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Articles

Correlation in Isolated Nuclei of Template-Engaged RNA Polymerase II, Ovalbumin mRNA Synthesis, and Estrogen Receptor Concentrations†

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ABSTRACT: Template-engaged and total RNA polymerase II molecules were quantitated in isolated nuclei at various stages of estrogen withdrawal and secondary stimulation by using [^{3}H]amanitin titration assays. Estrogen receptors, RNA transcriptional activity, and ovalbumin mRNA were also measured, and comparisons were made between these parameters to determine whether any significant correlations exist. In isolated nuclei, the highest positive correlations existed between template-engaged RNA polymerase II, ovalbumin mRNA synthesis in vitro, and estrogen receptor concentration. Interestingly, restimulation of estrogen-withdrawn chicks results in replenishment of RNA polymerase II activity to prewithdrawal levels within 4 h; however, the recovery of the numbers of template-engaged polymerase II molecules, ovalbumin gene transcription, and nuclear receptor binding lags behind. These findings suggest that the estrogen effect on RNA polymerase activity is more rapid than the increase in template-engaged RNA polymerase II and ovalbumin-specific gene transcription. The excellent correlation that exists between nuclear estrogen receptor concentrations, template-engaged RNA polymerase II, and ovalbumin gene transcription strongly supports the hypothesis that estrogen receptors mediate RNA polymerase II binding to sequences associated with preferential transcription of the ovalbumin gene.

One of the most dramatic and earliest recognized biochemical markers of estrogen action is the rapid induction of DNA-dependent RNA polymerase activity. The target tissue specificity of this response has been extensively investigated in the rat uterus (Gorski, 1964; Hamilton et al., 1968; Raynaud-Jammet et al., 1971; Glasser et al., 1972; Hardin et al., 1976) and chick oviduct (O'Malley et al., 1969; Spelsberg & Cox, 1976; Cox, 1976). The mechanisms involving hormonal regulation of gene transcription remain largely unknown, and the development of in vitro transcription systems to study these events under cell-free conditions has been a major focus of contemporary molecular biologists. Several models have been proposed to account for the induction of specific messenger RNAs by steroid hormones (O'Malley & Means, 1974; King & Mainwaring, 1974; Yamamoto & Alberts, 1975). They imply that steroid receptor complexes associate with high-affinity acceptor sites located in the nucleus and they produce conformational changes that modify transcriptional activity. This suggests that any study of these nuclear events should demonstrate a correlation between the number of nuclear estrogen receptors and the magnitude of specific inducible responses, i.e., the stimulation of RNA polymerase activity and the preferential transcription of certain genes.

Specific high-affinity estrogen receptor proteins have been identified in both cytoplasmic and nuclear compartments of chick oviduct cells (Cox et al., 1971; Harrison & Toft, 1975; Best-Belpomme et al., 1975; Teng & Teng, 1976; Mulvihill & Palmiter, 1977). However, the accurate quantitation of cytoplasmic and nuclear estrogen receptor molecules has recently been complicated by the discovery of estrogen receptor heterogeneity (Smith et al., 1979; Taylor & Smith, 1982a). To accurately quantitate receptor sites in chick oviduct cell fractions which also contain low-affinity and high-capacity estrogen binding macromolecules, it is necessary to use assays which do not overestimate estrogen receptor concentrations by discriminating estrogen receptors from low-affinity estrogen binding sites. Through the use of such assays, the two high-affinity estrogen receptors have been measured in the chick

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oviduct which have K_d's of 0.1 and 2 nM, and their concentration in oviduct nuclei is highly correlated ($p < 0.001$) with ovalbumin gene transcription in vitro (Taylor et al., 1980; Smith & Taylor, 1981).

