Isolation and Purification of a Hen Nuclear Oestrogen Receptor and its Effect on Transcription of Chick Chromatin

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An oestrogen receptor was isolated, characterized and purified from the nuclear fraction of the hen oviduct. The receptor sediments at 4.6S on glycerol gradients, has an equilibrium dissociation constant (K_d) of 1.1×10^{-10} M, an association constant (k_a) of 1.4×10^{-6} M⁻¹·s⁻¹, and a dissociation constant (k_d) of 5×10^{-5} s⁻¹. The receptor chromatographed from DEAE-cellulose as a single peak at 0.15 M-KCl and was not retained by phosphocellulose. Polyacrylamide-gel electrophoresis of the receptor in the presence of sodium dodecyl sulphate demonstrated two subunits with apparent mol.wts. of 74000 and 80000. The overall purification achieved was 90000-fold by using a combination of cell fractionation, (NH₄)₂SO₄ fractionation and affinity chromatography. This represents the first separation, isolation and purification of the highest-affinity binding component (K_d 10^{-10} M) of two high-affinity oestrogen-binding proteins present in both chick and hen oviduct cytosol and nuclei. To examine directly the effect of the purified receptor on transcription a reconstituted cell-free system was used, which contained the receptor–oestradiol complex, *Escherichia coli* RNA polymerase, rifampicin and chromatin prepared from hormone-withdrawn chick tissue. The receptor–hormone complex at a concentration of 0.1 nM stimulated transcription of oviduct chromatin by promoting an increase of 14000 sites for RNA-chain initiation, which is similar to the number of additional sites measured in the oviducts of diethylstilboestrol-stimulated immature chicks [Tsai, Schwartz, Tsai & O'Malley (1975) *J. Biol. Chem.* **250**, 5165–5174]. Oestradiol alone had no effect on transcription. Thus the data demonstrate that the purified nuclear oestradiol–receptor complex can regulate gene transcription *in vitro* in a manner similar to that observed in target cells *in vivo*.

There is considerable evidence to suggest that steroid hormones act on their target cells by the formation of high-affinity complexes with cytoplasmic receptor proteins (Toft & Gorski, 1966; Edelman & Fimognari, 1968; O'Malley *et al.*, 1970; Baxter & Tomkins, 1971), which in turn translocate to the nucleus and bind to acceptor sites on the chromatin (Gorski *et al.*, 1968; Jensen *et al.*, 1968; Kalimi *et al.*, 1973, 1975; Buller *et al.*, 1975). It is this nuclear binding which is implicated in the series of events leading to the physiological responses elicited by a particular hormone (O'Malley & Means, 1974; Clark *et al.*, 1973). It follows therefore that a study of the receptor form present in the nucleus will provide significant insight into the hormone-mediated responses of target cells.

The chick oviduct has been used as a model system to study the effects of steroid hormones on gene expression. The administration of oestrogen to an immature chick results in the appearance of tubular-

gland cells in the magnum of the oviduct and in the synthesis of cell-specific egg-white proteins *de novo* (O'Malley & McGuire, 1968, 1969; O'Malley *et al.*, 1969; Comstock *et al.*, 1972; Means *et al.*, 1972; Rosenfeld *et al.*, 1972). When oestrogen treatment is discontinued, the cells lose their ability to synthesize cell-specific proteins (Kohler *et al.*, 1969; Oka & Schimke, 1969; Palmiter *et al.*, 1970; Palmiter, 1972; Chan *et al.*, 1973; Harris *et al.*, 1975), so that after 12 days of hormonal withdrawal the rate of the synthesis of ovalbumin, which constitutes approx. 60% of the total protein, is less than 1% of that observed in the oestrogen-stimulated chicks (Kohler *et al.*, 1969; Chan *et al.*, 1973; Harris *et al.*, 1975). A corresponding decrease in the numbers of RNA-initiation sites on oviduct chromatin has also been measured (Tsai *et al.*, 1975; Kalimi *et al.*, 1976). On re-administration of oestrogen there is a rapid increase in RNA-initiation sites on chromatin, followed by increases in ovalbumin-mRNA synthesis. These changes in numbers of initiation sites for RNA synthesis correspond to the changes in concentrations

Abbreviation used: SDS, sodium dodecyl sulphate.

of nuclear oestrogen receptors (Kalimi *et al.*, 1976). Such results are consistent with the hypothesis that steroid-hormone-receptor complexes manifest their effect on RNA transcription through direct interactions with the chromatin of target-cell nuclei.

We have previously shown that there are two distinct high-affinity oestrogen-binding proteins in both chick oviduct and hen oviduct cytosols (Smith *et al.*, 1979). These two oestrogen-specific binding proteins also appear to be present in 0.4M-KCl nuclear extracts of hen oviducts and have K_d values of 1.1×10^{-10} M and 4×10^{-9} M. To investigate the role of each binding protein in systems *in vitro* they must be first separated and purified. We have succeeded in separating and purifying the highest-affinity oestrogen-binding protein, which has all the characteristics of a receptor.

In the present paper we describe the isolation, characterization and purification of the nuclear oestrogen receptor from the hen oviduct. Further, we discuss its effect on the cell-free transcription of chick chromatin obtained from chicks that have been withdrawn from oestrogen stimulation. We have not purified the lower-affinity oestrogen-binding protein or studied its effects on gene transcription. Our results are consistent with the proposal that the physiological effect of steroid hormones on their target tissues is mediated at the nuclear level by their prior interaction with specific receptors.

Materials and Methods

Female 7-day-old White Leghorn chickens were oestrogen primed with daily subcutaneous injections of 2.5 mg of diethylstilboestrol in sesame oil for 14–18 days. The chicks were then withdrawn from injections for 7–12 days before use. The animals were killed by cervical dislocation, and the tissues removed, stripped of connective tissue and rinsed in ice-cold 0.9% NaCl. Hen oviducts were obtained from White Leghorn laying hens. Non-radioactive steroids were purchased from Steraloids (Wilton, NH, U.S.A.) and [2,4,6,7- 3 H]oestradiol (100 Ci/mmol) was purchased from Amersham-Searle (Arlington Heights, IL, U.S.A.). Tris, $(\text{NH}_4)_2\text{SO}_4$, sucrose and [3 H]UTP (15 Ci/mmol) were purchased from Schwarz-Mann (Orangeburg, NY, U.S.A.). Nucleoside triphosphates were obtained from Sigma (St. Louis, MO, U.S.A.) and P-L Biochemicals (Milwaukee, WI, U.S.A.). *Escherichia coli* paste (strain B) was purchased from Miles Laboratories (Elkhart, IN, U.S.A.). Rifampicin was from Calbiochem (La Jolla, CA, U.S.A.) and all other reagents were of 'Fisher certified' (Pittsburgh, PA, U.S.A.) and 'Baker analyzed' (Phillipsburg, NJ, U.S.A.) grades. Protein concentrations were determined by the method of Lowry *et al.* (1951) and DNA concentrations were determined by that of Burton (1956).

