Correlation of nuclear acceptor sites for oestrogen receptors with gene transcription *in vitro*

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The interaction of oestrogen receptors with their nuclear acceptor sites was studied to ascertain whether these acceptor sites are involved in the regulation of ovalbumin-gene expression in the chick oviduct. As previously described, two distinct oestrogen-receptor species exist, and both are translocated into the nucleus after oestrogen administration *in vivo* [Smith, Clarke, Zalta & Taylor (1979) *J. Steroid Biochem.* 10, 31–35]. In the present investigation we observed that the tubular-gland-cell concentrations of cytoplasmic receptors (800–900/cell) do not vary with prolonged withdrawal, nor do the relative ratios of the two receptor types change; however, the nuclear accumulation and retention of these receptors after secondary oestrogen administration are attenuated in a time-dependent fashion. Chicks were withdrawn from oestrogen for 24–84 h. Some animals were then restimulated with oestrogen and killed after 4 h, when oviduct nuclei were isolated. These nuclei were assayed for nuclear receptor concentrations and for their capacity to synthesize ovalbumin mRNA *in vitro*. Although an equal number of cytoplasmic receptors appeared to be translocated, oestrogen-receptor occupancy within the nucleus was not equal, but was inversely proportional to the preceding length of withdrawal. This decrease in nuclear acceptor sites was accompanied by a similar decrease in the capacity of these same nuclei to transcribe ovalbumin mRNA *in vitro*. A statistical evaluation of nuclear oestrogen-receptor concentrations and ovalbumin-mRNA synthesis *in vitro* was made. Correlation analysis revealed a Pearson coefficient $r = 0.87$ ($P < 0.001$, $n = 17$), indicating that a high degree of correlation exists between these two parameters. These results are consistent with the hypothesis that nuclear oestrogen-receptor–acceptor complexes may correspond to initiation sites for RNA polymerase II transcription of an oestrogen-regulated gene.

The chick oviduct is an ideal model for the study of the modulation of gene expression by oestrogens. The administration of oestrogen to an immature chick results in proliferation and differentiation of the oviduct epithelium, with development of tubular-gland cells. These tubular-gland cells synthesize and secrete the egg-white proteins ovalbumin conalbumin, ovomucoid and lysozyme. This action is thought to be mediated through the formation and

nuclear accumulation of highly specific oestrogen-receptor complexes (Jensen *et al.*, 1968; Clark *et al.*, 1973). Although oestrogen may regulate post-transcriptional processes (Palmiter & Carey, 1974; Pennequin *et al.*, 1978), the weight of evidence suggests that the principal regulatory effect is at the transcriptional level (King & Mainwaring, 1974; O'Malley & Means, 1974; Yamamoto & Alberts, 1976; Swaneck *et al.*, 1979a,b). Our interest has been directed at defining the precise role of oestrogen-receptor complexes on gene transcription.

Specific high-affinity oestrogen-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; Smith *et al.*, 1979). However, the accurate quantification of cytoplasmic and nuclear oestrogen-

Abbreviation used: cDNA, complementary DNA.

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receptor molecules has more recently been complicated by the discovery of oestrogen-receptor heterogeneity (Smith *et al.*, 1979), a problem that is not restricted solely to chick oviduct tissue (Clark *et al.*, 1978; Gibbons *et al.*, 1979). To quantify accurately receptor sites in cell fractions which also contain low-affinity and high-capacity oestrogen-binding macromolecules, we have developed a precise assay which discriminates the two oestrogen receptors from low-affinity oestrogen-binding sites.

Several models have been proposed to account for the induction of specific mRNA species by steroid hormones (Yamamoto & Alberts, 1975, 1976). They imply that steroid-receptor complexes associate with high-affinity acceptor sites located in the nucleus and there they produce conformational changes that modify the transcriptional activity. This suggests that any study of these nuclear events should indicate a correlation between the number of nuclear oestrogen receptors and the magnitude of specific inducible responses, i.e. the preferential transcription of certain genes.

In the present study, nuclear and cytoplasmic oestrogen-receptor concentrations have been measured. Nuclei from the same specimens have been used for the synthesis of [³H]RNA *in vitro* and for the quantification of ovalbumin-mRNA sequences. These studies were performed under different conditions of oestrogen withdrawal and secondary stimulation in order to correlate nuclear acceptor-site concentrations with the ability of these nuclei to transcribe ovalbumin mRNA *in vitro*.

It is our ultimate goal to investigate the role of pure nuclear oestrogen receptors (Smith & Schwartz, 1979; Taylor & Smith, 1979) on gene transcription in reconstituted cell-free systems. The experiments described here are prerequisites of such reconstituted systems, since the optimization of oestrogen withdrawal allows a maximum number of nuclear acceptor sites to be available for occupancy by pure oestrogen-receptor complexes added *in vitro*. It is only through cell-free systems that the nature of the molecular link between steroid hormones, their specific receptors, and gene regulation can be effectively investigated.