In this report, we have extended our studies of the effects of total concentrations of nuclear receptors on in vitro gene transcription and have included other parameters of RNA synthesis. To examine RNA polymerase II concentrations, we have exploited the high-affinity and equimolar stoichiometry of amatoxin to these enzymes (Cochet-Meilhac & Chambon, 1974). The accuracy and specificity of this technique have been reviewed by Chambon and his collaborators (Chambon, 1974; Cochet-Meilhac et al., 1974; Courvalin et al., 1976) and successfully applied by several others (Brodner & Wieland, 1976; Kastern et al., 1979; Brown & Garrity, 1980). Our studies were performed in isolated nuclei under various conditions of estrogen stimulation and withdrawal; estrogen receptor concentrations, total RNA polymerase II levels, template-engaged RNA polymerase II levels, and polymerase II activity were measured in the same nuclei. In addition, the capacity of the nuclei at these various stages of estrogen stimulation to synthesize ovalbumin mRNA ($mRNA_{ov}$) was compared.

Experimental Procedures

Animals. Two-day-old white Leghorn pullets were obtained from Texas Animal Specialties, Humble, TX, given food and water ad libitum, and maintained in a 13:11-h light:dark environment. At 1 week of age, these chicks were implanted with two diethylstilbestrol (DES)-filled silicone implants as described (Taylor & Smith, 1979). After 14 days of estrogen stimulation, the implants were removed through cutaneous incisions, and withdrawal periods of 0–72 h ensued prior to sacrifice.

Tissue Preparations. Primarily stimulated, withdrawn, or secondarily stimulated chicks were sacrificed at the indicated times by decapitation. Oviduct tissue was removed, quickly trimmed of connective tissue, and rinsed in ice-cold 0.9% NaCl. Six to ten chick oviducts were pooled for each data point to minimize possible variation among different animals. In all cases, fresh tissue was used for nuclear isolation and transcription assays. All subsequent procedures were carried out at 4 °C unless noted otherwise.

Oviducts were weighed and homogenized in 9 volumes of 0.5 M sucrose, 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.4), 20 mM KCl, and 5 mM $MgCl_2$ (TKM) buffer with a Tissuemizer blender (Takmar Co., Cincinnati, OH) at 70 V for 1 min in short bursts. The homogenate was filtered through cheesecloth and organza, layered over 1 volume of 0.88 M sucrose TKM, and centrifuged at 2000*g* for 10 min. The supernatant was subjected to ultracentrifugation at 100000*g* for 60 min to furnish cytosol.

The crude pellet from the initial centrifugation was resuspended in 0.5 volume of 0.88 M sucrose TKM, homogenized in 6 volumes of 2.1 M sucrose TKM, and centrifuged at 12000*g* for 30 min to pellet nuclei. This pellet was finally resuspended in buffer containing 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 3 mM dithiothreitol, 12.5% (by volume) glycerol, 100 mM KCl, and 50 mM Tris-HCl (pH 7.8). Concentrations of nuclei were adjusted to 4×10^7 to 1×10^8 nuclei/mL (~100–250 μg/mL DNA). Nuclei prepared in this manner were free of cytoplasmic debris, were round or oval in shape, and contained one or two nucleoli as observed by phase-contrast light microscopy. Immediately after resuspension, purified nuclei were divided into four aliquots: (1) for nuclear estrogen receptor microassay; (2) for measurement of total and engaged RNA polymerase II; (3) for in vitro RNA synthesis; and (4) for DNA quantitation by the diphenylamine technique (Giles & Myers, 1965). The value of 2.5 pg of DNA/chick cell nucleus (Altman & Dittmer, 1973) was used to convert DNA concentrations to cellular or nuclear values.

Nuclear Estrogen Receptor Quantitation. Samples of oviduct nuclei were examined by using a nuclear exchange microassay reported previously (Taylor et al., 1980). [^{3}H]-Estradiol (Amersham/Searle, 100 Ci/mmol) was added to the suspended nuclei over a concentration range of 0.5–10 nM, and specific binding was assessed by Scatchard (1949) analysis to quantitate receptor concentrations.

Synthesis of [^{3}H]RNA in Isolated Nuclei. Suspensions of nuclei were kept on ice while 1 mM each of ATP, CTP, and GTP and 600 μM [5,6-^{3}H]UTP (Amersham/Searle, 20–35 Ci/mmol) were added. Transcription reactions were initiated by incubation at 37 °C, and 100-μL aliquots were removed at 5-min intervals.

