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# Effects of Highly Purified Estrogen Receptors on Gene Transcription in Isolated Nuclei†

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ABSTRACT: This report describes the direct effect of a highly purified hen oviduct estrogen receptor on the rates of RNA polymerase II dependent gene transcription in isolated nuclei. When this receptor was added to an in vitro transcription system containing oviduct nuclei from chronically estrogen-treated chicks withdrawn from estrogen for 0, 24, 36, 48, 60, or 72 h, the rates of incorporation of [$^3$H]UMP into RNA were increased by 1.4–3.8-fold. This effect was dependent upon receptor concentration, being half-maximal at 0.25 nM, and was inhibited by the addition of either $\alpha$-amanitin (1 $\mu$g/mL) or actinomycin D (40 $\mu$g/mL). The increase in transcription rates was not demonstrable with spleen or erythrocyte nuclei, and increases in polymerase II activity in vitro were not observed with estrogen alone. These results demonstrate that the increases in RNA polymerase II activity observed following estrogen administered in vivo are mediated by the action of estrogen–receptor complexes on oviduct nuclei. The synthesis of new RNA polymerase II is not essential. When the RNA synthesized in vitro was isolated and assayed for products of ovalbumin gene expression, the nuclei from chronically stimulated and 24-h estrogen-withdrawn oviducts showed 1.6–1.8-fold increases in ovalbumin gene expression in the presence of the highly purified receptor. Thus, within 24 h of estrogen withdrawal, exogenous receptor–estradiol complexes can mimic the effect of estrogen administered in vivo on ovalbumin mRNA synthesis. After 36 and 60 h of estrogen withdrawal, the addition of receptor did not produce increases in ovalbumin gene expression despite increases in RNA polymerase II activity. These results imply that this particular estrogen receptor requires other estrogen-dependent factors before it can directly cause specific initiation of the ovalbumin gene in nuclei isolated after 24 h of hormonal withdrawal.

The relationship between nuclear estrogen receptors and egg white protein gene transcription in vivo (Tsai et al., 1975; Mulvihill & Palmiter, 1977) has been recently confirmed and extended by using an in vitro transcription assay (Taylor et al., 1980; Smith & Taylor, 1981). This system utilizes relatively short pulses of [$^3$H]UTP labeling, allowing the detection of RNA molecules synthesized de novo. It is minimally affected by the rate of RNA degradation, a factor which is also hormonally modified (Palmiter & Carey, 1974). The optimization and validation of this assay, therefore, have provided an important tool for the investigation of class II transcriptional regulation under readily manipulated cell-free conditions.

It is our ultimate goal to characterize, purify, and examine in vitro the role of the particular chick oviduct proteins which confer the specificity of estrogenic responses at the genomic level. We have identified and characterized three distinct estrogen binding proteins in chick oviduct tissue, X, Y, and Z (Smith et al., 1979; Taylor & Smith, 1982), and have highly purified the higher affinity nuclear X receptor (Taylor & Smith, 1979; Smith & Schwartz, 1979).

To investigate the effect of addition of pure estrogen receptors on gene transcription in isolated nuclei, it was preferable to first validate that our cell-free system was capable of faithful transcription. In particular, it was pertinent to determine whether the extent of transcription in vitro was dependent upon the concentration of nuclear estrogen receptors. Validation of these parameters was achieved by the withdrawal and secondary stimulation in vivo of estrogen-treated chicks. These studies documented a highly significant relationship between nuclear acceptor site occupancy and

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ovalbumin mRNA ($mRNA_{ov}$) synthesis in vitro (Taylor et al., 1980). Most importantly, these experiments defined for us the optimal conditions for estrogen withdrawal and secondary stimulation to be used in subsequent receptor–nuclei reconstitution studies. The depletion of nuclear acceptor capacity after >60 h of diethylstilbestrol (DES) withdrawal constrained the conditions under which secondary estrogen administration was most sensitive and effective. Secondary stimulation of 60-h withdrawn chicks resulted in a 3-fold increase in nuclear receptor concentration and a concomitant doubling of $mRNA_{ov}$ transcription within 1 h after estrogen administration. In contrast, despite apparent mobilization of cytoplasmic receptors, secondary stimulation of 72-h withdrawn chicks effected no nuclear receptor accumulation or $mRNA_{ov}$ synthesis induction 1 h after DES injection. Thus, nuclei of choice for reconstitution studies were apparently those isolated after withdrawal from hormone for ≤60 h.

