~1 h before ovalbumin mRNA started to accumulate rapidly and, therefore, did not explain totally why there was a 2-h period after hormone treatment with relatively little increase in ovalbumin mRNA levels.

Ovalbumin mRNA Levels 24 h after Hormone Administration. The levels of ovalbumin mRNA 24 h after the various hormones treatments were determined to ascertain (1) whether there was any evidence of circannual rhythms and (2) whether they correlated with the levels of ovalbumin synthesis in oviduct magnum explants (Palmiter et al., 1973). At this time small variations in the initial lag period would not have an important influence on the results. The values reported in Table I represent the results obtained from chicks that were raised either in the fall (group A) or the late winter–early spring (group B). Group B chicks, raised during the winter and early spring, uniformly had lower levels of ovalbumin mRNA than the group A chicks. These data support the idea that the rate of ovalbumin accumulation may depend on a circannual rhythm (see Discussion).

The difference in ovalbumin mRNA levels between group A and group B chicks after a particular hormone treatment was often not what would be predicted from the rates of ovalbumin mRNA accumulation during the first few hours of secondary stimulation (Figures 2–6). Ovalbumin mRNA accumulation is not always linear throughout the first 24 h. This is especially apparent following the injection of a single hormone (see Figure 3). In fact, ovalbumin mRNA levels may even be declining 24 h after hormone treatment (J. F. Hoffmann, D. C. Van Eys-Fuchs, and S. S. Seaver, unpublished experiments). It is also interesting to note that in chicks raised during the winter, levels of ovalbumin mRNA after the administration of estrogen with progesterone or with testosterone are much higher than those found after the administration of estrogen alone. Less estrogen and progesterone receptor is translocated to the nucleus in the winter than in the summer–fall (Boyd & Spelsberg, 1979). Ovalbumin mRNA levels

are very sensitive to nuclear levels of the estrogen–receptor complex (Mulvihill & Palmiter, 1977). Since estrogen would not be as effective in stimulating ovalbumin mRNA during the winter, the effects of the second hormone on mRNA levels may become more apparent.

With the exception of the chicks treated with progesterone alone, the relative levels of ovalbumin mRNA 24 h after hormone treatment found in group A (fall) chicks did correlate well with the relative levels of ovalbumin synthesis in oviduct magnum after 4 days of in vivo hormone treatment (Palmiter et al., 1973; Palmiter & Smith, 1973). In both cases, ovalbumin mRNA or ovalbumin synthesis after estrogen treatment was not significantly altered by the combined administration of estrogen and progesterone or testosterone. However, after progesterone treatment the levels of ovalbumin mRNA relative to those found in estrogen-treated chicks were very much lower (2–3 times) than would have been predicted from the explant protein synthesis data of Palmiter et al. (1973). Why this is so is not clear. Progesterone may stimulate the translation of ovalbumin specifically (Palmiter, 1971; Robins & Schimke, 1978; Pennequin et al., 1978). Alternatively, the level of all the mRNAs could be lower in progesterone-treated chicks but the fraction of mRNA coding for ovalbumin may be only slightly lower. Ovalbumin mRNA levels in group B (winter) did not seem to correlate with explant data.

Discussion

We were interested in determining whether various combinations of hormones could influence the early accumulation of ovalbumin mRNA during secondary stimulation. In particular, we wanted to determine if the lag period between hormone treatment and a significant increase in ovalbumin mRNA levels could be shortened. Ovalbumin mRNA levels were measured by hybridization after the administration of estrogen or progesterone alone, the two hormones together, or either hormone with testosterone. We found that the lag period between hormone treatment and an increase in ovalbumin mRNA levels was 1.5–2.5 h and was independent of the hormone treatment and the time of year. This lag was not simply due to the slow delivery of hormone to the oviduct since we found that nuclear hormone–receptor levels had plateaued by 1 h. The lag period was also observed by others (McKnight et al., 1975; Hynes et al., 1979; Palmiter et al., 1976; Spelsberg & Cox, 1976; Roop et al., 1978) and for several other egg white protein mRNAs: lysozyme mRNA (Hynes et al., 1979), ovomucoid mRNA (Hynes et al., 1979; Tsai et al., 1978), and conalbumin mRNA (after progesterone treatment) (Palmiter et al., 1976), as well as with other mRNAs in other hormonally controlled systems (Palmiter et al., 1976; Deeley et al., 1977; Baker & Shapiro, 1978).

