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PURE ESTROGEN RECEPTOR COMPLEXES STIMULATE RNA TRANSCRIPTION IN VITRO

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SUMMARY

The high-affinity species (K_d = 0.2 nM) of the two estrogen receptors present in hen oviduct nuclei has been isolated, characterized, and purified to apparent homogeneity by affinity chromatography. An in vitro transcription system using isolated chick oviduct nuclei was developed and optimized to examine the effects of estrogen-receptor complexes on gene transcription. The capacity of nuclei to synthesize [^{3}H]RNA in vitro was enhanced by the addition of pure nuclear estrogen-receptor complexes.

INTRODUCTION

Current molecular models of estrogen action dictate that these steroid hormones exert their regulatory effects on target tissues by the formation of specific hormone-receptor complexes which translocate into the nucleus, resulting in new RNA synthesis (1, 2, 3). Early attempts to directly investigate the effects of steroid-receptor complexes on RNA synthesis in reconstituted cell-free systems demonstrated stimulation of transcriptional capacity in purified target tissue nuclei by the addition of relatively crude preparations of cytoplasmic receptors (4, 5). In two studies which documented highly purified receptor fractions, the template activity of chromatin was studied using E. coli RNA polymerase. Both progesterone (6) and estrogen (7) receptors were shown to significantly increase the number of rifampicin-resistant RNA initiation sites in chick oviduct chromatin. 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 (8).

To more clearly define the role of nuclear estrogen receptors in gene transcription we have isolated the classical estrogen receptor from hen nuclei and purified this protein to homogeneity. An in vitro transcription assay using purified chick oviduct nuclei, which does not require the addition of exogenous RNA polymerase, has been developed. This assay was used to study the enhancement of RNA synthesis by pure estrogen-receptor complexes.

Abbreviations:

TESH, 10 mM Tris, 1.5 mM EDTA, 12 mM monothioglycerol, pH 7.4; E_2, 17β-estradiol; DES, diethylstilbestrol; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl.

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MATERIALS AND METHODS

Nuclear Estrogen Receptor Purification:

White Leghorn hen oviducts were homogenized in 4 volumes of TESH buffer at 4°. The crude nuclear pellet was washed once with TESH and resuspended in a similar volume of TESH containing 0.4 M KCl. The suspension was shaken gently overnight at 4°. Following this extraction procedure, solubilized estrogen receptors were precipitated and partially purified in 30% $(NH_4)_2SO_4$. The pellets were washed and resuspended in a small volume of TESH. Endogenous hormone was stripped from the receptor by incubating the receptor preparation with a solution of dextran-coated charcoal in TESH at 42° for 15 minutes. Charcoal was removed by low speed centrifugation and the supernatant was centrifuged at 100,000 x g to remove insoluble denatured proteins.

This high-speed supernatant was then incubated with the E_2-17-hemisuccinyl-diaminodipropylamine-Sepharose 4B affinity gel at 4° for 18 hours at a ratio of 5:1 (v/v). The affinity gel was prepared by reacting 5 g of CNBr-activated Sepharose 4B (Pharmacia) with 0.6 mmol 3,3' diaminodipropylamine, pH 9.0, at 4° for 16 hours. Remaining CNBr-activated groups were inactivated by incubation with 50 mmol ethanolamine at 25° for 2 hours. Three mg of E_2-17-hemisuccinate (Steraloids) was coupled to the diaminodipropylamine side-chain using 10 mg of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide HCl (Sigma). This particular affinity adsorbent more consistently gave good yields of pure receptor than the adsorbent reported by us previously (7).

Affinity beads were separated from the suspension by centrifugation at 800 x g and nonspecifically adsorbed material was removed by washes in TESH and TESH containing 0.4 M KCl. Estrogen receptors were finally eluted by incubating the affinity gel with TESH containing 4 μ M estrogen ($[^3H]E_2$ or DES) at 37° for 15 minutes. The eluting buffer/gel volume ratio was 1:1.

Radiolabeled eluate was chromatographed on a 10 x 1 cm Sephadex G-75 column to separate $[^3H]E_2$-receptor complexes from free label. A parallel affinity gel sample, eluted with an identical concentration of unlabeled DES, was used to obtain pure estrogen-receptor complexes for the in vitro transcription assay.