[^{3}H]RNA synthesized in vitro was recovered by precipitation with ice-cold 10% Cl_3CCOOH containing 100 mM $Na_4P_2O_7$ and collected on nitrocellulose filters with a filtration apparatus (Millipore). Products of in vitro transcription were quantitated by liquid scintillation counting as described (Roop et al., 1978). Typically, [^{3}H]RNA synthesis was maximal after 30 min. Nuclear RNA synthesis sensitive to 1 μg/mL α-amanitin (Boehringer-Mannheim) was determined by the addition of this inhibitor to a replicate transcription assay. The difference in acid-insoluble [^{3}H]RNA synthesized in vitro was assumed to represent RNA polymerase II activity (Weinmann & Roeder, 1974).

[^{3}H]Amanitin Titration of RNA Polymerase II Enzymes. Samples of purified oviduct nuclei were resuspended in a [^{3}H]amanitin binding buffer consisting of 50 mM Tris-HCl (pH 7.9) 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM monothioglycerol, 100 mM $(NH_4)_2SO_4$, 200 μg/mL bovine serum albumin, and 10% (v/v) glycerol. The concentration of nuclei (~100 μg/mL DNA) was kept constant while *O*-[^{3}H]methyldemethyl-α-amanitin (5 Ci/mmol) concentrations were varied from 1 to 20 nM to titrate amanitin binding sites. The nitrocellulose filtration procedure of Cochet-Meilhac & Chambon (1974) was employed to separate bound from free [^{3}H]amanitin. In experiments designed to quantitate engaged RNA polymerase II molecules in isolated nuclei, the procedure of Yu (1975) was used to remove free polymerases. [^{3}H]Amanitin binding was assayed in the washed nuclear pellet as described above. Diphenylamine reactions (Giles & Myers, 1965) were used to determine concentrations of nuclei in representative samples. Initial experiments designed to validate this assay were performed as described, except that partially purified hen oviduct RNA polymerases I, II, and III (Tsai et al., 1980) were examined. Polymerase protein concentrations were adjusted to 100 μg/mL in these studies. Specific [^{3}H]amanitin binding was determined by comparison with identical samples which had been preincubated with a 10-fold excess of unlabeled α-amanitin. Scatchard analyses were resolved by bivariate linear regression analysis and used to quantitate binding site concentrations.

Synthesis of [^{3}H]RNA in Isolated Nuclei and Quantitation of ^{3}H-Labeled Ovalbumin mRNA Synthesized in Vitro. These experimental methods were performed as described previously (Taylor et al., 1980; Taylor & Smith, 1982b).

Results

Quantitation of Nuclear Estrogen Receptor Complexes. The microassay used to quantitate high-affinity estrogen receptor–acceptor site complexes in purified chick oviduct nuclei

Table I: Effects of Estrogen Withdrawal and Secondary Stimulation on Receptor Stimulation and Transcriptional Parameters of Oviduct Nuclei[a]

hormonal status	estrogen receptor/nucleus	RNA Pol II activity (fmol of [^{3}H]UMP incorporated/μg of DNA)	total RNA Pol II molecules/nucleus	engaged RNA Pol II molecules/nucleus	% $mRNA_{ov}$
stimulated	2780 ± 390	300 ± 10	34 500 ± 3000	31 800 ± 3000	0.27 ± 0.05
24-h withdrawn	1600 ± 250	184 ± 64	39 000 ± 5000	14 000 ± 5000	0.08 ± 0.02
48-h withdrawn	460 ± 150	150 ± 10	19 200 ● 3000	12 000 ± 1000	0.02 ± 0.01
48-h withdrawn + 4 h of DES	750 ± 70	310 ± 40	33 500 ± 2800	18 200 ± 1100	0.12 ± 0.01
72-h withdrawn	180 ± 40	130 ± 20	18 600 ± 2000	10 400 ± 800	<0.001
72-h withdrawn + 4 h of DES	600 ± 140	310 ± 80	25 000 ± 2200	16 000 ± 1100	0.06 ± 0.04