Isolation and partial purification of the nuclear receptor

Hen oviducts were minced and homogenized in 4 vol. of buffer A (10 mM-Tris/HCl, pH 7.4, 1.5 mM-EDTA, 12 mM-thioglycerol) with a Polytron PT-10 apparatus (Brinkmann Instruments, Houston, TX, U.S.A.) and rehomogenized with a Teflon-glass homogenizer (Glenco, Houston, TX, U.S.A.). The homogenate was centrifuged at 2000g for 15 min and the supernatant discarded. The pellet was resuspended in a similar volume of buffer A, gently homogenized with the Teflon-glass homogenizer and centrifuged again at 2000g for 15 min. This washing procedure was repeated before resuspension of the crude nuclear pellet in buffer A containing 0.4M-KCl. The suspension was then gently shaken at 4°C for 2 h. After this extraction procedure the suspension was centrifuged at 5000g for 15 min and the supernatant re-centrifuged at 100000g for 90 min. (In certain experiments, to remove any contaminating nucleic acids, the supernatant was then passed through a bed of DEAE-cellulose.) To the high-speed supernatant a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ in buffer A was added dropwise with stirring until a concentration of 30%-satd. $(\text{NH}_4)_2\text{SO}_4$ was attained. After stirring for a further 30 min the $(\text{NH}_4)_2\text{SO}_4$ solution was centrifuged at 5000g for 10 min and the supernatant discarded. The pellet was washed with water and redissolved in buffer A. Typically 2 ml of buffer A per hen oviduct was used, followed by 8 ml of dextran/charcoal suspended in buffer A. The charcoal suspension containing receptor was then heated to 42°C for 15 min to remove endogenous hormone. Charcoal was removed at low-speed centrifugation (2000g/10 min) and the supernatant was centrifuged at 200000g for 30 min to remove insoluble denatured proteins.

Charcoal-binding assay

This procedure is based on that described by Korenman (1969) for the measurement of oestrogen binding. The charcoal (100 g) is washed and centrifuged with 0.2M-HCl, water, 0.1M-NaOH, water, ethanol and ether, and then finally dried. Dextran-80 (15 mg) is dissolved in buffer A (300 ml) and washed charcoal (1.5 g) is added. The suspension is stirred for 6 h at room temperature and then left at 4°C overnight before use. This charcoal suspension was used routinely for steroid-binding assays as well as for stripping endogenous hormone from the nuclear oestrogen receptor. To determine the specificity of oestrogen binding the receptor preparation was incubated overnight at 4°C with [3 H]oestradiol (1 nM) in the presence of various unlabelled steroids (0.1 μ M). After incubation an equal volume of charcoal suspension was added to each preparation, the resulting suspension was thoroughly mixed and

left for 5 min before centrifuging for 5 min at 2000g. The supernatant was then decanted into 10 ml of Scintiverse LSC Cocktail (Fisher, Fairlawn, NJ, U.S.A.) and counted for ^3H incorporation.

Scatchard analyses

The receptor preparations were incubated with [^3H]oestradiol in the concentration range 0.1–16 nM either at 4°C for 18 h or at 30°C for 4 h (Kalimi *et al.*, 1976). To assess the total non-specific binding a similar series of assay tubes was set up simultaneously containing [^3H]oestradiol and either unlabelled oestradiol or diethylstilboestrol in the concentration range 0.02–3.2 μM . The total [^3H]oestradiol bound after incubation was measured by removing free oestradiol by charcoal absorption, or with hydroxyapatite (Erdos *et al.*, 1970). In order to confirm specificity, Scatchard analyses were also carried out with unlabelled progesterone (0.1 μM) and with unlabelled testosterone (0.1 μM) present in buffer A. The K_d values were determined by the method of Rosenthal (1967).

Determination of the rate of association of oestradiol to its receptor

To start the binding reaction [^3H]oestradiol (1 nM) was added to the partially purified receptor at 4°C. Portions of the receptor were removed at 2 min intervals up to 8 min and added to unlabelled oestradiol (1 μM) to quench the reaction. Each solution was then incubated with an equal volume of dextran-charcoal suspension for 5 min before centrifuging (2000g/5 min) to remove unbound hormone. The total radioactivity bound was then determined by measuring the ^3H content of the supernatant. The total amount of receptor present was determined by measuring the specific binding on saturation of receptor, assuming one binding site of hormone per receptor molecule. The concentrations of receptor and steroid at time t could then be computed. Although the overall receptor-hormone-binding reaction is reversible, for short time periods the dissociation is negligible; thus the reaction may be considered a second-order reaction. The equation of a second-order reaction in this case may be conveniently represented by eqn. (1):

$$-\ln\left(\frac{S-X}{R-X}\right) = (S-R) k_a t \quad (1)$$

where S = molarity of steroid, R = molarity of receptor, X = concentration that has reacted by time t (s), and k_a = rate constant of association. By rearranging the above equation and plotting $-\ln[(S-X)/(R-X)]$ versus t , k_a = slope/($S-R$), thus k_a can be readily calculated.