Materials and methods

[2,4,6,7-³H]Oestradiol (100 Ci/mmol) and [5,6-³H]UTP (35 Ci/mmol) were purchased from Amersham-Searle (Arlington Heights, IL, U.S.A.). Non-radioactive nucleoside triphosphates and diethylstilboestrol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 'Ultra Pure' Tris and 'Enzyme Grade' sucrose were provided by Schwarz/Mann (Orangeburg, NY, U.S.A.). All other chemicals were 'reagent grade' purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Hormone treatment

For stimulation, 2-week-old female White Leghorn chicks were subcutaneously injected with 2.5 mg of diethylstilboestrol in sesame oil. After 14 daily injections these treatments were discontinued to allow intervals of 'withdrawal' indicated in the text. Length of withdrawal refers to the number of elapsed hours after the last injection. In some cases, chicks were secondarily stimulated by an additional subcutaneous injection of 2.5 mg of diethylstilboestrol.

Tissue preparation

Primarily stimulated, withdrawn, or secondarily stimulated chicks were killed at the indicated times by decapitation. Oviduct tissue was removed, quickly trimmed of connective tissue and rinsed in ice-cold normal saline (0.9% NaCl). Six to ten chick oviducts were pooled for each data point, to minimize possible variation among different animals. All subsequent procedures were carried out at 4°C unless noted otherwise.

Oviducts were weighed and homogenized in 9 vol. of 0.5 M-sucrose in 50 mM-Tris/HCl (pH 7.4)/20 mM-KCl/5 mM-MgCl₂ (TKM buffer) with a Tissuemaker blender (Tekmar Co., Cincinnati, OH, U.S.A.) at 70V for 1 min in short bursts. The homogenate was filtered through cheesecloth and organza, layered over 1 vol. of 0.88 M-sucrose/TKM buffer, and centrifuged at 2000 *g*_{av} for 10 min. The supernatant was subjected to ultracentrifugation at 100 000 *g*_{av} for 60 min to furnish cytosol.

The crude pellet from the initial centrifugation was resuspended in 0.5 vol. of 0.88 M-sucrose/TKM buffer, homogenized in 6 vol. of 2.1 M-sucrose/TKM buffer and centrifuged at 12 000 *g*_{av} for 90 min to pellet nuclei. This pellet was finally resuspended in buffer containing 1 mM-ATP, -GTP and -CTP, 5 mM-MgCl₂, 2.5 mM-MnCl₂, 3 mM-dithiothreitol, 12.5% (v/v) glycerol, 100 mM-KCl and 50 mM-Tris/HCl (pH 7.8). Concentrations of nuclei were adjusted to approx. 4×10^7 – 1×10^8 nuclei/ml (100–250 µg of DNA/ml). Nuclei prepared in this manner were free of cytoplasmic debris, 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 three samples: (1) for nuclear oestrogen-receptor microassay, (2) for RNA synthesis *in vitro*, and (3) for DNA determination by the diphenylamine technique (Giles & Myers, 1965).

Cytoplasmic oestrogen-receptor assay

Cytosol fractions were obtained as described above and oestrogen receptors were measured by hormone-binding-site titration and Scatchard analysis by using the dextran-coated-charcoal adsorption technique described previously (Smith *et al.*, 1979). Cytosol (200 µl) was added to tubes

containing [^3H]oestradiol (concentration range 0.1–50 nM) and to corresponding tubes which also contained a 100-fold excess of unlabelled diethylstilboestrol. After incubation at 4°C for 18 h, 0.5 ml of the dextran-coated-charcoal solution was added, samples were incubated for 5 min, and the tubes were centrifuged at 800g for 10 min to pellet charcoal. In some cases cytoplasmic receptor assays were performed under exchange conditions (30°C for 3 h). Samples were then cooled to 4°C before the dextran-coated-charcoal solution was added. Supernatants were added to 7 ml of Scintiverse (Fisher) and counted for radioactivity at 27% efficiency. Specifically bound [^3H]oestradiol was determined by subtracting the non-specifically bound radioactivity from the total bound radioactivity. These data were plotted as described by Scatchard (1949) and the resulting specific-oestrogen-binding curves were resolved by the method of Rosenthal (1967). High-affinity receptor binding was verified by calculation of equilibrium dissociation constants, and the concentration of receptors was determined from the abscissa intercept. Values for cytoplasmic oestrogen receptors represent the sum of the 0.1 nM- and 4 nM- K_d species described previously (Smith *et al.*, 1979).

Nuclear exchange microassay

Small volumes of resuspended nuclei (100 μl) were incubated in the presence of increasing concentrations of [^3H]oestradiol under conditions of elevated temperature to effect hormone exchange (Anderson *et al.*, 1972). The conditions of the exchange reaction were optimized for maximum receptor detection and minimum receptor degradation and were idealized at 30°C for 3 h. [^3H]Oestradiol binding over the concentration range 0.5–20 nM was measured and Scatchard analysis was again utilized to quantify high-affinity specific binding.

A novel application of disc filtration was employed to separate bound from free [^3H]oestradiol by using glass-fibre filters on a Millipore (Bedford, MA, U.S.A.) filter apparatus. Reaction mixtures were filtered through the discs under low suction. The filters were washed three times with buffer containing 15 mM-Tris/HCl (pH 7.4), 10% (v/v) glycerol and 1% (v/v) Triton X-100 to remove free and membrane-bound [^3H]oestradiol. This procedure also removes any contaminating cytosol receptors. Filter discs were heat-dried for 15 min, treated with NCS solubilizer (Amersham) with gentle shaking for 15 min, and discs and eluates were counted for radioactivity in Liquifluor (New England Nuclear, Boston, MA, U.S.A.)/acetic acid/toluene (16:1:380, by vol.), at 25% efficiency. Specifically bound [^3H]oestradiol was determined by comparison with similar samples incubated as

above, but in the presence of a 100-fold excess of non-radioactive diethylstilboestrol.