It is obvious that oviduct nuclei isolated from chicks shortly following DES injections in vivo do not necessarily equate with nuclei reconstituted with pure estrogen–receptor complexes in vitro. However, the high correlation between nuclear receptor concentration and $mRNA_{ov}$ synthesis suggested that this in vitro transcription system was an appropriate one for attempting such reconstitution experiments. The high correlation observed was in the presence of both estrogen receptor forms X and Y (Smith & Taylor, 1981). Since both may affect nuclear events in an independent or interdependent manner, it is important that the effects of each be studied in suitably reconstituted systems. We chose to purify the higher affinity receptor X ($K_d$ = 0.1 nM). This report describes the direct effect of the highly purified estrogen–receptor complex X on both estrogen target and nontarget cell nuclei. The receptor was added to isolated nuclei, and its direct effects on RNA polymerase II activity and ovalbumin gene transcription were evaluated in vitro.

## Experimental Procedures

*Animals.* White Leghorn pullets were hormonally stimulated and withdrawn as described (Taylor et al., 1980).

*Preparation of Purified Chick Oviduct Nuclei.* A modification of the procedure of Knowler et al. (1973) was used to isolate highly purified oviduct nuclei as described previously (Taylor et al., 1980). The concentrations of nuclei were adjusted to (2–8) × $10^7$ nuclei/mL (50–200 μg of DNA/mL) by using the UV spectroscopic technique described by Lawson et al. (1980). Nuclear concentrations in this range had been previously shown to allow the most efficient transcription in vitro. Exact DNA concentration determinations were made by a modification of the diphenylamine test (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. In vitro transcription reactions were performed as described previously (Taylor et al., 1980).

*Estrogen Receptor Purification.* This procedure was performed as detailed in a previous report (Taylor & Smith, 1979). Briefly, nuclear estrogen receptors were extracted from hen oviduct nuclei at elevated ionic strength (0.4 M KCl). Ammonium sulfate fractionation (30%) and treatment at 42 °C with dextran–charcoal were followed by affinity chromatography on 17β-estradiol–17-hemisuccinyl–diaminodipropylamino–Sepharose 4B. Estrogen receptors were eluted with a solution of 4 μM DES in 10 mM Tris-HCl (pH 7.4) and 10% (v/v) glycerol buffer and stored at 0 °C. Receptor yields from each purification were quantitated by eluting small samples of affinity gel with 4 μM [$^3$H]estradiol (50 Ci/mmol), and the eluate was chromatographed on a Sephadex G-75 column to separate [$^3$H]estradiol–receptor complexes from free label.

*Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis.* This technique was used to evaluate the purity and molecular weight characteristics of purified estrogen receptor (X) proteins. The methods used were as described before (Smith & Schwartz, 1979; Taylor & Smith, 1979).

*Detection of Protease Activity with Azoalbumin.* The release of amino acid linked azo groups from azoalbumin (Sigma, St. Louis, MO) was used to measure proteolytic enzyme activity (Tomarelli et al., 1949). Azoalbumin was incubated with pure estrogen–receptor complexes (0.5–1.0 nM) or trypsin (0.5–500 ng/mL) at 22 °C for 60 min as described previously (Smith & Schwartz, 1979). Released azo groups were measured by the spectroscopic absorbance at 440 nm.

*Nuclear Reconstitution with Pure Estrogen–Receptor Complexes.* After the elution and quantitation of pure estrogen receptors were accomplished, the amount of receptor necessary to reach total concentrations of 0.25–1.0 nM in the final in vitro transcription assay was calculated. Concentrations of nuclei and transcription buffer were adjusted accordingly so that the final concentrations were as described above. Receptors were preincubated with nuclei for 10 min at 0 °C before the addition of nucleoside triphosphates and incubation at 37 °C for 30 or 60 min. For each receptor reconstitution experiment, an identical sample of control nuclei was prepared in which an equal volume of 4 μM DES elution buffer (instead of pure receptor) was added. Several pilot experiments in which nuclei isolated after 24–60 h of estrogen withdrawal were resuspended in transcription buffer with or without 4 μM DES indicated that DES alone had no effect on [$^3$H]RNA synthesis in vitro. Nonetheless, transcription buffer containing 4 μM DES was used as a control in all experiments where receptor–DES complexes were used. Nuclear transcription in receptor-treated samples was compared to matched controls and analyzed by the paired Student's $t$ test.

*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 by using a filtration apparatus (Millipore). Products of in vitro transcription were quantitated by liquid scintillation counting as described (Taylor et al., 1980). The reproducibility of this assay was such that the coefficient of variation using replicate samples never exceeded 18%. Typically [$^3$H]RNA synthesized 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).