Determination of the rate of dissociation of oestradiol from its receptor

The receptor was incubated with 1 nM-[^3H]oestradiol for 2 h at 4°C. At zero time, unlabelled oestradiol was added at a concentration of 0.3 μM . Duplicate samples of the mixture were immediately taken and treated with dextran-charcoal for 5 min. They were then centrifuged at 2000g for 5 min and [^3H]oestradiol in the supernatant was determined. Samples were taken at 15, 30, 45, 60, 90, 120, 150, 180 min and 18 h and were treated similarly to determine the amount of [^3H]oestradiol bound at these time points. Non-specific binding was determined by a simultaneous assay in the presence and absence of unlabelled oestradiol and the specific bound hormone was computed at each time point. Since a large excess of unlabelled oestradiol was added to prevent reassociation of [^3H]oestradiol, the dissociation reaction becomes first-order with respect to receptor-[^3H]oestradiol complex. By using the first-order equation $\ln RH = k_d t + \ln RH_0$, where RH = concentration of receptor-hormone complex at time t and RH_0 = concentration of receptor-hormone complex at zero time, and k_d = rate constant of dissociation. A plot of $\ln RH$ versus t (s) will yield a straight line with its negative slope equal to k_d .

Glycerol density gradients

Linear gradients of 10–30% (v/v) glycerol in buffer A containing 5 mg of heparin/ml were prepared in 5 ml polyallomer tubes (Beckman) and placed in a cold-room at 4°C for 2 h. The receptor solutions (0.3 ml), which had been incubated for 2 h with [^3H]oestradiol in the presence and absence of unlabelled oestradiol and then treated with dextran-charcoal, were layered on the gradients together with [^{14}C]ovalbumin and ^{14}C -labelled bacterial alkaline phosphatase prepared as described by Rice & Means (1971). The gradients were centrifuged for 18 h at 55000 rev./min in a SW 65L Ti rotor (Beckman) at 2°C. After centrifugation the gradients were fractionated (0.2 ml) and [^3H]oestradiol was measured in each fraction.

Oestradiol succinyl-polylysyl-polyalanyl-Sepharose

The affinity resin was prepared by a method based on that described by Sica *et al.* (1973). The poly-DL-alanyl-poly-L-lysine was provided by Miles Biochemicals (Elkhart, IN, U.S.A.). The most useful resin was one having a capacity of 1.4 μg of oestradiol-17 β -hemisuccinate/ml of packed resin. In many cases it was necessary to dilute the prepared resin by as much as 10-fold in order to obtain good yields on elution of the pure receptor.

Purification of the oestrogen receptor

The partially purified 'nascent' receptor was added to the affinity resin up to a maximum volume of 15 ml of receptor solution/ml of packed resin, the actual volume of receptor solution depending on the receptor concentration. In general each ml of resin has the capacity to bind 0.1 nmol of receptor. The receptor+affinity resin were shaken overnight at 4°C and then centrifuged at 800g for 5 min. The supernatant was treated with dextran-charcoal for 5 min at 4°C to remove any endogenous oestradiol that may have been dissociated from the resin and then centrifuged at 2000g for 5 min. The supernatant was then incubated with [³H]oestradiol in the presence and absence of unlabelled oestradiol at 30°C for 4 h, to determine the amount of specific oestrogen binding remaining after incubation with the affinity resin. By comparing the amount of specific binding of [³H]-oestradiol before and after affinity chromatography, the amount of receptor retained by the affinity resin was calculated. Thus the yield after subsequent elution was then estimated. Each ml of pelleted resin from the above treatment was washed with 4 × 50 ml of 0.4 M-KCl in buffer A to remove loosely associated proteins, followed by 50 ml of buffer A. The receptor was then eluted from one-third of the resin by using buffer A containing 4 μM-[³H]oestradiol. The remainder of the resin was eluted with an equivalent volume of eluent containing unlabelled oestradiol. The eluate containing non-radioactive oestradiol was used for transcription *in vitro*. The fraction eluted with [³H]oestradiol was examined by Sephadex G-75 gel filtration to determine the amount of recovered oestrogen receptor. In general yields of 80–100% were obtained and the procedure yielded an eluate containing oestrogen-receptor complex at a concentration of 1 nM. This quantitative analysis of the receptor was assumed to hold for the remaining 66% of the preparation eluted with non-radioactive oestradiol.

DEAE-cellulose chromatography

Whatman DE-52 DEAE-cellulose was washed extensively with buffer A to remove fines and until the conductivity and pH of the eluate were the same as those of the buffer. The ion-exchange resin was used in a column of dimensions 1.3 cm × 7 cm. The bed height used was 3–5 cm for partially purified preparations and 0.5 cm whenever pure receptor was chromatographed. Receptors were eluted with a linear gradient (80 ml) of 0–0.4 M-KCl in buffer A.

Phosphocellulose chromatography

Whatman P-11 phosphocellulose was equilibrated in buffer A. Column sizes and elution conditions were similar to those described for DEAE-cellulose.

Preparation of chromatin

Chromatin was isolated from oviduct, liver and spleen by the protocol of Spelsberg *et al.* (1973) with minor modifications (Tsai *et al.*, 1975). Chromatin preparations obtained by the above procedure were found to have protein/DNA ratios in the range 2–2.7:1 (w/w). The chromatin was stored as approx. 1 mg of DNA/ml in 1.5 mM-NaCl/0.15 mM-sodium citrate, pH 7.0, at –20°C. Erythrocyte chromatin was prepared from the blood of 2-year-old hens by the procedure of Seligy & Neelin (1970).

Purification of RNA polymerase

E. coli RNA polymerase containing the σ subunit was isolated from late-exponential-phase *E. coli* B paste by the procedure of Burgess (1969) as modified by Bautz & Dunn (1969). The enzyme was stored at 20°C in 10 mM-Tris/HCl (pH 7.9)/10 mM-MgCl₂/0.1 mM-dithiothreitol/0.1 mM-EDTA/0.1 M-KCl/50% (v/v) glycerol at a protein concentration exceeding 6 mg/ml. The enzyme obtained in this way was 95% pure as determined by SDS/polyacrylamide-gel electrophoresis. The RNA polymerase was assayed by using calf thymus DNA as a template, and the enzyme specific activity was determined to be 700 units/mg of protein, where 1 activity unit of polymerase incorporates 1 nmol of AMP in 10 min of incubation at 37°C (Burgess, 1969).