The entire washing procedure was carried out at 4°C and required less than 10 min for each sample assayed. This assay time is far less than the half-life of dissociation of the oestrogen-receptor complex (Smith & Schwartz, 1979). Under these assay conditions, quantification of nuclear receptors was determined with high precision (coefficient of variation 10–12%), and contamination by low-affinity [^3H]oestradiol binding was minimized.

The tubular-gland-cell concentrations of cytoplasmic and nuclear oestrogen receptors were calculated by estimating the proportion of such cells in representative oviduct-magnum cross-sections. Tissue specimens isolated at different stages of hormone withdrawal were fixed in Bouin's fixative and 5 μm sections were stained with haematoxylin and eosin for microscopic examination. It was assumed that oestrogen receptors were confined to the tubular-gland cells and were absent from surface columnar epithelial cells.

Synthesis in vitro and extraction of [^3H]RNA

Purified nuclei isolated and resuspended as described above were incubated at 37°C for 30 min after the addition of 0.04 mM-[^3H]UTP. The reaction was terminated by the addition of bovine pancreatic deoxyribonuclease I (20 $\mu\text{g}/\text{ml}$), and this solution was incubated for 30 min at 37°C. The deoxyribonuclease I had been affinity-column-purified as described by Maxwell *et al.* (1977). Isolation of [^3H]RNA synthesized *in vitro* was accomplished by the addition of 0.5% sodium dodecyl sulphate and 2 mM-EDTA, and digestion with proteinase K (200 $\mu\text{g}/\text{ml}$) for 30 min at 37°C as described by Roop *et al.* (1978). The RNA was extracted by adding an equal volume of phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.). After vigorous mixing and centrifugation (2000g_{av}, for 6 min), the aqueous fraction was recovered. This extraction procedure was repeated twice. The nucleic acids mixture was adjusted to 150 mM-sodium acetate and precipitated with 2 vol. of ethanol at –20°C. Precipitated nucleic acids were resuspended in a buffer containing 10 mM-Tris/HCl (pH 7.5), 10 mM-NaCl and 3 mM-MgCl₂, and again treated with deoxyribonuclease I for 15 min at 37°C. The solution was subjected to a final phenol extraction and the aqueous phase was chromatographed on Sephadex G-50 to separate macromolecular RNA from free nucleotides, with distilled water as eluent. The eluate was freeze-dried and stored at –20°C until hybridization reactions were performed.

Preparation of ovalbumin-cDNA filters

Ovalbumin cDNA was synthesized by incubating DNA precursors (deoxyribonucleoside triphos-

phates) with purified hen oviduct ovalbumin mRNA in the presence of avian-myeloblastosis-virus reverse transcriptase. The cDNA obtained was incorporated into the bacterial plasmid pMB9, and this was used to transform *Escherichia coli* strain χ 1776 (McReynolds *et al.*, 1977). A positive clone, pOV230, was obtained, which contained 1860 of the 1872 base sequences in ovalbumin mRNA. Nitrocellulose filters containing immobilized pOV230 DNA were prepared as described by Roop *et al.* (1978) and were a gift from Dr. M.-J. Tsai and Dr. B. W. O'Malley, Department of Cell Biology, Baylor College of Medicine, Houston, TX, U.S.A.

Hybridization of [³H]RNA synthesized in vitro to ovalbumin-cDNA filters

Hybridization-reaction recoveries were monitored by the inclusion of a small quantity of ³²P-labelled ovalbumin mRNA as an internal standard. Freeze-dried [³H]RNA (1.5×10^6 – 4.5×10^6 c.p.m.) and ³²P-labelled ovalbumin mRNA (2500–3000 c.p.m., 1–2 Ci/ μ mol) were heated in silicone-treated glass vials at 80°C for 5 min in 40 μ l of 50% (v/v) formamide/210 mM-NaCl/21 mM-sodium citrate (pH 7.0) to denature any double-stranded structures. In some cases 7.5 μ g of purified competitor ovalbumin mRNA was included. Hybridization reactions were started by addition of ovalbumin-cDNA filters and incubation was at 42°C for 18 h. Conditions of filter washing and scintillation counting were as described by Tsai *et al.* (1978).

Results

Effects of oestrogen withdrawal on receptor localization and transcription of ³H-labelled ovalbumin mRNA

After 14 daily diethylstilboestrol injections, chicks were withdrawn from hormone for various intervals before they were killed. At each of these time points, cytoplasmic oestrogen receptors, nuclear oestrogen receptors and ovalbumin-mRNA transcription *in vitro* were quantified.