*Quantitation of $^3$H-Labeled Ovalbumin mRNA Synthesized in Vitro.* The in vitro expression of this specific gene was quantitated by hybridization to nitrocellulose filters containing cloned ovalbumin cDNA (pOV230). Samples of oviduct nuclei were resuspended as described above and contained either the affinity column elution buffer or highly purified receptor. After incubation at 37 °C for 30 min, RNA was isolated, and ovalbumin mRNA was quantitated as described

Table I: Physicochemical Properties of Crude and Purified Hen Oviduct Nuclear Estrogen Receptor

| property | crude | purified |
|---|---|---|
| sedimentation coefficient[a] (S) | 4.2 | 4.8 |
| Stokes radii[b] (nm) | 4.0, 5.6 | |
| mol wt | 73 000, 100 000[c] | 74 000, 80 000[d] |
| hormone binding specificity | $E_2$ = DES >>> $P_4$ = T[e] | $E_2$ >>> T ⩾ $P_4$ |

[a] Based on sucrose density gradient centrifugation. [b] Based on agarose 1.5 m gel filtration. [c] Computed from sedimentation coefficient and Stokes radii (Siegel & Monty, 1966). [d] Determined by $NaDodSO_4$–polyacrylamide gel electrophoresis (Weber & Osborn, 1969; Laemmli, 1970). [e] $E_2$, 17β-estradiol; DES, diethylstilbestrol; $P_4$, progesterone; T, 5α-dihydrotestosterone.

previously (Taylor et al., 1980).

## Results

*Purification of Nuclear Estrogen–Receptor Complexes.* The methodology of this approach relies heavily on the powerful technique of affinity chromatography. Using two different linkage strategies, we have successfully and reproducibly purified the higher affinity component (X) of the two hen oviduct estrogen receptors to apparent homogeneity (Smith & Schwartz, 1979; Taylor & Smith, 1979). By use of both types of affinity gels, a high affinity estrogen binding protein was eluted which has molecular weights, sucrose gradient sedimentation coefficient, and hormone binding specificity characteristic of the crude nuclear X component of the estrogen receptor in this species. These properties are summarized in Table I. Yields in the range of 20–30% with purification >10 000-fold were consistently observed. The synthesis of relatively low-capacity affinity gels (150–300 pmol of 17β-estradiol hemisuccinate/mL of gel) obviated the necessity for supersaturated eluent solutions which often give spurious gel chromatography results.

Under denaturing conditions in Laemmli (1970) and Weber & Osborn (1969) sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoretic systems, the highly purified receptor preparation was resolved into two bands which migrated as proteins of 74 000 and 80 000 daltons (Figure 1). Although we have no absolute proof that both or either of the bands detected by $NaDodSO_4$–polyacrylamide gel electrophoresis represents the purified receptor X, evidence supporting this hypothesis is the following: (i) similarity in molecular weight as determined by a combination of gel filtration and sucrose gradient centrifugation (Table I); (ii) increases in receptor yield were accompanied by increased density of staining in both bands; (iii) when high-capacity affinity chromatography gels were utilized, the absence of any receptor in the column eluate was accompanied by a lack of these two protein bands on $NaDodSO_4$–polyacrylamide gel electrophoresis.

It is also possible that only one, or neither, of the bands is receptor protein. They could be contaminants that coelute with the receptor. Nonetheless, the properties of the affinity chromatography eluate show that the estrogen receptor is indeed present and highly purified. The studies described herein were performed with pure nuclear estrogen–receptor complexes isolated by this technique (Taylor & Smith, 1979) which typically yielded highly purified receptors in concentrations of 1–2 nM, with gel electrophoretic profiles as shown in Figure 1.

*Effects of Pure Estrogen–Receptor Complexes on Nuclear Transcription in Vitro.* Pure estrogen–receptor complexes were added to suspensions of oviduct nuclei under in vitro transcription conditions at 37 °C. A 2–3-fold stimulation of [$^3$H]RNA synthesis over that of control samples was noted after 30 min, while lower levels of stimulation persisted up to a 60-min incubation. Pilot experiments demonstrated that when the transcription reactions were performed at 42 °C stimulation of [$^3$H]RNA synthesis in vitro did not continue beyond 15 min, presumably due to the heat lability of the pure estrogen–receptor complexes. Incubation of the receptor-reconstituted nuclei at 30 °C did not result in any greater enhancement of [$^3$H]RNA synthesis than that observed at 37 °C. Therefore, the subsequent assays were routinely performed at 37 °C for 30 min. The effect of the addition of pure