Measurement of RNA synthesis in the presence of pure oestrogen receptor

Various amounts of oestrogen-receptor complexes in buffer A were added in a maximum volume of 100 μl to 25 μl of chromatin suspension (5 μg of DNA). Incubations were carried out in glass tubes for 30 min at 22°C. A saturating amount of *E. coli* RNA polymerase (15 μg) was then added together with 20 μmol of Tris/HCl (pH 7.9), 1.0 μmol of MnCl₂, 0.5 μmol of mercaptoethanol and 100 μg of bovine serum albumin. The mixture was incubated for an additional 30 min at 22°C. After this preincubation period RNA synthesis was initiated by the addition of 37.5 nmol each of ATP, GTP, CTP and UTP, and 10 μCi of [³H]UTP, 10 μg of rifampicin, 0.1 μmol of potassium phosphate (pH 7.0) and 200 μg of heparin. Incubation was continued at 37°C for 15 min. The reaction was stopped either by precipitation with trichloroacetic acid (Tsai *et al.*, 1975) or by pipetting 100 μl samples on to DEAE-cellulose filters (Whatman DE 81, in duplicate). The filters were processed by the procedure of Roeder (1974), and were counted for radioactivity in scintillation fluid (Liquofluor; Amersham-Searle). Incorporated [³H]UMP had a specific radioactivity of 160 c.p.m./pmol. The determination of RNA-chain length and the calculation for numbers of new

initiation sites were done as described previously (Tsai *et al.*, 1975).

Detection of proteinases with azoalbumin

The release of amino acid-linked azo groups from azoalbumin (Sigma) can be used to measure minute quantities of proteolytic-enzyme activity (Tomarelli *et al.*, 1949). Azoalbumin (100 µg) was incubated for 1 h at 22°C together with either receptor protein fractions or with trypsin concentrations ranging from 1 µg to 0.1 ng in 1.5 mM-NaCl/0.15 mM-sodium citrate, pH 7.0, in a volume of 300 µl. The reaction was stopped by the addition of 50 µl of 100% trichloroacetic acid, and the mixture cooled to 4°C and centrifuged at 800g for 30 min. The A_{440} of the clarified supernatant fraction containing released azo groups was measured. This method was sufficiently sensitive to detect ng amounts of trypsin.

SDS/polyacrylamide-gel electrophoresis

The procedure described by Laemmli (1970) was used with 10% acrylamide gels.

Results

Extraction and isolation of the nuclear receptor

Although a similar nuclear receptor for oestrogen can be isolated from diethylstilboestrol-stimulated chick oviducts by extraction with 0.4 M-KCl, it was considered more efficient to use hen oviducts to supply larger amounts of receptor, especially since the yield of extractable receptor is variable. After extraction of the crude nuclear pellet with 0.4 M-KCl the receptor was partially purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$. The receptor obtained at this stage contained endogenous oestrogen, and all attempts to purify it by affinity chromatography were unsuccessful.

Numerous attempts were made to remove endogenous oestrogen by using dextran-charcoal at various temperatures. Interestingly, it was not until the approximate basal body temperature of the hen was reached that good yields of nascent receptor were obtained. The optimal conditions for the removal of endogenous oestrogen without completely denaturing the receptor were found to be 42°C for 15 min. It is remarkable that the receptor still retains activity after such drastic conditions, but our observations have been confirmed by Best-Belpomme *et al.* (1975), who used 45°C for 15–30 min to remove endogenous oestrogen. A similar observation has been made with the nuclear oestrogen receptor from human uteri (Clarke & Smith, 1976).

Binding specificity was examined by adding 0.1 µM-unlabelled steroids in the presence of 1 nM-[³H]-

oestradiol to the receptor and incubating for 18 h at 4°C. After the incubation free steroid was removed by charcoal adsorption and the amount of [³H]-oestradiol bound to the receptor was determined. Table 1 shows that unlabelled oestradiol, oestradiol-17α, diethylstilboestrol, and 1-{2-[p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl}pyrrolidine hydrochloride (Nafoxidin) compete well with [³H]oestradiol for binding, whereas oestrone, 5α-dihydrotestosterone, corticosterone and progesterone do not compete significantly.

Scatchard analysis of the equilibrium dissociation constant

The relationship of binding to hormone concentration was studied over a 40-fold concentration range. The binding sites were saturable (Fig. 1), and a Scatchard plot of the data over this particular concentration range indicated a single class of binding sites with a dissociation constant, K_d , of 1.1×10^{-10} M. This K_d is 20 times lower than that reported by Kalimi *et al.* (1976). This implies either that the receptor has been altered in some way by the charcoal treatment or that the presence of endogenous hormone changes the measured K_d . To determine whether this difference was explained by endogenous oestrogen, a portion of the receptor was incubated with oestradiol (10 nM) for 10 min at 37°C. Excess oestradiol was then removed by charcoal adsorption at 4°C. Scatchard analyses were then performed on both the oestrogen-treated and untreated receptor solutions at the same protein concentrations under exchange conditions (Kalimi *et al.*, 1976). Fig. 1 shows that both solutions contained the same concentrations of high-affinity binding sites at saturation, but the equilibrium dissociation constants differed by a factor of 30. Thus it can be concluded that the observed differences in the K_d of the nuclear receptor are explained by the presence of endogenous hormone.

Table 1. Inhibition of binding of [³H]oestradiol (1 nM) to the nuclear receptor by various competitors (0.1 µM)

The assays were performed as described in the Materials and Methods section.

Competitor	Inhibition (%)
None	0
Oestradiol-17β	100
Oestradiol-17α	90
Oestrone	11
Oestriol	87
5α-Dihydrotestosterone	<1
Corticosterone	10
Progesterone	<1
Diethylstilboestrol	100
Nafoxidin	54

Fig. 1. Scatchard analyses of oestradiol binding to the nuclear oestrogen receptor

The Scatchard analyses of the specific binding of oestradiol was performed in the concentration range 0.1–4 nM-[³H]oestradiol. Incubations with [³H]-oestradiol were carried out for 4 h at 30°C and the amount of [³H]oestradiol bound was determined by using the charcoal-adsorption assay. Specifically bound oestradiol is plotted versus bound/free ratio. Charcoal-stripped nuclear receptor (○) is compared with stripped receptor that was incubated with oestradiol for 10 min at 37°C, followed by removal of unbound oestradiol by charcoal adsorption before Scatchard analysis (●). The protein concentrations of the receptor preparations were equalized before assay.