As previously demonstrated (Smith *et al.*, 1979), cytosol fractions of oestrogen-withdrawn chick oviduct homogenates contain two distinct oestrogen-receptor molecules, having equilibrium dissociation constants (K_d) of 0.1 and 4 nM. In oestrogen-stimulated oviducts, no cytoplasmic oestrogen receptors could be demonstrated, even when [³H]-oestradiol-binding assays were performed under exchange conditions. Thus it is unlikely that our failure to detect receptors in cytosol was due to the presence of excess diethylstilboestrol. Low concentrations of cytoplasmic receptors were detectable after 24–36 h. After 48–54 h of oestrogen withdrawal, these receptors reached maximum concentrations in cytosol, representing 800–900

molecules/tubular-gland cell. After longer periods of withdrawal (up to 96 h) the cellular concentrations, K_d values and relative ratios of the two cytoplasmic receptors remained constant. Histological examination of oviduct tissue specimens during these stages of withdrawal revealed minimal changes in the number of tubular-gland cells, although evidence of secretory activity in these cells appeared to wane dramatically after 60 h of oestrogen withdrawal.

Highly purified nuclei isolated from chick oviducts at different stages of hormone withdrawal were assayed for nuclear oestrogen receptors by using the sensitive filter assay described in the Materials and methods section. As shown in Table 1, a precipitous fall in nuclear receptor concentration was observed immediately after hormone withdrawal. Oviduct nuclei from chicks withdrawn from diethylstilboestrol for over 60 h had a constant basal content of nuclear receptors (150–200 molecules/tubular-gland-cell nucleus), which comprised less than 10% of the nuclear receptor concentration of chronically stimulated chicks. This nuclear-receptor nadir corresponded to maximum cytoplasmic receptor concentrations, as indicated above. The rapid depletion of nuclear receptors during oestrogen withdrawal was accompanied by oviduct involution and loss of ovalbumin-gene expression as measured by transcription *in vitro* (Fig. 1, Table 1). To verify that cytoplasmic receptors could not be detected in this nuclear receptor assay, [³H]oestradiol-labelled cytosol from hormone-withdrawn chick oviducts was applied to duplicate filters, which were washed in the usual manner. No [³H]oestradiol was retained by the filters.

Hybridization of [³H]RNA synthesized *in vitro* to nitrocellulose filters containing cloned pOV230 DNA was performed under conditions of high stringency to completion of RNA-DNA annealing (Roop *et al.*, 1978). Efficiencies of hybridization were monitored with a ³²P-labelled ovalbumin-mRNA internal standard transcribed from ovalbumin cDNA. Hybridizations were performed in a 10–100-fold DNA excess to maximize assay sensitivity. Experiments performed to confirm the validity of this transcription assay revealed that [³H]RNA synthesis was linear with respect to DNA concentration and was inhibited 60–70% by α -amanitin (1 μ g/ml) at a concentration that specifically inhibits eukaryotic RNA polymerase II activity (Weinmann & Roeder, 1974). Ovalbumin-gene transcription was inhibited by over 90% at this concentration of α -amanitin and was DNA-template-specific, since actinomycin D (40 μ g/ml) inhibited synthesis of ³H-labelled ovalbumin mRNA by over 95%. Transcription of this gene *in vitro* was appropriately asymmetric, as over 88% of ovalbumin-specific sequences synthesized in stimulated nuclei could be competed from binding to an

Table 1. *Effects of oestrogen withdrawal on receptor localization and transcription of ^3H -labelled ovalbumin mRNA* Oviducts were excised from six to ten chicks for each data point at the indicated stages of hormone withdrawal and/or secondary stimulation. Cytoplasmic and nuclear oestrogen receptors were quantified by Scatchard (1949) analysis. Tubular-gland-cell concentrations of these receptors were calculated by estimating the proportion of these cells in representative histological cross-sections of oviduct tissue. Transcription *in vitro* was determined as [^3H]UMP c.p.m. incorporated into RNA capable of hybridization to ovalbumin-cDNA filters. Non-specific binding was determined by hybridization in the presence of excess unlabelled ovalbumin mRNA. Specific filter binding was corrected for recovery by using a ^{32}P -labelled ovalbumin-mRNA internal standard. The amount of ^3H -labelled ovalbumin mRNA transcribed *in vitro* is presented as a percentage of the total [^3H]RNA synthesized.

	No. of cytoplasmic receptors/tubular- gland cell	No. of nuclear receptors/tubular- gland cell	^3H hybridized (c.p.m.)	^{32}P recovery (%)	Corrected ^3H hybridized (c.p.m.)	$10^{-6} \times$ Input ^3H (c.p.m.)	Ovalbumin mRNA (%)
Stimulated	<50	3100	405	10.2	3510	1.55	0.23
+ competitor mRNA			47				
24 h withdrawn	<50	1420	332	19.8	1465	1.77	0.08
+ competitor mRNA			42				
36 h withdrawn	<50	1340	251	18.4	957	2.20	0.04
+ competitor mRNA			75				
48 h withdrawn	840	560	85	16.2	401	1.72	0.02
+ competitor mRNA			20				
60 h withdrawn	800	180	48	16.4	152	1.42	0.01
+ competitor mRNA			23				
72 h withdrawn	910	190	35	14.9	20	2.05	<0.001
+ competitor mRNA			32