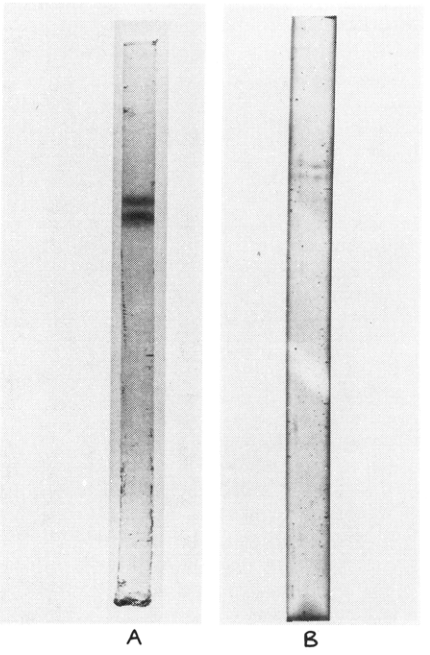


FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of purified nuclear estrogen receptor X. Highly purified nuclear X receptors prepared by the affinity chromatography procedures of Smith & Schwartz (1979) (gel A) and Taylor & Smith (1979) (gel B) were subjected to electrophoresis according to Laemmli (1970) and Weber & Osborn (1969), respectively. Under both sets of $NaDodSO_4$–polyacrylamide gel electrophoresis conditions, and using two different affinity gel designs, protein bands of molecular weights 74 000 and 80 000 were observed.

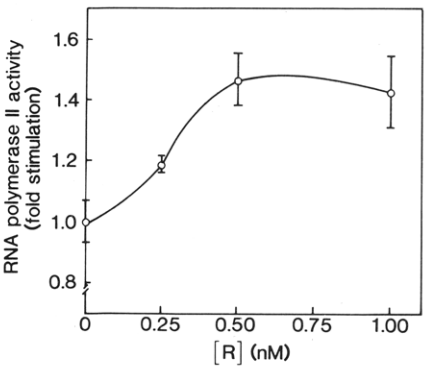


FIGURE 2: Concentration dependence of transcriptional stimulation by purified estrogen–receptor complexes. Oviduct nuclei isolated from chicks withdrawn from estrogen for 24 h were incubated under usual in vitro transcription conditions in the presence of increasing concentrations of purified estrogen–receptor complexes. A statistically significant ($p < 0.02$) stimulation of RNA polymerase II activity was observed in the receptor-treated nuclei. [$^3$H]RNA synthesis which was sensitive to 1 μg/mL α-amanitin was assumed to reflect RNA polymerase II mediated transcription. Error bars describe the range of duplicate determinations.

Table II: Effects of Pure Receptor Solutions and Various Control Solutions on [$^3$H]RNA Synthesis in Isolated Nuclei[a]

| additive | RNA polymerase II activity (fmol of [$^3$H]UMP incorporated/ $\mu$g of DNA) | $x$-fold stimulation |
|---|---|---|
| control buffer (no DES) | 140 | 1.0 |
| 4 $\mu$M DES elution buffer | 144 | 1.0 |
| low-capacity affinity gel eluted with control buffer (sham eluted) | 136 | 1.0 |
| low-capacity affinity gel eluted with 4 $\mu$M DES elution buffer (1 nM estrogen receptor recovered) | 408 | 2.9 |
| high-capacity affinity gel eluted with 4 $\mu$M DES elution buffer (no receptor detectable) | 144 | 1.0 |

[a] Oviduct nuclei were isolated from 36-h withdrawn chicks and incubated with the indicated additives under in vitro transcription conditions. These values represent the means of duplicate determinations in a single experiment. Coefficients of variation among replicates in these and subsequent determinations were $\leq$18% (see Experimental Procedures).

receptors on RNA polymerase II catalyzed transcription was determined by the use of the specific inhibitor $\alpha$-amanitin in replicate experiments at a concentration of 1 $\mu$g/mL.

The concentration dependence of added estrogen receptors on class II gene expression was examined in the experiment depicted in Figure 2. The results showed a saturable response which was maximal at a final receptor concentration of 0.5 nM.

The receptor-mediated specificity of this response was confirmed by control experiments in which the addition of estrogen alone, or "sham" (estrogen absent) eluted affinity gel samples, did not stimulate [$^3$H]RNA synthesis. Furthermore, if high-capacity affinity gels were used and eluted in the usual fashion with 4 $\mu$M DES solutions, hormone–receptor complexes were refractory to elution and undetectable by both gel exclusion chromatography and $NaDodSO_4$–polyacrylamide gel electrophoresis. The addition of these eluates to the in vitro transcription system effected no change in the transcriptional capacity of isolated nuclei (Table II). Although Table II shows the results obtained by using nuclei obtained after 36 h of estrogen withdrawal, similar experiments using nuclei after 48, 60, and 72 h of withdrawal showed that stimulation of RNA polymerase II activity only occurred in the presence of receptor.