[a] Oviduct nuclei were isolated from chicks after the indicated periods of estrogen withdrawal and secondary restimulation. Nuclear estrogen receptor concentrations, RNA polymerase (Pol) II activity, total and engaged RNA Pol II molecules, and percentage of ovalbumin mRNA ($mRNA_{ov}$) were quantitated as described under Experimental Procedures. The results are expressed as the means of two independent experiments. Estrogen receptor concentrations and concentrations of RNA polymerase II were determined by Scatchard analysis of equilibrium binding data.

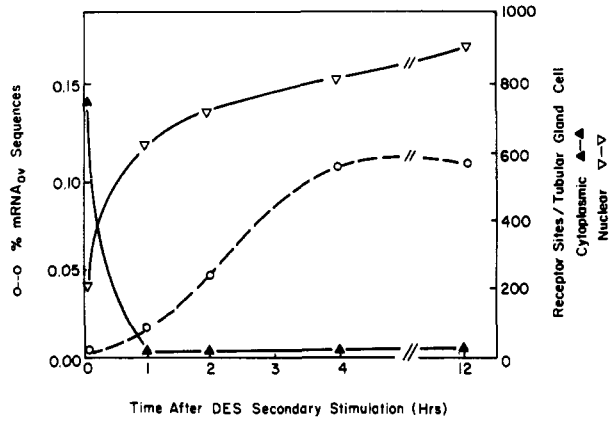

FIGURE 1: Estrogen receptor concentration and ovalbumin mRNA synthesis following secondary DES stimulation. Chicks were withdrawn from DES for 60 h following 14-day stimulation with DES. Oviducts were harvested and nuclei were isolated as described under Experimental Procedures.

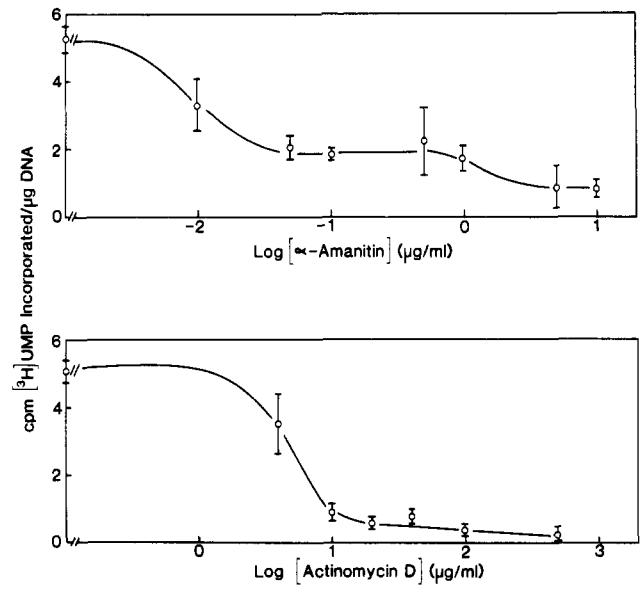

FIGURE 2: Concentration dependence of RNA synthesis inhibitors on in vitro transcription. Nuclear transcription assays were performed in the presence of increasing concentrations of α-amanitin (above) and actinomycin D (below). [^{3}H]RNA synthesis was quantitated after 30 min at 37 °C.

has been described previously. This method, which is based on Scatchard analysis, has a high level of precision (coefficient of variation = 12%) and detects estrogen receptor complexes that are tightly associated with nuclei. Because of the presence of low-affinity estrogen binding which is not attributable to receptor binding (Eriksson et al., 1978; Taylor et al., 1980), it is imperative to use Scatchard analysis to avoid the overestimation of receptor sites inherent in single concentration assays. Contaminating cytoplasmic receptors and [^{3}H]estradiol attached to the outer nuclear membrane (Jackson & Chalkley, 1974) are removed by a final washing with 1% Triton X-100 and hence do not interfere in this assay. With this microassay, mean nuclear estrogen receptor concentrations ranged from 180 molecules/nucleus in hormone-withdrawn chick oviducts to 2780 molecules/stimulated oviduct nucleus (Table I). These concentrations reflect the total sums of the two estrogen receptors (K_d's of 0.1 and 2 nM).