Measurement of the rates of association and dissociation of oestradiol

For the formation of the steroid (S)–receptor (R) complex, assuming a single class of binding sites per molecule of receptor:

The rate of association, for short reaction times, may be considered as that of a second-order irreversible reaction and may be measured as described in the Materials and Methods section. The rate of dissociation can be studied by preventing the re-association of labelled hormone to the receptor and may be plotted as a first-order reaction, again as discussed in the Materials and Methods section. Fig. 2 shows the plots obtained in the determinations of k_a and k_d at 4°C. The k_a measured over the first 8 min of association was $1.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, and the k_d was $5.0 \times 10^{-5} \text{ s}^{-1}$ when measured for the first 6 h of dissociation. At 30°C the k_d was $2.3 \times 10^{-3} \text{ s}^{-1}$.

The dissociation curves were biphasic at both 4°C and 30°C. The second component of the curves became evident after 6 h at 4°C and after 40 min at

Fig. 2. Measurement of the rates of association (k_a) and dissociation (k_d) of oestradiol with the nuclear receptor

(a) The rate of association was determined for the first 8 min of the reaction at 4°C. For this short reaction time the reaction may be plotted as a second-order irreversible reaction. (b) Determination of the dissociation rate constant at 4°C, which was measured for the first 6 h of dissociation and is plotted as a first-order reaction. Reassociation of [³H]-oestradiol was masked by performing the dissociation experiment in the presence of excess of unlabelled oestradiol as described in the Materials and Methods section. The k_a was $1.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and k_d $5.0 \times 10^{-5} \text{ s}^{-1}$.

30°C. The half-time ($t_{1/2}$) of oestrogen dissociation at 4°C and 30°C was 230 min and 5 min respectively.

Sedimentation on glycerol gradients

After incubation of the nuclear receptor with [³H]oestradiol a single binding component was observed of sedimentation coefficient 4.6S. In the presence of 0.4 M-KCl no change in this value was observed, indicating that no salt-dependent dissociation of subunits was occurring. This lack of subunit dissociation is in contrast with that reported for the cytoplasmic receptors for oestrogen in the chick (Harrison & Toft, 1975) and calf (De Sombre *et al.*, 1969) and the progesterone receptor from chick (Schrader *et al.*, 1972). Approx. 50% of the radioactivity applied to the gradients sedimented at the bottom of the gradient tubes. When receptor containing unlabelled oestradiol together with [³H]-oestradiol was sedimented under identical conditions

both the 4.6S and the aggregated forms of the receptor were significantly displaced (Fig. 3).

Purification of the nuclear receptor

Purification of the receptor was achieved by affinity chromatography. The affinity resin consisted of polyalanyl-polylysine coupled to CNBr-activated Sepharose to which oestradiol was linked via its 17-hemisuccinate by using a water-soluble carbodiimide. The preparation of the resin was similar to that described previously (Sica *et al.*, 1973). The concentration of polyalanyl-polylysine coupled was 0.3 mg/ml of Sepharose. The amount of steroid coupled to the resin was determined by removing the steroid by base hydrolysis, extracting the hydrolysate with ether and determining oestradiol by radioimmunoassay. The amount of steroid linked to the polyalanyl-polylysyl side chain was 5 nmol/ml of resin, of which 0.04 nmol was available for binding to the receptor. This limited availability of the steroid for receptor binding is in agreement with previous

observations (Sica *et al.*, 1973; Smith *et al.*, 1975; Kuhn *et al.*, 1975).

We have shown previously that, before attempting affinity chromatography on columns containing a steroid coupled by an ester linkage, it is necessary to remove esterase activity by using $(\text{NH}_4)_2\text{SO}_4$ fractionation (Smith *et al.*, 1975; Kuhn *et al.*, 1975). The nuclear receptor had already been partially purified by using $(\text{NH}_4)_2\text{SO}_4$ precipitation before removal of endogenous hormone, so further precautions were not necessary before incubating the receptor with the oestradiol resin.

The nuclear receptor was incubated with the affinity resin overnight at 4°C to ensure that equilibrium between immobilized oestradiol and the receptor was established. The mixture was then centrifuged, and the supernatant removed. After the resin was thoroughly washed, the receptor was displaced from the immobile oestradiol on the resin with excess $[^3\text{H}]$ oestradiol at 37°C for 10 min. The material eluted from the affinity column was examined by Sephadex G-75 gel filtration to determine the amount of oestrogen-binding activity recovered. Samples of the pure receptor were incubated with excess unlabelled oestradiol, diethylstilboestrol and progesterone. Only diethylstilboestrol and oestradiol displaced significant amounts of the bound $[^3\text{H}]$ -oestradiol, demonstrating the specificity of the receptor for oestrogens. When the receptor-hormone complex was applied to phosphocellulose the receptor passed through the column. The receptor was, however, retained by DEAE-cellulose and was eluted at $0.15 \pm 0.02 \text{ M-KCl}$ on salt-gradient elution. These ion-exchange properties of the pure receptor were shared by the receptor before its purification by affinity chromatography. Glycerol-gradient analyses of the pure receptor showed a small peak at 4.6S together with aggregated receptor at the bottom of the gradient.

The material eluted from the affinity column was purified 90000-fold over the 0.4 M-KCl crude nuclear extract (Table 2). This purification is reported as a minimum value, since the amount of protein determined was at the detection limit of the assay and it was assumed that all receptor isolated had retained its capacity to bind oestradiol. The pure receptor appeared to have retained the properties of the partially purified form and bound oestradiol specifically. There was no evidence of subunit structures on ion-exchange chromatography, although the material gave two bands on SDS/polyacrylamide-gel electrophoresis, corresponding to mol.wts. 74000 and 80000 (Fig. 4). All attempts to determine the molecular weight of the receptor under non-denaturing conditions proved abortive because of aggregation and the relatively rapid dissociation of oestradiol; therefore these molecular-weight estimates for the receptor remain tentative.