Hormone Treatment:

Two silicone tubings (5 cm in length containing 25-26 mg each of DES) were implanted subcutaneously into the legs of 2-3 week old female White Leghorn chicks. Two weeks following implantation the implants were removed and the chicks were withdrawn from hormone for 36 hours prior to sacrifice.

In Vitro Transcription Assay:

Chick oviduct nuclei were prepared as described by Roop et al. (9). $[^3H]$RNA was synthesized in isolated nuclei in reaction mixtures of 250 μl that contained 1 mM each of ATP and GTP, 0.6 mM each of $[^3H]$UTP and $[^3H]$CTP (35.4 Ci/mmol and 22.0 Ci/mmol, respectively), 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 3 mM dithiothreitol, 100 mM KCl, 50 mM Tris, pH 7.8, 10% (v/v) glycerol and 1-2 x 10^7 nuclei. Incubations were for 0-60 minutes at 37° and at each time point 10% TCA-insoluble $[^3H]$RNA was collected on Millipore filters.

Hormone Binding Assay:

Saturation analysis of the partially purified nuclear estrogen receptor was performed in the presence of $[^3H]E_2$ of final concentrations ranging from 0.2 to 8.0 nM. Incubations were for 18 hours at 4° and assayed as described previously (10).

Post-Labeled Sucrose Density Gradient Centrifugation:

Linear gradients of 5-20% sucrose (w/v) in TESH or TESH containing 0.4 M KCl were prepared with a Beckman gradient former. 300 μl of partially-purified nuclear estrogen receptor were layered on each gradient. Centrifugation and collection were performed as described by Clark et al. (11). Labeling was carried out using a final concentration of 1 nM $[^3H]E_2$. Nonspecific binding was determined in the presence of a 200-fold molar excess of DES.

Protein Determinations:

Protein was quantitated by the Coomassie Blue method of Bradford (12).

SDS-Polyacrylamide Disc Gel Electrophoresis:

Disc gels containing a final acrylamide concentration of 7% and a final SDS concentration of 0.1% were electrophoresed according to the method of Weber and Osborn (13).

RESULTS AND DISCUSSION

The characteristics of the two estrogen receptors present in 0.4 M KCl extracts of hen oviduct nuclei have been described previously (10). Because of the relative heat lability of the low-affinity binding protein, $(NH_4)_2SO_4$ precipitation of the crude nuclear extract followed by incubation at 42° with dextran-coated charcoal resulted in a single class of saturable binding sites having a K_d characteristic of the high-affinity receptor (Fig. 1A). Due to the rapid rate of dissociation ($k_d = 5 \times 10^{-5}$ sec^{-1}) of this receptor (7), its sedimentation characteristics were determined using post-labeled sucrose density gradients (Fig. 1B). A single peak of specifically bound $[^3H]E_2$ at 4.2S was observed both in the presence and absence of 0.4 M KCl. This is consistent with previous reports of the sedimentation behavior of hen oviduct nuclear estrogen receptors (7, 14).

While cytosol preparations contain esterases and proteases which can destabilize the linkage groups used in a variety of estrogen affinity adsorbents (15, 16), the partially-purified nuclear receptor contained no such degradative factors. Hence, this preparation