To determine the time following secondary stimulation at which transcriptional relationships might be stabilized following a single DES injection, nuclear receptor concentrations were compared to the changes in ovalbumin gene transcription as a function of time. Figure 1 illustrates the effect of DES administration to 60-h DES-withdrawn chicks on soluble estrogen receptors and immobilized nuclear receptors and their relationship to the percent ovalbumin mRNA synthesized in vitro. This particular time of withdrawal was selected since it was intermediate between the two withdrawal times selected for subsequent secondary stimulation studies (48 and 72 h). Figure 1 demonstrates that cytoplasmic (soluble) receptor concentration falls precipitously in the first hour and this is accompanied by a gradual nuclear receptor immobilization which plateaus within 4 h. Similarly, the rates of ovalbumin gene transcription also plateau in this time period. Thus, 4 h was selected as a suitable time for the subsequent measurement of nuclear receptor levels and transcriptional parameters.

Secondary stimulation of 48- and 72-h withdrawn chicks with DES for 4 h resulted in an increase in the concentration of the nuclear receptors. However, the resultant increase was not the same and in neither case reached the levels observed in the chronically stimulated chicks. The absolute concentration of estrogen receptors bound to these nuclei was reduced according to the time of withdrawal preceding the secondary stimulation (Table I).

Characteristics of [^{3}H]RNA Synthesis in Isolated Oviduct Nuclei. Oviduct nuclei were isolated from tissue homogenates as described under Experimental Procedures. This procedure employs sedimentation through hypertonic sucrose-containing buffers and has been previously shown to optimally maintain the purity, structural integrity, and endogenous RNA polymerase II activity in these nuclei (Knowlet et al., 1973). Nuclei were incubated in a transcription buffer containing 600 μM [^{3}H]UTP, a concentration that exceeds the K_m for the eukaryotic RNA polymerase II enzyme (Chambon, 1974) and that is not rate limiting in the transcription reaction. Under the described assay conditions, the incorporation of [^{3}H]UMP into acid-insoluble RNA was linear with respect to DNA concentration (data not shown).

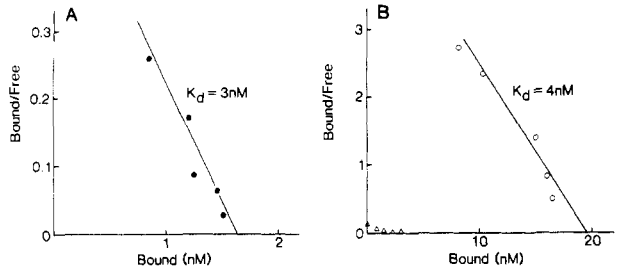

FIGURE 3: Scatchard analysis of [^{3}H]amanitin binding. [^{3}H]Amanitin binding site titration assays were performed as described in the text, analyzed according to Scatchard (1949). Purified whole nuclei (panel A, ●) had essentially the same equilibrium dissociation constant (K_d), as purified hen oviduct RNA polymerase II (panel B, ○). Purified hen oviduct RNA polymerase I (panel B, △) revealed minimal [^{3}H]amanitin binding.

The inhibitory effects of α-amanitin and actinomycin D on in vitro transcription are shown in Figure 2. These results suggest that 62% of [^{3}H]RNA synthesized in these nuclei is the product of RNA polymerase II, while 20% and 18% are attributable to polymerases I and III, respectively. The reaction is inhibited >90% by actinomycin D (40 μg/mL) which suggests that synthesis is DNA template dependent. In vitro transcription required the addition of all four ribonucleoside triphosphates, and the products of the reaction were sensitive to pancreatic RNase (20 μg/mL) digestion.