Fig. 1. *Effects of oestrogen withdrawal on oviduct involution, nuclear oestrogen-receptor depletion and ovalbumin-mRNA synthesis in vitro*

Chicks were killed and oviducts excised at various intervals after oestrogen withdrawal (times in h below the columns). (a) Oviducts were trimmed of connective tissue and weighed. The mean weights ($\pm$ S.E.M.) of at least ten oviducts/time point are presented ($\boxtimes$). (b) Nuclei were isolated and purified from oviduct homogenates. Nuclear receptors were quantified by Scatchard (1949) analysis by using a nuclear exchange microassay, and were corrected for numbers of tubular-gland cells. Mean concentrations of nuclear receptors/tubular-gland cell ($\pm$ S.E.M. of duplicate experiments) are presented ($\boxplus$). (c) Identical samples of purified nuclei were assayed for ovalbumin-mRNA synthesis *in vitro* as described in the Materials and methods section. Results are the means of duplicate experiments $\pm$ S.E.M., expressed as percentage of total [^3H]RNA synthesized *in vitro* ($\square$).

ovalbumin-cDNA filter with an excess of purified unlabelled ovalbumin mRNA. Under these conditions, a fall in specific ovalbumin-gene transcription was observed, from 0.23% of total [^3H]RNA synthesized in diethylstilboestrol-stimulated chick oviduct nuclei to undetectable values (<0.001%) after 72 h of oestrogen withdrawal (Fig. 1, Table 1).

Effects of secondary stimulation on oestrogen-receptor translocation and synthesis of ^3H -labelled ovalbumin mRNA

The above experiments demonstrated that after 48–60 h of oestrogen withdrawal cytoplasmic oestrogen-receptor concentrations were maximized,

whereas nuclear receptors were at a basal concentration. Changes in nuclear receptor concentrations and transcription of ^3H -labelled ovalbumin mRNA after secondary stimulation were examined by withdrawing chicks from oestrogen for 54–84 h and then administering an additional hormone injection. At 4 h after secondary stimulation, these chicks were killed and oviduct tissue was fractionated. In each group no cytoplasmic receptors were detected. The amounts of nuclear receptors in the secondarily stimulated chicks were at least 3-fold less than those concentrations in chronically stimulated chicks. We observed an inverse relationship between nuclear receptor contents and the preceding length of withdrawal (Table 2). This observation was in contrast with the finding that cytoplasmic receptors were fully depleted after secondary stimulation, despite the preceding length of withdrawal.

The transcription of ovalbumin mRNA *in vitro* by samples of nuclei isolated under the conditions of withdrawal and secondary stimulation closely paralleled the concentrations of nuclear receptors. In agreement with the trend in nuclear receptor concentrations, ovalbumin-gene expression in nuclei prepared from secondarily stimulated chicks was significantly less than in chronically stimulated chick oviduct nuclei analysed simultaneously. Again, transcription of ovalbumin mRNA *in vitro* was inversely proportional to the length of withdrawal before secondary stimulation (Table 2).

Kinetics of nuclear oestrogen-receptor accumulation and induction of ovalbumin-mRNA transcription after secondary stimulation

Results from the above experiments demonstrated that cytoplasmic and nuclear oestrogen-receptor concentrations were at a maximum and a minimum, respectively, after 60 h of hormone withdrawal. It was also evident, however, that secondary stimu-

lation was attenuated if intervals of withdrawal of over 72 h were allowed. The conditions under which hormone-withdrawn chicks were most sensitive to secondary stimulation fell within this narrow time period. Having determined these optimal conditions, we proceeded to examine the kinetics of nuclear-receptor accumulation and ovalbumin-gene expression during secondary stimulation.

Chicks withdrawn from oestrogen for 60 h were secondarily stimulated for periods of 0–24 h. Oviduct-tissue homogenates were assayed for cytoplasmic receptors, nuclear receptors and transcription of ovalbumin mRNA *in vitro* at each time point. Under these conditions a stoichiometric translocation of oestrogen receptors from the cytoplasmic to nuclear fractions was documented. Mobilization and depletion of cytoplasmic receptors, as observed above, were complete within 30 min of secondary stimulation, and nuclear accumulation of these receptors occurred over the ensuing 4–12 h period (Table 3). Although no cytoplasmic receptors were detected after 30 min (indicating that no replenishment of receptors in the cytoplasm had occurred), a substantial increase in the number of nuclear oestrogen receptors was noted between 12 and 24 h. These additional receptors most probably represent nascent molecules immediately translocated by hormone released from the subcutaneous deposit of diethylstilboestrol.

Transcription of ovalbumin mRNA *in vitro* was also measured during secondary stimulation (Table 3). Nuclei isolated from chicks that had received no oestrogen for 60 h synthesized very small amounts of ovalbumin mRNA (0.01%). After secondary stimulation the induction of transcription of ovalbumin mRNA *in vitro* proceeded linearly for 4 h, and then appeared to remain constant until 12 h. Between 12 and 24 h a 2-fold increase in ovalbumin-mRNA synthesis was observed, reaching a value approx. 50% of the ovalbumin-mRNA concentration

Table 2. *Effects of secondary stimulation on oestrogen-receptor translocation and synthesis of ^3H -labelled ovalbumin mRNA*

For experimental details see legend to Table 1. Abbreviation: DES, diethylstilboestrol.