The purified estrogen receptor preparation was next examined for activities which may have spuriously caused increased RNA polymerase activity. Incubation in the presence of a chicken DNA template, 600 $\mu$M [$^3$H]UTP, and the appropriate cofactors (Tsai et al., 1980) revealed that the preparation contained no intrinsic or contaminating RNA polymerase activity (Figure 3). Pure estrogen–receptor complexes incubated with azoalbumin substrate indicated that nonspecific proteolytic activity was not responsible for the augmented RNA synthetic capacity of receptor-treated nuclei (Smith & Schwartz, 1979).

One of the potential artifacts accounting for stimulation of RNA synthesis in vitro is digestion of the chromatin template of these nuclei by contaminating DNase activity. For examination of this possibility, nick-translated [$^3$H]DNA (Roop et al., 1978) was incubated with the purified receptor preparation for 30 min at 37 °C. A parallel reaction containing [$^3$H]DNA and elution buffer (4 $\mu$M DES) alone was also examined. Both reaction mixtures were treated with ice-cold 10% $Cl_3CCOOH$ to precipitate macromolecular [$^3$H]DNA. No difference in acid-soluble radioactivity was noted in the two samples. This result argues against major DNase contamination in the receptor preparations. In addition, the chromatin transcription experiments of Smith & Schwartz (1979) indicated that the purified receptor did not contain single-stranded DNA nicking activity. The possibility of contamination of the estrogen–receptor complex with RNA or DNA was excluded by orcinol (Schneider, 1957), diphenylamine (Giles & Myers, 1965), and ethidium bromide binding (Sharp et al., 1973) reactions. Since receptor-mediated stimulation of nuclear [$^3$H]RNA synthesis is blocked by actinomycin D (40 $\mu$g/mL) and $\alpha$-amanitin (1 $\mu$g/mL), synthesis from a DNA template via RNA polymerase II enzymes is strongly suggested.

Having demonstrated a saturable, specific transcriptional response to added nuclear estrogen–receptor complexes, we proceeded to examine this effect in nuclei isolated from different tissues of the chicken. Spleen and erythrocyte nuclei were isolated, and nuclear estrogen–receptor reconstitution in vitro was performed in the usual manner. The results of these experiments (Table III) indicated that the effect of estro-

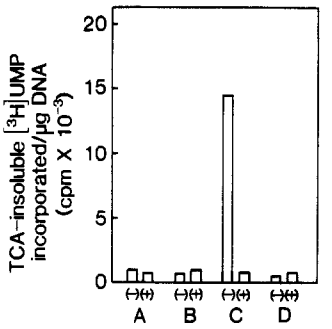


FIGURE 3: Investigation of RNA polymerase activity in purified estrogen receptor preparations. Purified estrogen–receptor complexes eluted from affinity gels as described in the text were assayed for intrinsic or contaminating RNA polymerase activity. Two individual receptor preparations (A and B) were incubated with 80 $\mu$g/mL chick DNA, 600 $\mu$M [$^3$H]UTP, and other reagents as described (Tsai et al., 1980). The final concentrations of purified estrogen receptors were 0.3 and 0.7 nM, respectively. Hen oviduct RNA polymerase II [0.5 mg/mL (C)] and bovine serum albumin [0.5 mg/mL (D)] were included as controls. Each sample was assayed in the absence (−) and presence (+) of 1 $\mu$g/mL $\alpha$-amanitin.

Table III: Target Tissue Specificity of Estrogen Receptor Mediated Stimulation of [$^3$H]RNA Synthesis in Vitro[a]

| tissue source | | RNA polymerase II activity (fmol of [$^3$H]UMP incorporated/ $\mu$g of DNA) | $x$-fold stimulation |
|---|---|---|---|
| oviduct nuclei | −R | 160 ± 52 ($N$ = 3) | |
| | +R | 392 ± 40 | 2.5 ($p < 0.002$) |
| spleen nuclei | −R | 48 ± 15 ($N$ = 3) | |
| | +R | 55 ± 12 | 1.1 (ns) |
| erythrocyte nuclei | −R | 10 ± 3 ($N$ = 3) | |
| | +R | 10 ± 7 | 1.0 |

[a] Oviduct, spleen, and erythrocyte nuclei were isolated from chicks sacrificed after 24 h of estrogen withdrawal. Nuclei were incubated with pure estrogen receptors at a concentration of 0.6 nM (+R) or with 4 $\mu$M DES elution buffer (−R) at 37 °C for 30 min. [$^3$H]RNA synthesis was determined in the absence and presence of 1 $\mu$g/mL $\alpha$-amanitin by 10% $Cl_3COOH$ precipitation as described. Values for RNA polymerase II activity represent the means of duplicate or triplicate determinations in isolated nuclei ± standard deviations in $N$ experiments. $x$-fold stimulation describes the overall effect of the addition of purified estrogen receptor on this activity. ns = not significant.