In all subsequent studies, nuclear transcription assays were conducted in parallel with identical samples containing 1 μg/mL α-amanitin. By comparison, the transcriptional activity of RNA polymerase II could be estimated. The RNA polymerase II activity decreased from 300 fmol of [^{3}H]UMP incorporated/μg of DNA in oviduct nuclei to 130 fmol of [^{3}H]UMP incorporated/μg of DNA in oviduct nuclei from chicks withdrawn from DES for 72 h. Restimulation with DES increased the polymerase II activity to stimulated levels within 4 h following DES administration as shown in Table I.

Quantitation of RNA Polymerase II by [^{3}H]Amanitin Titration. We have used a modification of the nitrocellulose filter binding technique described by Cochet-Meilhac & Chambon (1974). Optimal specific [^{3}H]amanitin binding to whole nuclei and partially purified RNA polymerase II occurred between 2 and 6 h of incubation at 4 °C. Nonspecific binding was calculated by preincubating samples and excess unlabeled α-amanitin for 2 h prior to adding the [^{3}H]amanitin. Under these conditions, specific [^{3}H]amanitin binding was directly proportional to the number of nuclei or the polymerase concentration.

Figure 3 demonstrates the Scatchard (1949) derived equilibrium dissociation constants obtained for [^{3}H]amanitin binding to purified chick oviduct nuclei (A) and purified hen oviduct nuclear RNA polymerase II (B). Partially purified preparations of hen oviduct RNA polymerases I and III revealed minimal [^{3}H]amanitin binding under the labeling conditions described. Similar Scatchard plots of α-[^{3}H]amanitin binding to RNA polymerase II molecules in isolated whole nuclei from stimulated, withdrawn, and secondarily stimulated chicks were performed to quantitate the total concentrations of RNA polymerase II enzymes present in each nucleus. Our estimate in the estrogen-stimulated chick oviduct nucleus (34 500 ± 3000) closely corroborated the values (37 000–41 000) reported by Chambon (1974) for this tissue. Following withdrawal from DES, the levels of RNA polymerase II fell, in an appropriately logarithmic fashion, to 18 600 molecules/nucleus with 72 h. Restimulation afforded a significant increase which approached the levels measured in nuclei from chronically stimulated chicks (Table I).

Since transcriptional regulation may be modulated by quantitative alterations between pools of free and engaged RNA polymerase molecules, [^{3}H]amanitin binding was employed to measure the concentration of template-engaged RNA polymerase II. Oviduct nuclei isolated as described previously were gently homogenized and washed, as described by Yu (1975), to remove free RNA polymerase. The nuclear pellet, retaining all the engaged enzyme, was resuspended in [^{3}H]amanitin labeling buffer, and engaged RNA polymerase II molecules were quantitated (Table I). Results using stimulated nuclei revealed 31 800 engaged enzymes/nucleus. After 72 h of estrogen withdrawal, engaged polymerase II concentrations were reduced to 10 400 molecules/nucleus. This value is in close agreement with the 10 000–15 000 elongating RNA polymerase II enzymes/nucleus of withdrawn chick oviduct determined by Cox (1976) using end-group analysis.

Groups of chicks withdrawn from DES implants for 48 or 72 h were secondarily stimulated by subcutaneously injecting 2.5 mg of DES/chick. Four hours after the hormone injection, these animals were sacrificed, and the results displayed in Table I were obtained. This secondary stimulation resulted in a 2-fold increase in polymerase II activity, a 1.3–1.8-fold increase in total polymerase II, and a 1.5–1.6-fold increase in nuclear concentrations of engaged RNA polymerase II.

Ovalbumin mRNA Synthesis in Isolated Nuclei. Nuclei were isolated and purified from chick oviducts stimulated with DES for 14 days and then withdrawn for 24, 48, and 72 h from DES; certain groups were secondarily stimulated with DES for 4 h. These nuclei were resuspended and subjected to in vitro transcription at 37 °C for 30 min. The reaction was terminated by the addition of purified bovine pancreatic deoxyribonuclease I. The [^{3}H]RNA synthesized in vitro was isolated, and the amount of ovalbumin mRNA was quantitated by hybridization to filters containing immobilized ovalbumin cDNA as described under Experimental Procedures. The results shown in Table I demonstrated that oviduct nuclei lose their capacity to synthesize ovalbumin mRNA in a manner which is dependent upon the period of estrogen withdrawal.