	No. of cytoplasmic receptors/tubular- gland cell	No. of nuclear receptors/tubular- gland cell	^3H hybridized (c.p.m.)	^{32}P recovery (%)	Corrected ^3H hybridized (c.p.m.)	$10^{-6} \times$ Input ^3H (c.p.m.)	Ovalbumin mRNA (%)
Stimulated + competitor mRNA	<50	2530	588 40	9.1	6022	2.01	0.30
54 h withdrawn + 4 h DES + competitor mRNA	<50	800	340 72	12.0	2233	1.51	0.15
60 h withdrawn + 4 h DES + competitor mRNA	<50	750	642 110	13.7	3883	3.75	0.10
72 h withdrawn + 4 h DES + competitor mRNA	<50	730	280 71	13.0	1608	2.04	0.08
84 h withdrawn + 4 h DES + competitor mRNA	<50	500	260 111	12.7	1173	4.57	0.03

Table 3. Kinetics of nuclear oestrogen-receptor accumulation and induction of ovalbumin-mRNA transcription after secondary stimulation

For experimental details see legend to Table 1. Abbreviation: DES, diethylstilboestrol.

	No. of cytoplasmic receptors/tubular- gland cell	No. of nuclear receptors/tubular- gland cell	³ H hybridized (c.p.m.)	³² P recovery (%)	Corrected ³ H hybridized (c.p.m.)	10 ⁻⁶ × Input ³ H (c.p.m.)	Ovalbumin mRNA (%)
60h withdrawn	760	170	58	12.7	118	1.52	0.01
+ competitor mRNA			43				
60h withdrawn + 1h DES	<50	620	71	7.2	306	1.51	0.02
+ competitor mRNA			49				
60h withdrawn + 2h DES	<50	700	113	12.9	527	2.05	0.03
+ competitor mRNA			45				
60h withdrawn + 4h DES	<50	790	297	13.1	1732	2.09	0.08
+ competitor mRNA			70				
60h withdrawn + 12h DES	<50	900	297	12.6	1278	1.96	0.07
+ competitor mRNA			136				
60h withdrawn + 24h DES	<50	1370	439	10.2	2843	2.14	0.13
+ competitor mRNA			149				

synthesized in chronically stimulated chick oviduct nuclei. Similar results have been reported by McKnight & Palmiter (1979), who have examined the synthesis of ovalbumin mRNA during secondary stimulation, using an assay similar to our own.

Correlation analysis of nuclear oestrogen-receptor concentrations with ovalbumin-gene expression *in vitro*

Nuclear-receptor-binding sites were plotted versus ovalbumin mRNA synthesized *in vitro* for all nuclear assays performed as displayed in Tables 1, 2 and 3 ($n = 17$). The resulting scattergram demonstrated a good approximation of a straight line. Linear regression and correlation analysis of the data showed that a high degree of correlation existed between these two variables, with a Pearson correlation coefficient $r = 0.87$ ($P < 0.001$). Moreover, when the data were statistically analysed by non-parametric methods, rankings of each of the variables again revealed a high correlation: the Spearman rank-order correlation coefficient $\rho = 0.87$ ($P < 0.001$) and the Kendall rank order coefficient $\tau = 0.71$ ($P < 0.001$). Thus these data clearly show that a tight correlation exists between the number of nuclear acceptor sites for oestrogen receptors and the quantity of ovalbumin mRNA synthesized by these nuclei *in vitro*.

Discussion

This study has explored the localization and quantification of specific high-affinity oestrogen-receptor molecules and their relationship to the expression *in vitro* of a specific gene in the same specimen of isolated chick oviduct nuclei. Evidence is presented here which supports the hypothesis of receptor translocation or immobilization in the

nucleus. After secondary stimulation with oestrogen, cytoplasmic receptors appear to be rapidly mobilized from the cytosol fraction and subsequently become tightly associated in the nucleus, presumably at chromatin acceptor sites. Although some investigators have argued that apparent translocation may reflect an artifact of tissue homogenization and disruption of normal equilibria *in vivo* (Gorski & Gannon, 1976; Sheridan *et al.*, 1979), the work of many investigators indicates that oestrogen-receptor complexes move from the cytoplasm to the nuclei of target cells (Jensen *et al.*, 1968; Shyamala & Gorski, 1969; Clark *et al.*, 1973; Katzenellenbogen *et al.*, 1973; Sutherland & Baulieu, 1976). Regardless of the exact mechanism of oestrogen transport in target cells *in vivo*, stimulation by these steroid hormones directs a redistribution or altered equilibrium of oestrogen molecules bound to specific oestrogen receptors. Although artifacts of homogenization might obscure the true localization of receptors in hormone-withdrawn tissue, oestrogen stimulation clearly results in the association of these receptor proteins with nuclear chromatin. We have chosen to retain the terminology of translocation, understanding that it signifies the process of oestrogen-receptor immobilization within the nucleus after hormone binding.