Fig. 3. Glycerol-gradient analysis of the nuclear receptor

The receptor solutions (0.3 ml) labelled with $[^3\text{H}]$ -oestradiol were loaded on gradients of 10–30% glycerol in buffer A containing 5 mg of heparin/ml and then centrifuged for 18 h as described in the Materials and Methods section. The sedimentation-coefficient markers used were $[^{14}\text{C}]$ ovalbumin (3.6S) and $[^{14}\text{C}]$ -labelled bacterial alkaline phosphatase (6.3S). Gradient analyses with $[^3\text{H}]$ oestradiol alone (●) or $[^3\text{H}]$ oestradiol in the presence of 100-fold excess of diethylstilboestrol (○) are shown. In the presence of 0.4 M-KCl no change in the sedimentation coefficient of the receptor was observed.

Table 2. *Purification of the nuclear oestrogen receptor*

Step	Total protein (mg)	Radioactivity (c.p.m./mg of protein)	Yield (%)	Purification (fold)
0.4M-KCl extract of nuclear pellet	875	—	—	—
(NH ₄) ₂ SO ₄ fractionation and charcoal stripping	110	3000	~30	8
Elution from affinity column	~0.001*	~3.3 × 10 ⁷	100	~9.0 × 10 ⁴

* Protein concentration <0.005 when measured by method of Lowry *et al.* (1951). The value of 0.001 was obtained by comparison with stained protein standards on SDS/polyacrylamide gels.

Fig. 4. *SDS/polyacrylamide-gel electrophoresis*

The procedure described by Laemmli (1970) was used with 10% acrylamide gels. Accompanying gels were run containing the molecular-weight standards bovine serum albumin (68000), ovalbumin (45000), chymotrypsinogen (25000) and cytochrome *c* (17500). The log of the molecular weight of the standards was plotted versus their relative mobility in the gels. The resulting straight line constructed through these points was used to estimate the molecular weight of the receptor. The two bands shown migrated at mol.wts. of 74000 and 80000.

Effect of the pure oestrogen receptor on transcription of chromatin in vitro

Tsai *et al.* (1975) have shown previously that on re-administration of oestrogen to hormone-withdrawn chicks the number of RNA-initiation sites on

oviduct chromatin doubles within 30 min of a single dose of diethylstilboestrol and reaches a plateau at a maximum of 27000 sites/pg of DNA within 1–2 h. This rapid increase in transcription is consistent with the hypothesis that steroid–receptor complexes enter target cells and regulate gene transcription. Now that we have purified the receptor from oviduct nuclei we are able to test whether the oestradiol–receptor complex can act directly on oviduct chromatin to cause this increase in RNA transcription.

A fixed concentration of oviduct chromatin from hormone-withdrawn chicks was preincubated with increasing quantities of purified oestrogen receptor (1 nM) for 30 min at 22°C. The mixtures were then incubated for an additional 30 min with an excess of RNA polymerase to allow the formation of pre-initiation complexes. A solution containing ribonucleotides, rifampicin and heparin was then added and incubation was continued at 37°C for 15 min. RNA synthesis under these conditions increased in a dose-dependent manner, as shown in Fig. 5. The half-maximal stimulation was found to occur at an oestrogen–receptor concentration of approx. 0.5 nM. This increase in numbers of initiation sites reached a plateau at 26000 sites/pg of DNA, which is similar to the plateau value observed after a single injection of diethylstilboestrol to hormone-withdrawn chicks (Kalimi *et al.*, 1976).

To determine whether the stimulation of transcription was perhaps caused by an artifact from the affinity column, three different eluates were examined for activity in the transcription assay. The first was obtained by collecting the washings from the affinity column just before the normal elution with oestradiol. For the second eluate the column was eluted with oestradiol in the usual way at 37°C for 10 min, and the final eluate was obtained as a post-oestradiol elution by using buffer A at 37°C for 10 min. The results (Table 3) confirm that only the material eluted with oestradiol had significant activity in the transcription assay. It appears that hormone-binding activity is necessary for a transcriptional response under these conditions. Furthermore, if the pure receptor was boiled for 10 min transcription was decreased by 70%.

Fig. 5. Effect of oestrogen receptor on initiation sites for RNA synthesis in oviduct chromatin

Chromatin was isolated from oviducts of chicks withdrawn from diethylstilboestrol treatment for 7 days. Portions of the chromatin (5µg of DNA) were incubated with increasing concentrations of highly purified oestrogen receptor for 30min at 22°C. RNA polymerase (15µg) was added and the incubation was continued for a further 30min. Nucleoside triphosphates with [³H]UTP, rifampicin and heparin were then added and incubation was continued at 37°C for 15min. The amount of RNA synthesized was determined from [³H]UTP incorporation into trichloroacetic acid-precipitable material. The numbers of new RNA initiation sites were calculated as described by Tsai *et al.* (1975). The horizontal straight line represents control values. These assays were repeated at least five times per point. The ranges in the Figure are ±s.e.m. and the significance of the receptor-directed increase in transcription was *P*<0.01 as determined by the standard difference of the means of two populations.

Concn. of receptor (nM)	No. of initiation sites/pg of DNA
0	11 500
0.4	13 500
0.8	21 400
2	25 400
2.8	25 800
4	22 800

In order to examine further whether some activating factor was binding non-specifically to the oestradiol affinity column and was being fortuitously eluted with oestradiol, the receptor preparation was incubated with 1 nM-oestradiol in the presence of the affinity resin. In this way the receptor was prevented from binding to the oestradiol covalently attached to the affinity resin. Washing and elution of this column were accomplished in the usual way, and a transcription assay was performed on this material and the results were compared with the results obtained from material that had not been incubated with oestradiol. Only the material purified in the absence of oestradiol gave a significant increase in initiation sites. Thus, if a factor other than the receptor is responsible for the increases in numbers of initiation sites, either such a factor, should it exist, binds oestradiol with high affinity, or the factor itself has a high affinity for the receptor. These results are also confirmed by the fact that purification of an active oestradiol receptor could not be achieved by affinity chromatography unless the endogenous oestrogen was first removed by charcoal treatment. The transcription assay was also carried out on material that had been chromatographed on DEAE-cellulose to remove any contaminating nucleic acids before purification by affinity chromatography. This material was still 'active' in the transcription assay, showing that quantities of low-molecular-weight RNA or DNA that may have contaminated the receptor preparations were not responsible for the observed increase in RNA-initiation sites.