Discussion

When estrogen-stimulated chicks are withdrawn from subcutaneous hormone injections or DES-filled silicone implants, a dramatic involution of oviduct tissue ensues, which is accompanied by a decrease in several biochemical markers of estrogen action (Taylor et al., 1980). The data in Table I demonstrate the concurrent and progressive attenuation of nuclear estrogen receptor concentrations, RNA polymerase II concentration, and RNA polymerase II activity after estrogen receptor withdrawal. Nuclear receptor concentrations fell to 5% of the stimulated level, although only a 53% decrease in RNA polymerase II activity ensued over this time interval. Hence, a significant proportion of class II gene transcription continues in this tissue in the absence of receptor-mediated estrogen stimulation. With the [^{3}H]amanitin binding assay, total RNA polymerase II enzyme concentrations fell to 53% of stimulated levels during the 72-h period, and template-engaged RNA polymerase II concentration was reduced by 66%.

The five parameters measured as a function of time of withdrawal from DES (Table I) were subjected to a correlation matrix analysis (Langley, 1967). This analysis showed that of all the parameters a significant positive correlation exists only between three variables: engaged RNA polymerase II concentrations are correlated with both the percentage ovalbumin mRNA synthesized in vitro ($p < 0.05$) and the nuclear estrogen receptor concentrations ($p < 0.05$). These results

suggest but, of course, do not prove that estrogen directs the binding of transcriptionally active RNA polymerase II and that this effect is mediated via nuclear receptor occupancy. These results, therefore, extend our previous findings which demonstrated that nuclear receptor occupancy was highly correlated with ovalbumin mRNA synthesis in vitro (Taylor et al., 1980; Smith & Taylor, 1981).

Secondary stimulation of 48- and 72-h withdrawn chicks resulted in a 2- and 3-fold increases in nuclear estrogen receptors, respectively, consistent with our previous observations (Taylor et al., 1980). These increases were accompanied by dramatic increases in the concentrations of total RNA polymerase II and polymerase II activity, the latter reaching levels similar to those observed in chronically stimulated nuclei. Secondary stimulation was also associated with 1.5- and 1.6-fold increases in template-engaged RNA polymerase II enzymes. Interestingly, while RNA polymerase II activity was restored to prewithdrawn levels within 4 h of secondary stimulation, a similar restoration in template-engaged enzyme, ovalbumin gene transcription, and nuclear receptor binding lagged behind (Table I). When these data are subjected to a correlation matrix analysis, all parameters are highly correlated with each other ($p < 0.05$). This is hardly surprising, since secondary stimulation markedly affects each variable and the sample size is small. However, when the results from these experiments are combined with those obtained from DES withdrawal, the correlation matrix again shows a significant correlation ($p < 0.05$) between engaged RNA polymerase II, ovalbumin mRNA synthesized in vitro, and nuclear estrogen receptor occupancy.

Table I shows that estrogen administration causes an increase in the nuclear concentration of RNA polymerase II, but this effect temporally precedes the relative increase in the measured level of nuclear receptors. The observed rapid increase in activity of this enzyme might occur at a different class of acceptor sites than those responsible for ovalbumin-specific gene transcription. We have previously shown in the same nuclear transcription system that addition of the highly purified high-affinity (K_d = 0.1 nM) chick estrogen receptor to oviduct nuclei results in an approximate 2-fold stimulation of RNA polymerase II activity, but under these conditions, there was no preferential increase in $mRNA_{ov}$ synthesis (Taylor & Smith, 1982b). Thus, increases in polymerase II activity to prewithdrawal levels can occur without an increase in the concentration of these enzymes and might occur before a stable, high-affinity nuclear receptor–acceptor complex (detectable under the stringent conditions of our receptor assay) is formed. Mechanistically, the increased activity is perhaps due to a destabilizing effect of the receptor hormone complex on chromatin (Smith & Schwartz, 1979) or might be associated with a direct effect on the RNA polymerase itself.