Although there are several explanations for this lag period (Palmiter et al., 1976), we favor the idea that it is reasonable to expect such a lag period. The accumulation of ovalbumin mRNA probably depends on many interrelated processes, which are not operating at maximal efficiency in withdrawn chick oviduct. The rapid transcription of ovalbumin mRNA may depend on processes which increase the half-life of mRNAs; processes involved in stabilizing mRNAs may in turn depend on hormonally controlled transcription. It will take time after hormone treatment for these processes to attain some critical level or rate at which the transcription of ovalbumin mRNA will lead to a rapid increase in ovalbumin mRNA.

Very unexpectedly we found that the rate of ovalbumin mRNA accumulation seemed to depend more on the time of year in which the experiments were conducted and less on the actual hormone(s) administered. This seasonal dependence was also observed in ovalbumin mRNA levels 24 h after hormone treatment. Ovalbumin mRNA levels increased faster in chicks receiving hormone(s) during the summer than during the winter and early spring (Figures 2, 3, and 5 and Table I). Spelsberg and co-workers have noticed a strong correlation between seasons and the binding of progesterone receptor to the nuclear protein–acceptor DNA complex, oviduct weight, oviduct protein levels, amount of nuclear [^{3}H]progesterone or estrogen binding in vivo, and the levels of RNA polymerase II (the polymerase responsible for transcribing mRNA) but not the binding of progesterone receptor to naked DNA (Boyd & Spelsberg, 1979; Spelsberg et al., 1979). The period of peak activity, acrophase, occurred during the summer and fall months centering around the autumnal equinox while the period of decreased activity, bathophase, occurred during a 5-month period anywhere between late November and early May. These data fit this circannual rhythm within a 95% confidence limit. In the case of the progesterone receptor which has been shown to consist of two forms, A and B (Schrader & O'Malley, 1978), Boyd & Spelsberg (1979) have evidence that the amount of the A form decreases during the bathophase while the level of the B form remains fairly constant. Since we had not considered the existence of a circannual rhythm while designing these experiments, we do not have enough data to construct the type of correlation curves used by Spelsberg et al. (1979). However, the rate of ovalbumin mRNA accumulation probably does follow a circannual rhythm, since it is known that ovalbumin mRNA levels can be sensitive to a decrease in the amount of nuclear receptor (Mulvihill & Palmiter, 1977; our unpublished observations). Such circannual rhythms may help explain why some groups have observed that ovalbumin mRNA levels increase faster after progesterone administration than estrogen administration (Palmiter et al., 1976; McKnight et al., 1975). If data obtained from chicks treated with progesterone during the summer and fall (Figure 3, closed squares) are compared to data obtained from chicks treated with estrogen during the winter and early spring (Figure 2, open circles), it appears that ovalbumin mRNA levels increase somewhat sooner and definitely faster after progesterone treatment than after estrogen treatment. Yet when ovalbumin mRNA accumulation is measured after estrogen or progesterone treatment when both experiments are done simultaneously or in the same season, there is no difference between either hormone treatment until after 5 h, at which time ovalbumin mRNA accumulates faster in estrogen-treated chicks.

These seasonal differences may also help explain why the effects of combined hormone treatment were so much more pronounced during the bathophase than the acrophase. During the bathophase the administration of a single hormone may not elevate nuclear receptor levels to as high a level and for as long a time as during the acrophase. This will lead to a decreased rate of ovalbumin mRNA accumulation and would make the effects of the second hormone on ovalbumin mRNA accumulation more apparent (see Table I). Thus, it may be important when comparing the effects of different hormones on the chick oviduct to conduct all the pertinent experiments on the same group of chicks, if possible, or at least during the same season. Even after the administration of the same hormone(s) the results obtained during the acrophase may be quantitatively different from those obtained during the bathophase.

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