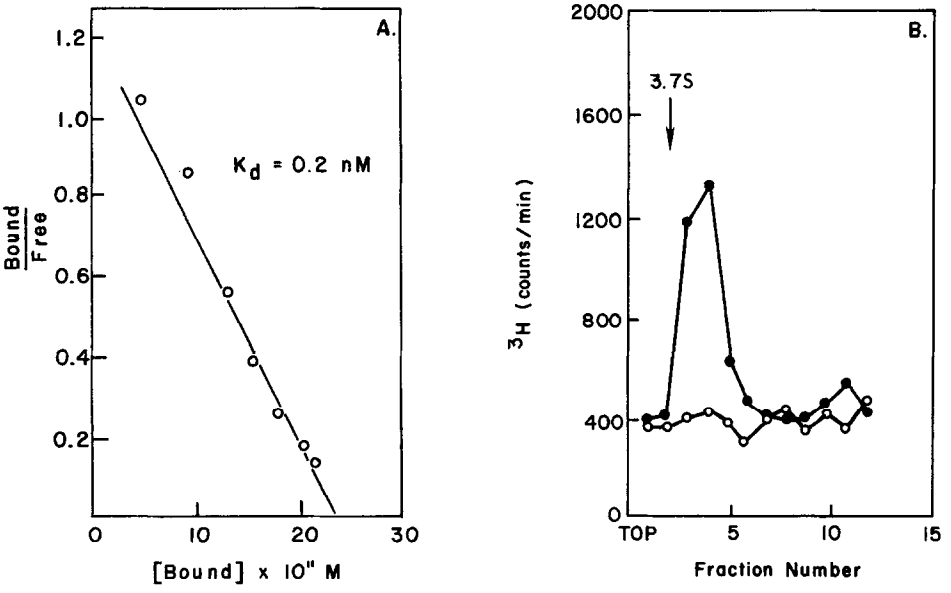

Fig. 1A. Scatchard analysis of specific $[^3H]E_2$ binding of hen oviduct nuclear estrogen receptor.

Fig. 1B. Post-labeled sucrose density gradients of hen oviduct nuclear estrogen receptor: total $[^3H]E_2$ binding (●) and nonspecific binding (○).

TABLE 1

Purification of Hen Oviduct Nuclear Estrogen Receptor

purification step		total volume	total protein	total cpm	cpm/mg protein	yield	purification
(1)	0.4 M KCl - TESH extract of hen oviduct nuclei	250 ml	2.2 g	1.2×10^7	5.4×10^3	100%	1 x
(2)	$(NH_4)_2SO_4$ precipitation and charcoal adsorption	20 ml	144 mg	4.7×10^6	3.3×10^4	39%	6 x
(3)	Affinity and Sephadex G-75 chromatography	4 ml	< 20 µg	1.5×10^6	$> 7.4 \times 10^7$	30%	> 13,800 x

could be applied directly to the E_2-17-hemisuccinyl-diaminodipropylamine-Sepharose 4B affinity gel as described in Materials and Methods.

After the receptor-adsorbed gel was washed free of nonspecifically bound material, estrogen receptors were eluted with a solution containing 4 µM [^{3}H]E_2. Sephadex G-75 chromatography of the eluate was used to quantitate macromolecular bound [^{3}H]E_2. Elution of the receptor in the presence of unlabeled DES quantitatively reduced the peak of radioactivity, while the addition of an excess of unlabeled progesterone had no effect.

Stepwise purification of the estrogen receptor is shown in Table 1. In all cases binding to the affinity gel was carried out under conditions of receptor excess. The calculated yield for this final purification step is corrected for the proportion of the receptor (40-50%) not retained by the affinity gel. The protein content of the purified receptor was difficult to quantitate accurately as its absorption value fell below that of the lowest sample on the standard curve. SDS-polyacrylamide gel electrophoresis was employed to qualitatively determine the purity of the eluted receptor as well as to obtain an estimate of its size. Two bands of molecular weights $\sim$ 75,000 and $\sim$ 80,000 were observed (Fig. 2). Whether this heterogeneity represents a possible contaminant, subunit structure, or metabolic products of the receptor has not yet been determined, however DEAE-cellulose chromatography of the pure receptor reveals only a single hormone-binding species eluting at 0.15 M KCl. Attempts to examine the native receptor by electrophoresis under non-denaturing conditions are currently in progress. The size of the receptor is in agreement with recent estimates of the calf uterine estrogen receptor (16, 17). Incubations with the appropriate substrates revealed that this protein contained no DNase, protease, or RNA polymerase activities.

The incorporation of [^{3}H]ribonucleotides into RNA under the conditions described is linear with respect to the concentration of nuclei, inhibited 80-90% by Actinomycin D