The observation was also made, in agreement with our previous findings, that the ability of withdrawn nuclei to bind receptors with high affinity was reduced according to the time of withdrawal preceding secondary stimulation. This effect was accompanied by a similar attenuation in the capacity of the nuclei to synthesize ovalbumin mRNA (Table I; McKnight & Palmiter, 1979; Taylor et al., 1980). Additional information provided by our present study indicates that this attenuated response of nuclear estrogen receptor accumulation and ovalbumin gene expression is also accompanied by a reduction in template-engaged RNA polymerase II levels. Since all the secondary stimulation experiments were conducted on chicks withdrawn from estrogen for up to 72 h, our data cannot be explained by deficiencies associated with a reported 2-h lag in the induction of ovalbumin gene transcription or by the loss of tubular gland cells (McKnight & Palmiter, 1979).

The concentrations of nuclear estrogen receptors obtained in our studies differ from those reported by McKnight & Palmiter (1979). These differences are explained by the different methodologies employed, since our studies utilized Scatchard analysis to avoid overestimation of receptor sites caused by the presence of low-affinity, high-capacity estradiol binding sites (Smith & Taylor, 1981). Irrespective of the reported differences in absolute values, the relative concentrations of nuclear receptors at different times following secondary estrogen stimulation are similar. Thus, in agreement with the speculation of McKnight & Palmiter (1979), it appears that the time-dependent occupancy of nuclear sites by estrogen receptors determines the rate of expression of the ovalbumin gene. However, the data in Table I show that the nuclear concentration of estrogen receptors is also related to the template engagement of RNA polymerase II.

A concentration of 3000 receptors/cell is associated with maximum rates of transcription of the ovalbumin gene, whereas following 72 h of withdrawal from DES the total receptor concentration is only 1100/cell (Taylor et al., 1980). This implies that an approximate 3-fold increase in estrogen receptors is required before the rate of ovalbumin gene transcription is maximized. Such an increase is a minimum value, since this estimate does not account for receptors not bound tightly to nuclei. We have demonstrated previously that even after 24 h of secondary stimulation in chicks withdrawn from estrogen for 60 h, both nuclear receptor occupancy and ovalbumin gene transcription are only 50% of those attained in the maximally stimulated state. Thus, it appears that increases in the intracellular concentrations of estrogen receptors to maximum levels require >24 h. Although rates of synthesis and the half-life of estrogen receptors in the chick oviduct have not been measured, studies in our laboratory using androgen-responsive cells have shown that following withdrawal from hormone for only 48 h, approximately 24 h of exposure to androgens is required to increase androgen receptor concentrations 3-fold (Smith et al., 1984; Syms et al., 1985). If a similar slow increase in receptor concentrations occurs in the withdrawn chick oviduct following estrogen exposure, it suggests that this increase may be the rate-determining step in the increased template binding of RNA polymerase II and subsequent expression of the ovalbumin gene. Since previous studies have demonstrated that oviduct cells withdrawn from DES for 48 h contain higher concentrations of free receptors than cells withdrawn for 72 h (Taylor et al., 1980), it would be predicted that upon secondary stimulation of these two groups with DES, higher levels of estrogen receptors and template-engaged polymerase would be present in the nuclei from the 48-h withdrawn chicks. The data in Table I confirm this predicition.

The above rationalization of our findings does not, of course, exclude more complex mechanisms such as alterations in the concentrations of nuclear receptor acceptor proteins which might accompany these increases in receptor concentrations. However, the positive correlation that exists between immobilized nuclear estrogen receptors, template-engaged RNA polymerase II, and ovalbumin gene transcription supports the notion that estrogen receptors preferentially increase RNA polymerase II binding to ovalbumin gene sequences.

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