Table IV: Effect of Time of Estrogen Withdrawal on Pure Estrogen Receptor Induced Stimulation of RNA Polymerase II Activity[a]

| estrogen withdrawal (h) | | RNA polymerase II activity (fmol of [$^3$H]UMP incorporated/ μg of DNA) | x-fold stimulation |
|---|---|---|---|
| 0 | −R | 464 ± 304 ($N$ = 5) | |
| | +R | 656 ± 504 | 1.4 (ns) |
| 24 | −R | 192 ± 32 ($N$ = 4) | |
| | +R | 376 ± 24 | 2.0 ($p < 0.001$) |
| 36 | −R | 136 ± 40 ($N$ = 3) | |
| | +R | 512 ± 144 | 3.8 ($p < 0.01$) |
| 48 | −R | 144 ($N$ = 1) | |
| | +R | 248 | 1.7 |
| 60 | −R | 156 ± 40 ($N$ = 3) | |
| | +R | 284 ± 30 | 1.8 ($p < 0.01$) |
| 72 | −R | 112 ($N$ = 1) | |
| | +R | 176 | 1.6 |

[a] Oviduct nuclei isolated at the indicated times after estrogen withdrawal were incubated with 0.5–0.8 nM estrogen–receptor complexes (+R) or 4 μM DES elution buffer (−R) under in vitro transcription conditions as described. Values for RNA polymerase II activity represent the means of duplicate or triplicate experiments in isolated nuclei ± standard deviations in $N$ independent experiments. x-fold stimulation describes the overall effect of the addition of purified estrogen receptor on this activity. ns = not significant.

Table V: Effects of Estrogen Receptor Reconstitution on [$^3$H]mRNA$_{ov}$ Transcription in Vitro[a]

| hormonal status | | % mRNA$_{ov}$ synthesis in vitro | x-fold stimulation |
|---|---|---|---|
| stimulated | −R | 0.30 | |
| | +R | 0.54 | 1.8 |
| stimulated | −R | 0.24 | |
| | +R | 0.39 | 1.6 |
| 24-h withdrawn | −R | 0.11 | |
| | +R | 0.20 | 1.8 |
| 36-h withdrawn | −R | 0.08 | |
| | +R | 0.07 | 0.9 |
| 60-h withdrawn | −R | <0.001 | |
| | +R | <0.001 | 1.0 |
| 60-h withdrawn | −R | <0.001 | |
| | +R | <0.001 | 1.0 |

[a] Oviduct nuclei isolated under the indicated hormonal conditions were incubated with 0.5–0.8 nM estrogen–receptor complexes (+R) or 4 μM DES elution buffer (−R) for 30 min at 37 °C. Total [$^3$H]RNA synthesized in vitro was extracted and hybridized to nitrocellulose filters containing ovalbumin cDNA. The percentage of the total [$^3$H]RNA which remained hybridized to the filters was calculated as described previously (Taylor et al., 1980) to obtain the percent mRNA$_{ov}$ synthesis in vitro.

gen–receptor complexes on [$^3$H]RNA transcription was specific for an estrogen target tissue. These data provide additional evidence that the stimulation of gene expression in oviduct nuclei cannot be ascribed to nonspecific template nicking, protease activity, or polymerase contamination.

The stimulatory effect of added estrogen–receptor complexes on nuclear class II gene transcription was next investigated in relation to the length of hormone withdrawal. The results of these in vitro reconstitution studies are shown in Table IV. Samples of oviduct nuclei isolated from 0–72-h withdrawn chicks all demonstrated a stimulation in RNA polymerase II activity when pure nuclear estrogen–receptor complexes were added in vitro at a final concentration ranging from 0.5 to 0.8 nM (~3000–5000 receptors/nucleus). Statistical analysis by the paired Student's $t$ test revealed a significant stimulation in receptor-reconstituted nuclei as compared to the untreated, matched controls, regardless of the length of hormonal withdrawal. At discrete times of estrogen withdrawal (Table IV), the stimulation of nuclear RNA polymerase II activity was significant at levels of $p < 0.001$ (24 h) and $p < 0.01$ (36 and 60 h).