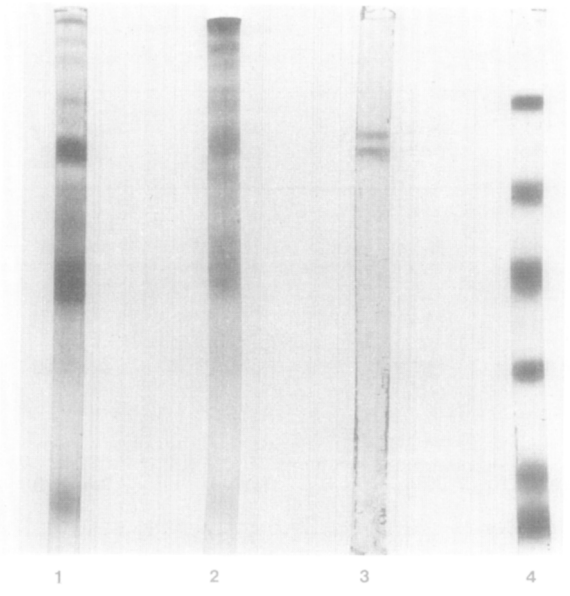

Fig. 2. Parallel SDS-polyacrylamide disc gel electrophoresis. Gels 1, 2 and 3 correspond to those steps in the purification procedure (Table 1). Gel 4 contains 1 μg each of phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme (top to bottom). Based on the migration of these standards, the apparent molecular weights of the two protein bands in the purified receptor (gel 3) were ∿75,000 and ∿80,000.

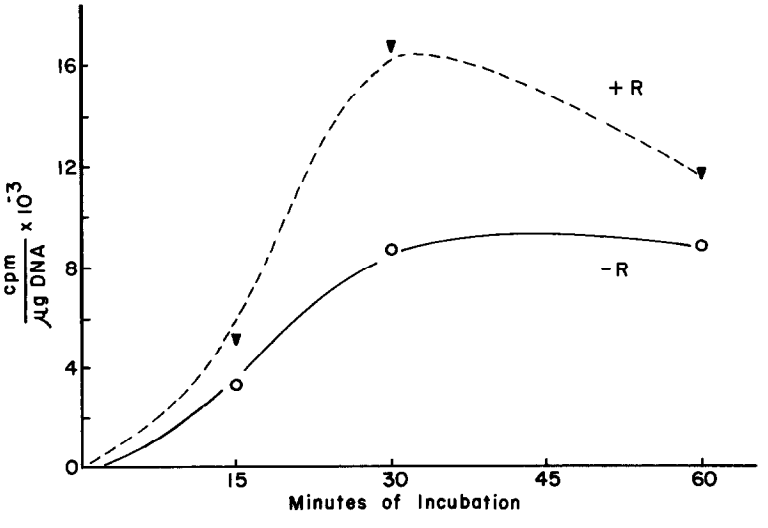

Fig. 3. Incorporation kinetics of [^{3}H] ribonucleotides into 10% TCA-insoluble RNA. Oviduct nuclei were isolated from chicks withdrawn from estrogen for 36 hours and [^{3}H]RNA synthesis was measured in the presence (▼) or absence (o) of added pure estrogen-receptor complexes (R).

(30 μg/ml) and 60-70% by α-amanitin (1 μg/ml). The kinetics of [^{3}H]RNA synthesis by nuclei isolated from withdrawn chick oviducts are illustrated in Fig. 3. Typically the level of [^{3}H] RNA accumulation was greatest after 30 minutes at 37°. Pure DES-receptor complexes eluted from the affinity gel were added to the transcription reaction mixtures at a final concentration of 0.5-0.8 nM. A similar volume of the 4 μM DES-elution buffer was added to control mixtures to rule out direct estrogen-mediated effects. In three independent experiments using freshly prepared nuclei and receptor preparations, nuclear [^{3}H]RNA synthesis in the presence of pure estrogen-receptor complexes was enhanced 2-3-fold over the control values. Stimulation of transcription by added estrogen-receptor complexes was reduced to control levels in the presence of 1 μg/ml α-amanitin, implying that the enhancement was mediated via RNA polymerase II.

The observation that in vitro RNA synthesis was enhanced by the addition of pure estrogen-receptor complexes cannot be ascribed to contamination of the receptor fraction by RNA polymerase. Moreover, the pure receptor contained no proteolytic or DNA nicking activities which could have artifactually produced a stimulation of RNA synthesis by altering the template activity of nuclear chromatin. These findings support the hypothesis that the nuclear estrogen-receptor complex may have a direct role in the regulation of gene transcription in the chick oviduct. Further studies are directed towards an understanding of the interactions between the estrogen receptor and other chromosomal proteins.

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