When the hormone-stimulation/withdrawal regimen described in the Materials and methods section is used, stoichiometric translocation can be attained when chicks receive a single 2.5 mg dose of diethylstilboestrol after a 60h withdrawal interval. Under these conditions, nuclear receptors accumulate at putative acceptor sites, reaching a maximum steady-state concentration within 2–4 h, to account for essentially all of the translocated cytoplasmic oestrogen receptors. This value is

maintained until 12h after secondary stimulation, when a second increase in nuclear receptors is observed. As no cytoplasmic receptors are detectable at this time, the latter increase in nuclear receptors would reflect nascent oestrogen-receptor synthesis and immediate translocation. The apparent lack of stoichiometry of cytoplasmic receptor translocation after 1h can be explained by the slower kinetics of the formation of high-affinity nuclear receptor complexes. Any loosely bound nuclear receptor complexes would not be detected in the nuclear exchange assay, since these complexes would be washed from the nuclei during their isolation and washing with Triton-containing buffer. The lag in cytoplasmic receptor replenishment after oestrogen withdrawal and depletion of nuclear receptors implies that new synthesis rather than recycling is the mechanism of cytoplasmic receptor replenishment.

When withdrawal intervals of over 60h are allowed, secondary stimulation results in a sub-maximal accumulation of nuclear receptors (Table 2), and this decreased response is time-dependent. During the time period investigated (54–84h) tubular-gland morphology and tubular-gland-cell number/oviduct undergo no significant changes (Oka & Schimke, 1969; R. N. Taylor, unpublished work). Concentrations of cytoplasmic receptors likewise appear to be constant during such withdrawal periods, and these receptors are fully capable of mobilization from the cytosol fraction. The ratio of the two receptor types also does not change in these withdrawal periods; therefore the diminished response is not due to a preferential loss of one of these receptors. The observed differences in the nuclear accumulation of these receptors would indicate that prolonged nuclear retention (>4h) of oestrogen receptors requires that functional nuclear acceptor sites be present. Although these acceptor sites appear to retain stoichiometric receptor-binding capacity up to 60h after oestrogen withdrawal, intervals of hormone withdrawal of over 60h result in the progressive depletion of functional acceptors.

The relative amounts of synthesis of ^3H -labelled ovalbumin mRNA were compared at ≤ 24 , 60, 72 and 84h after oestrogen withdrawal, and after a short interval (4h) of secondary stimulation. Initial rates of synthesis were estimated on the basis of $d(\% \text{ ovalbumin mRNA})/dt$, assuming linear kinetics and negligible mRNA degradation during this short interval. Although no absolute values can be attached to these estimates, the relative rates of ovalbumin-mRNA synthesis were 10:10:2:1 (all approx.) for chicks ≤ 24 , 60, 72 and 84h withdrawn respectively. These estimates are in agreement with the finding that nuclear acceptor sites appear to be maintained for up to 60h of oestrogen withdrawal, but are decreased after longer periods

without oestrogen. These rates also reflect the observation that nuclear acceptors for oestrogen receptors are maintained at a steady-state low number (150–200/nucleus) for periods of withdrawal of over 72h. After secondary stimulation, transcription of ovalbumin mRNA is promptly initiated, but at an apparently low rate. As the length of secondary oestrogen exposure is prolonged, the extent of ovalbumin-mRNA synthesis approaches that in the stimulated oviduct. Translocation of cytoplasmic receptors into the nucleus seems to require a time-dependent resynthesis or change in structural conformation of acceptor sites before nuclear receptors can be detected. Increase in these nuclear sites occur in a quantal, rather than linear, manner, and are observed until the nuclear receptor content of the chronically stimulated chick (2500–3000/tubular-gland cell) is reached. Presumably this phenomenon is linked to cell-cycle changes, as a characteristic 8–12h interval is noted, which may be related to the mitogenic schedule of oestrogen (Socher & O'Malley, 1973).

The variation in results obtained from the assay of transcription *in vitro* on replicate samples from oestrogen-withdrawn oviducts was much greater (coefficient of variation 25%) than that for the quantification of nuclear receptors (coefficient of variation 10–12%). These relative variations made it important to perform multiple assays ($n = 17$) over different times of hormonal withdrawal and restimulation, so that these data could be appropriately evaluated statistically. Three independent statistical treatments of the data showed that a very high correlation ($P < 0.001$) existed between the numbers of nuclear acceptor sites for oestrogen receptors and ovalbumin-gene transcription *in vitro*.

The rate and extent of ovalbumin-mRNA synthesis, accumulation in chick oviduct nuclei, and ultimately processing and transport into tubular-gland-cell cytoplasm, form a complex regulatory mechanism under sophisticated co-ordinate control by several steroid hormones and probably by many other intracellular modulators. We have attempted to understand the first of a series of regulatory steps by the precise quantification of nuclear oestrogen receptors and synthesis of ovalbumin-mRNA *de novo* in identical samples of chick oviduct nuclei. Although these nuclei have been subjected to manipulations necessary for their isolation and purification, both the nuclear receptor microassay and the assay of transcription *in vitro* were performed on identical samples from each preparation. Although the relationship existing between these two parameters confirms previously reported correlations derived from less-direct methods, our results link the oestrogen-mediated induction of ovalbumin-gene expression with the occupancy of nuclear acceptor sites.