*Effects of Pure Estrogen–Receptor Complexes on Ovalbumin mRNA Transcription in Vitro.* In an attempt to define the role of the estrogen–receptor complex on the induction of a specific chicken gene, we have also used this transcription system to examine the rate of ovalbumin gene expression in vitro. Nuclei isolated at different stages of hormone withdrawal were examined by using the in vitro transcription assay after the addition of 0.5–0.8 nM pure estrogen–receptor complexes. Matched control samples were assayed in parallel. As described previously (Taylor et al., 1980), [$^3$H]RNA synthesized in nuclear suspensions was extracted, purified, and hybridized to nitrocellulose filters containing cloned ovalbumin cDNA (pOV230). The [$^3$H]RNA which specifically hybridized to these filters represented ovalbumin mRNA sequences transcribed in vitro and was quantitated as a percentage of the total [$^3$H]RNA synthesized in vitro. The results of these experiments are shown in Table V. The data clearly indicate that the stimulation of ovalbumin gene expression by added estrogen–receptor complexes is dependent upon the time of estrogen withdrawal preceding receptor reconstitution. Apparently, the loss of important nuclear factors after 24 h of estrogen withdrawal renders the ovalbumin gene in these nuclei insensitive to transcriptional restimulation by receptor addition.

## Discussion

This study is the first in which the effect of a highly purified estrogen receptor has been investigated in a validated receptor concentration dependent, in vitro transcriptional system utilizing endogenous RNA polymerase II. Early attempts to directly investigate the effects of estrogen–receptor complexes on RNA synthesis in reconstituted cell-free systems demonstrated stimulation of transcriptional capacity in target tissue nuclei by the addition of crude receptor preparations. Raynaud-Jammet & Baulieu (1969) showed a 3-fold stimulation of [$^3$H]RNA synthesis in isolated calf uterus nuclei following the addition of calf uterus cytosol and 1 nM estradiol. Nuclear transcriptional stimulation required both the hormone and the soluble receptor fraction, and the effect was blocked if the cytosol was heated at 60 °C for 1 h. In a similar study, Arnaud et al. (1971) prepared estrogen receptor fractions from calf endometrium and incubated these with isolated uterine nuclei. These investigators observed that estradiol-charged cytoplasmic receptors (7.7 S) effected no stimulation of nuclear [$^3$H]RNA synthesis. However, the addition of heat-transformed cytosol receptors (5.0 S) and extracted nuclear receptors both stimulated a doubling of nuclear transcription in vitro. These experiments suggested that the stimulation was mediated by increased transcriptional activity of engaged RNA polymerase molecules. In fact, a decrease in the free polymerase pool was observed.

The results of Arnaud et al. (1971) were confirmed by Mohla et al. (1972), who also showed that nuclear or transformed receptors were required to increase RNA synthesis in calf and rat uterus nuclei. The latter investigators also claimed a target tissue specificity of this effect. Incubation of trans-

formed cytosol and estradiol with rat uterus nuclei caused a 2.7-fold increase in [$^3$H]RNA synthesis, whereas kidney and liver nuclei manifested no stimulation of transcriptional capacity. In view of more recent data (Chamness et al., 1975; Murono et al., 1979), it is probably incorrect to consider liver and kidney as nontarget tissues for estrogen, as both appear to possess estrogen receptors. Nevertheless, the action of estrogen–receptor complexes on the transcriptional apparatus of these tissues may differ remarkably from the uterus which manifests dramatic hypertrophy and hyperplasia in response to estrogen.

In a previous study using highly purified receptor fractions, the template activity of chromatin was studied by using *Escherichia coli* RNA polymerase. Chick oviduct estrogen receptors were shown to significantly increase the number of rifampicin-resistant RNA initiation sites on chick oviduct chromatin (Smith & Schwartz, 1979). While the fidelity and authenticity of chromatin transcription by prokaryotic RNA polymerase have been questioned, there is evidence that the initiation sites recognized by *E. coli* RNA polymerase may be the same as those utilized by homologous hen oviduct RNA polymerase II (Tsai et al., 1976). Irrespective of the interpretation of these reproducible results, it must be concluded that the addition of highly purified steroid receptor–hormone complexes causes changes in chromatin structure and template activity in a tissue-specific and concentration-dependent manner.