We have found that crude (NH₄)₂SO₄ precipitates of cytosol fractions of eukaryotic cells can often spuriously stimulate RNA synthesis by a template-independent process which is unrelated to hormone receptors. To rule out such an effect in the present study, we investigated the sensitivity of rifampicin-resistant incorporation of [³H]UMP into RNA under various conditions and in the presence of inhibitors (Table 4). Table 4 shows that RNA synthesis in the presence of receptor and chromatin was completely dependent on template and was inhibited by actinomycin D. Furthermore, none of the components

Table 3. Effect of various affinity-column eluates on RNA synthesis from oviduct chromatin
RNA synthesis was assayed in the presence of nucleotides and rifampicin as described in the Materials and Methods section.

Components added	Rifampicin-resistant radioactivity (c.p.m.)
Control	2480
Purified oestrogen receptor	5400
Affinity-column elution buffer containing oestradiol	2580
Final fraction of column wash before elution	2770
Post-elution wash of column with buffer	3070
Affinity-column eluent not containing a binding component for oestradiol	2510

Table 4. *Effect of oestrogen receptor on RNA synthesis in vitro from oviduct chromatin*

RNA synthesis was assayed in the presence of nucleotides and rifampicin as described in the Materials and Methods section.

Components added to assay	[³ H]UMP incorporated into RNA	
	(c.p.m./5 µg of chromatin DNA)	(% of control)
Background		
RNA polymerase only	230	9
Oestrogen-receptor complex plus RNA polymerase	330	13
Chromatin alone	200	8
Control		
Chromatin plus RNA polymerase	2550	100
Add 10 nM-oestrogen alone	2640	103
Experimental		
Chromatin plus RNA polymerase plus 0.4 nM-oestrogen-receptor complex	5440	213
Add α -amanitin (10 µg)	5410	212
Add actinomycin D (10 µg)	200	13

alone contained significant synthetic activity, and hence neither the chromatin, polymerase, nor receptor were contaminated with other enzymes that were capable of incorporating [³H]UTP into acid-insoluble material. In these experiments, the oestrogen receptor preincubated with chromatin in the presence of *E. coli* RNA polymerase increased RNA synthesis by 100% over control values. With the addition of α -amanitin, a potent inhibitor of eukaryotic RNA polymerase II, there was little effect on the oestradiol-receptor-directed stimulation of RNA transcription. These data show that the stimulation of RNA synthesis was not due to the activation of endogenous RNA polymerase, which could have been a contaminant in chromatin preparations, but rather was due to transcription by the added *E. coli* enzyme.

When the pure receptor preparation was incubated with azoalbumin to measure proteinase activity, none could be detected. The possibility of extremely low amounts of proteinase (<10 ng) could not be ruled out by using this method. However, the fact that the measured increase in initiation sites reached a plateau, rather than continuing to increase with increasing concentration of receptor, implies that the observed transcription was unlikely to be the effect of some non-specific contaminant.

The tissue specificity for the role of the oestrogen receptor on transcription is shown in Fig. 6. The response is in the order oviduct > liver >> erythrocyte and spleen. Both the oviduct and liver are known target tissues for oestradiol; thus the response is exactly as predicted. The receptor preparation had no effect on DNA, which implies that prior interaction of the receptor with a chromosomal protein is necessary before transcription can occur.

Fig. 6. *Tissue specificity for the oestrogen-receptor effect on initiation sites for RNA synthesis*

Chromatin from oviduct (●), liver (○), spleen (□) and erythrocytes (△) was incubated with increasing doses of purified oestrogen receptor. Conditions for RNA synthesis were identical with those described in Fig. 5 and in the Materials and Methods section. Data are expressed as the difference of [³H]UTP radioactivity (c.p.m.) incorporated between control and oestrogen-receptor-treated chromatin.

Discussion

Two distinct high-affinity oestrogen-binding macromolecules have been measured in both the cytoplasm (Fig. 7) and nucleus of chick and hen oviduct tissues (Smith *et al.*, 1979). Since both macromolecules are found in the nucleus, it might be predicted that both have a unique function at the

Fig. 7. Scatchard analysis of chick oviduct cytosol. Oviduct cytosol was obtained from chicks stimulated for 14 days with diethylstilboestrol followed by withdrawal from diethylstilboestrol for 8 days before assay. The assay was carried out by using [³H]-oestradiol in the concentration range of 0.1–16 nM as described in the Materials and Methods section. The exact value for the equilibrium dissociation constant (K_d) of each component, 0.1 nM and 4 nM, was obtained from a graphical solution of the curve as described by Rosenthal (1967).

nuclear level. A study of their distinct functions requires the complete separation and purification of each, followed by a careful evaluation of their biological responses in cell-free systems. Although it has not proved possible to separate the two, we have been able to destroy preferentially the oestradiol-binding capacity of the component having the lower affinity for oestradiol (K_d approx. 10^{-9} M). This has enabled us to study the properties and to purify the remaining higher-affinity receptor (K_d approx. 10^{-10} M) by affinity chromatography.

The high-affinity oestrogen receptor isolated from oviduct nuclei of diethylstilboestrol-stimulated chicks or hens appeared similar in properties to a component present in oviduct cytosol from chicks withdrawn from diethylstilboestrol for 7 days (Fig. 7). The equilibrium dissociation constants for oestradiol binding were the same ($K_d = 1.1 \times 10^{-10}$ M) and the rates of dissociation were the same ($k_d = 5 \times 10^{-5}$ s⁻¹). The similar high specificity of oestrogens for both the cytoplasmic and nuclear receptors was confirmed by using competitive binding assays. Thus it is conceivable that the nuclear oestrogen receptor had been translocated from the cytoplasm after first complexing with oestradiol. The rate of dissociation of oestradiol from the nuclear receptor is different from that described by Best-Belpomme

et al. (1975). It is also different from that described by Harrison & Toft (1975) for the chick oviduct cytosol receptor. This apparent discrepancy, however, is explained readily by the fact that these groups of investigators labelled their respective receptor preparations by incubating with [³H]oestradiol overnight at 4°C. A shorter incubation time was considered adequate, since we had shown that the association of oestradiol to the high-affinity binding sites was essentially complete after 30 min at 4°C. Increasing the incubation time of the receptor with oestradiol before measurement of dissociation of oestradiol dramatically changes the slopes of the dissociation curves.