Although many questions about the mechanism of oestrogen action on ovalbumin-gene expression remain unanswered, this investigation has demonstrated that isolated oviduct nuclei contain functional nuclear acceptors for oestrogen receptors which are closely correlated with the synthesis of ovalbumin-mRNA sequences *in vitro*. With this information, the cell-free conditions that are most sensitive to oestrogen restimulation can be selected for oestrogen-receptor reconstitution experiments. Under such conditions, using pure nuclear oestrogen-receptor complexes (Smith & Schwartz, 1979; Taylor & Smith, 1979), the correlations between nuclear receptor-acceptor-site binding and specific gene transcription may be extended to ascertain the precise role of oestrogen receptors in gene expression.

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References

- Anderson, J., Clark, J. H. & Peck, E. J. (1972) *Biochem. J.* **126**, 561–567
- Best-Belpomme, M., Mester, J., Weintraub, H. & Baulieu, E.-E. (1975) *Eur. J. Biochem.* **57**, 537–547
- Clark, J. H., Anderson, J. & Peck, E. J. (1973) in *Receptors for Reproductive Hormones* (O'Malley, B. W. & Means, A. R., eds.), pp. 15–59, Plenum Press, New York
- Clark, J. H., Hardin, J. W., Upchurch, S. & Eriksson, H. (1978) *J. Biol. Chem.* **253**, 7630–7634
- Cox, R. F., Catlin, G. H. & Carey, N. H. (1971) *Eur. J. Biochem.* **22**, 46–56
- Gibbons, W. E., Buttram, V. C., Besch, P. K. & Smith, R. G. (1979) *Am. J. Obstet. Gynecol.* **135**, 799–803
- Giles, K. W. & Myers, A. (1965) *Nature (London)* **206**, 93
- Gorski, J. & Gannon, F. (1976) *Annu. Rev. Physiol.* **38**, 425–450
- Harrison, R. W. & Toft, D. O. (1975) *Endocrinology* **96**, 199–205
- Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W. & DeSombre, E. R. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **59**, 632–638
- Katzenellenbogen, J. A., Johnson, H. J. & Carlson, K. E. (1973) *Biochemistry* **12**, 4092–4099
- King, R. J. B. & Mainwaring, W. I. P. (1974) *Steroid-Cell Interactions*, pp. 190–262, University Park Press, Baltimore
- Maxwell, I. H., Maxwell, F. & Hahn, W. E. (1977) *Nucleic Acids Res.* **4**, 241–246
- McKnight, G. S. & Palmiter, R. D. (1979) *J. Biol. Chem.* **254**, 9050–9058
- McReynolds, L. A., Catterall, J. F. & O'Malley, B. W. (1977) *Gene* **2**, 217–230
- Mulvihill, E. R. & Palmiter, R. D. (1977) *J. Biol. Chem.* **252**, 2060–2068
- Oka, T. & Schimke, R. T. (1969) *J. Cell Biol.* **43**, 123–137
- O'Malley, B. W. & Means, A. R. (1974) *Science* **183**, 610–620
- Palmiter, R. D. & Carey, N. H. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2357–2361
- Pennequin, P., Robins, D. M. & Schimke, R. T. (1978) *Eur. J. Biochem.* **90**, 51–58
- Roop, D. R., Nordstrom, J. L., Tsai, S. Y., Tsai, M.-J. & O'Malley, B. W. (1978) *Cell* **15**, 671–685
- Rosenthal, H. E. (1967) *Anal. Biochem.* **48**, 317–338
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Sheridan, P. J., Buchanan, J. M., Anselmo, V. C. & Martin, P. M. (1979) *Nature (London)* **282**, 579–582
- Shyamala, G. & Gorski, J. (1969) *J. Biol. Chem.* **244**, 1097–1103
- Smith, R. G. & Schwartz, R. J. (1979) *Biochem. J.* **184**, 331–343
- Smith, R. G., Clarke, S. G., Zalta, E. & Taylor, R. N. (1979) *J. Steroid Biochem.* **10**, 31–35
- Socher, S. H. & O'Malley, B. W. (1973) *Dev. Biol.* **30**, 411–417
- Sutherland, R. L. & Baulieu, E.-E. (1976) *Eur. J. Biochem.* **70**, 531–541
- Swaneck, G. E., Nordstrom, J. L., Kreuzaler, F., Tsai, M.-J. & O'Malley, B. E. (1979a) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1049–1053
- Swaneck, G. E., Kreuzaler, F., Tsai, M.-J. & O'Malley, B. W. (1979b) *Biochem. Biophys. Res. Commun.* **88**, 1412–1418
- Taylor, R. N. & Smith, R. G. (1979) *Biochem. Biophys. Res. Commun.* **91**, 136–142
- Teng, C. S. & Teng, C. T. (1976) *Biochem. J.* **154** 1–9
- Tsai, S. Y., Roop, D. R., Tsai, M.-J., Stein, J. P., Means, A. R. & O'Malley, B. W. (1978) *Biochemistry* **17**, 5773–5780
- Weinmann, R. & Roeder, R. G. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1790–1794
- Yamamoto, K. R. & Alberts, B. M. (1975) *Cell* **4**, 301–310
- Yamamoto, K. R. & Alberts, B. M. (1976) *Annu. Rev. Biochem.* **45**, 721–746