For a more clear definition of the role of one of the nuclear estrogen receptors in gene transcription, pure receptors have now been used to reconstitute isolated oviduct nuclei in an in vitro transcription assay which does not require the addition of exogenous RNA polymerase. The addition of pure estrogen–receptor complexes to isolated nuclei in vitro provides an experimental system which is significantly less complex than the administration of estrogens to intact chicks in vivo. Any effect of these pure estrogen receptors on the transcriptional capacity of isolated nuclei reflects a direct action on the nuclear template and/or the RNA polymerase apparatus itself. To ensure the validity of these experiments, we have conscientiously addressed the potential artifacts of spurious stimulators of transcription (Buller et al., 1976) and excluded contamination of the receptor preparation by RNA polymerase, protease, DNase, DNA, or RNA. We have demonstrated a physiologically appropriate target tissue specificity and dose–response effect on RNA polymerase II activity by using purified receptors. The maximal response to the added receptor was observed at a concentration of approximately 0.5 nM, similar to that required for the in vitro saturability of hen nuclear acceptor sites by partially purified estrogen receptors (Kon et al., 1980). These concentrations are also similar to those required for a half-maximal response in rifampicin-resistant initiation site assays, when chick chromatin was titrated with pure estrogen–receptor complexes (Smith & Schwartz, 1979).

The addition of saturating concentrations of pure estrogen receptors to isolated oviduct nuclei caused 1.4–3.8-fold increases in endogenous RNA polymerase II activity. Similar effects on class II gene expression have been observed following estrogen administration in vivo (Spelsberg & Cox, 1976; Hardin et al., 1976). Increases in RNA polymerase II activity after secondary stimulation in vivo have been previously shown by us to be associated with a doubling in engaged RNA polymerase II concentrations (R. N. Taylor and R. G. Smith, unpublished experiments). This finding is compatible with the hypothesis that estrogen stimulation, and the nuclear accumulation of estrogen receptors, increases the template binding of RNA polymerase II molecules.

We have also demonstrated that when nuclei are withdrawn from estrogen for ≤24 h the addition of the highly purified estrogen receptor results in an apparent 1.6–1.8-fold stimulation of ovalbumin-specific gene expression. While such an increase in $mRNA_{ov}$ synthesis in nuclei is not dramatic, these nuclei are already transcribing $mRNA_{ov}$ at near-maximum rates (Hynes et al., 1979; Swaneck et al., 1979; Taylor et al., 1980). The stimulation observed in the chronically stimulated nuclei suggests that during the isolation of these nuclei some endogenous nuclear receptors may dissociate from their acceptors, making these vacant sites available for exogenous estrogen–receptor complex binding in the reconstitution studies. Alternatively, excess functional nuclear acceptor sites may exist under this hormonal condition. In the experiment of Swaneck et al. (1979) when nuclei were isolated from short-term withdrawn chicks, 1 h after secondary DES administration in vivo, a similar significant increase in ovalbumin gene expression above that of control nuclei was observed. Thus, at least in the 24-h estrogen-withdrawn state, the apparent estrogen-induced increase in ovalbumin mRNA synthesis observed in vivo can be mimicked in vitro by the addition of highly purified receptors to isolated nuclei. However, when nuclei were obtained from chicks withdrawn from estrogen for 36 and 60 h, no increase in ovalbumin mRNA synthesis was induced by addition of receptors. This implies that the loss of an additional factor(s) during hormonal withdrawal may render the ovalbumin gene in these nuclei insensitive to selective initiation by the addition of the receptor alone. The effect of this receptor–hormone complex may therefore be on elongation rates rather than ovalbumin specific initiation, so that already initiated chains are completed more rapidly. Thus, RNA polymerase II activity was significantly stimulated by exogenous receptor complexes, but no increase in percent $mRNA_{ov}$ synthesis was apparent.

The experiments reported here have directly addressed the mechanisms by which the transcription of estrogen-regulated genes is controlled. We have shown that reconstitution with a highly purified estrogen receptor increases RNA polymerase II activity in isolated nuclei in a concentration-dependent and tissue-specific manner. We postulate that the receptor may increase RNA synthesis by increasing the affinity of this polymerase for its DNA template and/or by enhancing its rate of elongation. The former is suggested since the addition of pure estrogen–receptor complexes to isolated oviduct nuclei seems to increase the number of RNA polymerase II molecules bound to the chromatin template (unpublished results). Finally, the stimulatory effect of this pure receptor on class II gene transcription in vitro does not appear to be by specific initiation of the ovalbumin gene, since the percent $mRNA_{ov}$ synthesis is enhanced only after short withdrawal intervals (≤24 h).

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