There is very good evidence to suggest that a conformational change occurs in the receptor molecule on incubation with oestradiol. The measured rate of dissociation of oestradiol becomes slower with increasing preincubation time, but the numbers of high-affinity limited-capacity oestradiol-specific binding sites do not change. It appears that the steroid is becoming enveloped by the receptor molecule. *In vivo* a similar process probably occurs more rapidly because of the higher basal temperature and the constant availability of oestradiol. This possibility is supported by the fact that the rate of exchange of [³H]oestradiol with endogenous oestradiol in diethylstilboestrol-stimulated chick oviduct, or hen oviduct nuclei, is much slower than would be predicted from the dissociation experiments described above. For example, after 12 h at 4°C only 20% exchange occurs, and even at 30°C 4 h is required for complete exchange (Kalimi *et al.*, 1976). When a 0.4 M-KCl nuclear extract is used rather than intact nuclei for exchange, a similar phenomenon is observed. The possibility of irreversible aggregation or denaturation of oestrogen-binding sites is precluded by the fact that removal of the endogenous oestradiol by charcoal treatment seems to reverse the conformation change. It is perhaps pertinent that both in our laboratory and in those of other workers the efficient removal of endogenous oestradiol from nuclear receptors requires temperatures close to the basal body temperature of the hen. It follows that envelopment of the steroid hormone by the receptor is a most important conformational change which may prove to be essential for the control of gene expression by steroid hormones. Such an activation occurs when the pure nuclear oestrogen receptor is eluted from the affinity column at 37°C in the presence of diethylstilboestrol.

The receptor purified by affinity chromatography exhibited similar high specificity for oestrogens, similar sedimentation properties, and similar properties on ion-exchange chromatography, to those of the crude receptor. We have used the pure oestrogen-receptor-hormone complex to demonstrate changes in RNA-initiation sites in oviduct chromatin which

are dependent on the presence of purified oestrogen receptor. The oestrogen-receptor-hormone complex can regulate directly the transcription of chromatin *in vitro* by increasing the number of initiation sites for RNA synthesis available to RNA polymerase. Such results are not consistent with models for the mechanism for steroid-hormone action which invoke a requirement for RNA or protein intermediates to be induced, which could then exert secondary effects on transcription. Macromolecular synthesis cannot occur during preincubation of chromatin with the receptor, and, at the time of nucleotide addition, the simultaneous addition of rifampicin permits only a single RNA chain per initiation site to be synthesized. The hormone-receptor complex appears to affect only the number of initiation sites rather than increasing RNA-chain size or elongation rates.

The tissue-specificity studies summarized in Fig. 6, coupled with the fact that the receptor-hormone complex does not cause an increase in initiation sites in DNA, imply that the hormone-receptor complex must first interact with a chromatin acceptor protein before new initiation sites are exposed. Thus collectively our data support the conclusion that the primary locus of steroid hormone action is direct transcriptional control.

The receptor-hormone complex at a concentration of 0.5 nM stimulated transcription of oviduct chromatin by promoting an increase of 14000 initiation sites, similar to the number of additional sites measured in the oviducts of oestrogen-stimulated chicks (Tsai *et al.*, 1975). Oestradiol alone had no effect on transcription. The measurement of oestrogen-receptor-stimulated initiation sites for *E. coli* RNA polymerase in chromatin from oestrogen-stimulated chick oviduct correlates fairly well with estimates of the total poly(A)-containing-RNA sequence complexity of stimulated chick oviduct RNA (Monahan *et al.*, 1976). At present, however, it is impossible to demonstrate that the initiation sites measured *in vitro* correspond to actual sites *in vivo* for RNA-chain initiation. Until this question can be resolved, measurement of chromatin initiations can be considered only as an approximation of the DNA sequences in chromatin available for transcription and as an analytical tool for quantifying the overall extent of gene expression.

The use of a prokaryotic polymerase to study the initiation of RNA synthesis on eukaryotic DNA and chromatin may be questioned. Evidence by Tsai *et al.* (1976) indicates that *E. coli* RNA polymerase and hen oviduct polymerase II utilize the same set of initiation sites in chromatin. At the present time, it is not possible to prove that these initiation sites *in vitro* are identical with the actual initiation sites utilized *in vivo*. Nevertheless, the ability of different RNA polymerases to utilize common sites would be

consistent with the hypotheses that chromosomal proteins are major determinants of initiation sites for RNA polymerase in chromatin. Finally, we have performed experiments similar to those described in the present paper, but instead using endogenous oviduct RNA polymerase II. These results are in agreement with the data obtained with bacterial polymerase and indicate a similar extent of receptor-mediated stimulation of chromatin transcription.

We are cognizant of the problem of production as artifacts of false initiation sites. In a previous publication (Schwartz *et al.*, 1976) we addressed ourselves to this problem, in which progesterone receptors might cause nucleolytic cleavage or nicking, thus creating false initiation sites. We have shown (Tsai *et al.*, 1975) that rifampicin-resistant initiation sites in chromatin have certain properties similar to those observed for polymerase initiation on single-strand or nicked DNA. Therefore it was possible that the receptor might act by nicking the DNA in chromatin and thereby spuriously increasing the number of available RNA-chain-initiation sites. We tested this possibility; DNA was isolated and purified separately from both hormone-withdrawn chick chromatin and receptor-treated chromatin and then utilized as the template in the RNA-polymerase titration assay. It is known that *E. coli* RNA polymerase binds strongest to nicked or single-strand regions of DNA, followed by binding of the polymerase to promoter sites and then less tightly to non-specific or random-binding sites. Since it has previously been documented that the number of nicks in DNA correlated directly with the amount of RNA polymerase required to saturate the initial part of the curve, it was possible to use this assay to determine whether the receptor caused nicks in the DNA of chromatin (Schwartz *et al.*, 1975). We previously showed that chromatin incubated with progesterone-receptor complex supported a 50% increase in RNA synthesis in comparison with control chromatin, whereas no discernible increase in the amount of bound enzyme could be detected within the nicked region of the curve. Therefore the increased quantity of RNA-chain initiation caused by receptors does not appear to be related to fragmentation of the chromatin's DNA. We have performed similar experiments with the oestrogen receptor and have found no increase in DNA nicking. In fact receptor with DNA failed to increase transcription rates or the amount of nicks.

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