Progesterone-binding Protein of Chick Oviduct

VI. INTERACTION OF PURIFIED PROGESTERONE-RECEPTOR COMPONENTS WITH NUCLEAR CONSTITUENTS*

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SUMMARY

Components A and B of the chick oviduct progesterone-binding protein system have been prepared by chromatography on DEAE-cellulose. Both components are shown to bind [3H]progesterone in vivo and to accumulate in oviduct nuclei in vitro. Neither component preparation contains detectable enzymatic activity for metabolizing progesterone in vitro. Each component can enter nuclei separately where both are strongly retained bound to nuclear chromatin. In vitro assays for the interaction of the components with DNA and chromatin acidic proteins were used to study binding specificity of the components. Component A binds to DNA from several sources but Component B does not bind to DNA. Conversely, Component B binds to purified oviduct chromatin and retains target organ specificity, but Component A does not bind to purified chromatin. The two types of binding activity characteristic of crude cytosol progesterone receptor, namely, the association with DNA and with target cell chromatin proteins, have thus been resolved and shown to reside in receptor Components A and B, respectively.

METHODS

Scintillation Counting—Radioactivity was determined in 0.6-nl aqueous samples by adding 5 ml of scintillation fluid containing toluene, Triton X-100 (Beckman), and Spectrafluor...
(Amersham-Searle), 1000:521:42 (v/v/v). Counting efficiency for $^{3}H$ was 33%. Nonaqueous samples were counted in scintillation fluid containing toluene and Spectrafluor, 1000:42 (v/v). Counting efficiency was 51% in a Beckman LS-230 scintillation spectrometer.

Chemicals—Sources of all chemicals have been described previously (1-5).

Isolation of Purified Progesterone Receptor: Resolution into Components A and B—High speed supernatant fractions (cytosol)$^1$ of oviducts from chicks stimulated 15 days with diethylstilbestrol (DES; 5 mg per day subcutaneously) were used as the source of progesterone-binding proteins as described previously.

Progesterone-binding proteins in 0.01 M Tris.-0.001 M Na$_2$EDTA-0.012 M 1-thioglycerol, pH 7.4 (TESH buffer) were labeled with $^{3}H$progesterone (33.5 Ci per mmole) for 1 hour at 0°. The cytosol was brought to 30% saturation in ammonium sulfate (neutralized 100% saturated stock solution) and the precipitated binding protein was collected by centrifugation. The precipitated protein was dissolved in 0.15 M KCl containing $[^3]H$progesterone (10 ng equivalent) equiva-
ted to 0.3 M KCl, respectively, as described previously (2).

Sucrose Gradient Analysis—Receptor preparations (0.2 ml) were layered on 5 to 20% sucrose gradients in TESH buffer or in TESH-0.3 M KCl. The samples were centrifuged and handled as previously described (4, 5) in a Spinco SW 50.1 rotor for 16 hours at 45,000 rpm.

Assay of Receptor Components in Crude Fractions—When it was necessary to measure the presence of Components A and B in crude cytosol or nuclear extracts, a shortened isolation procedure was used. Aliquots of cytosol or nuclear extracts were diluted with TESH to a KC1 concentration less than 0.03 M. The samples were then applied directly to a 5-ml DEAE-cellulose column equilibrated in TESH and eluted stepwise with TESH-KCl as described above.

Chromatography of Progesterone Metabolites—Metabolic activity of the binding protein preparation was studied in 1.0-m1 samples of cytosol, Component A or Component B labeled with $[^3]H$progesterone in TESH-0.15 M KCl. The samples were incubated for 30 min at either 0 or 37°. NADPH (0.001 M) was added in parallel experiments. After the incubations, progesterone metabolites were extracted into 15 ml of dichloromethane (Mallinckrodt) by vigorous shaking for 2 min at room temperature. The upper aqueous phase was discarded by suction from the interface and lower organic phase. The organic phase and interface were backwashed with 1.0 ml of H$_2$O which was also discarded by aspiration. The remaining dichloromethane was collected by filtration through Pasteur pipettes stopped with glass wool, and evaporated to dryness.

Samples were resuspended in small volumes of dichloromethane and chromatographed on previously coated silica gel thin layer chromatography plates (Merck) developed with benzene-ethyl acetate, 4:1 (v/v).$^2$ No attempt was made in this study to identify all of the possible progesterone metabolites. Instead, six regions were defined on the thin layer chromatography plates by $R_F$ comparison with selected steroid standards chromatographed at the same time in adjacent lanes. The regions were (a) $R_F = 0$, the origin (highly polar material); (b) $R_F = 0$ to 0.18, mixed diols (marker: 20$^5$-hydroxy-4-pregnen-3-one); (c) $R_F = 0.18$ to 0.37, mixed monols; (d) $R_F = 0.37$, progesterone; (e) $R_F = 0.52$, 5P-pregnane-3,20-dione; and (f) $R_F = 0.58$, 5a-pregnane-3,20-dione. Some monols cochromatographed with progesterone. The progesterone spots were therefore acetylated after extraction and rechromatographed in the same thin layer chromatography system. The authentic progesterone derivative separated from the acetylated monols at this step. Thus the technique permitted isolated of authentic progesterone and the two pregnane diones free of other contaminants. The silica gel was scraped from the plates in each region, collected by suction into plugged Pasteur pipettes, and the steroids eluted from the silica gel with dichloromethane. The dichloro-

methane was evaporated to dryness, and the samples counted for $^{3}H$ in toluene-Spectrafluor. Extraction efficiency was about 90%. Recovery from the plate was assumed to be constant for each class of metabolites for all experiments.

Extraction of Binding Proteins Labeled with $[^3]H$progesterone in Vitro—Each of three chicks stimulated with DES for 15 days received 200 µCi (0.3 ml) of $[^3]H$progesterone in 0.9% NaCl solution-10% ethanol by intravenous injection into the left-wing vein. After 30 min the animals were killed and the oviducts were removed and processed as described above to prepare binding proteins.

Nuclear Binding Protein Components from Oviduct Slices Incubated with $[^3]H$progesterone in Vitro—Oviduct slices from DES-primed chicks were rinsed in cold 0.9% NaCl solution and 2.4-g batches were incubated at 0° in 6 ml of Basal Eagle’s Medium (BEM) containing $^{3}H$progesterone. After 5 min the slices were transferred to fresh BEM without progesterone and incubated 20 min at 37°. The slices were then transferred to 10 ml of cold TESH buffer, homogenized, and centrifuged at 800 × g. The supernatant fraction was centrifuged at 105,000 × g for 1 hour to prepare cytosol. The nuclear pellet was resuspended and washed successively in 5 ml of TESH, 5 ml of TESH containing 0.2%, Triton X-100, and 5 ml of TESH. Nuclear binding proteins were extracted at 0° for 40 min in 3 ml of TESH containing 0.4 M KCl. The nuclear pellet was removed by cen-
trifugation and the soluble extract diluted 1:10 with TESH for chromatography on DEAE-cellulose.

Uptake of Cytoplasmic Binding Proteins by Isolated Oviduct Nuclei—For each binding protein uptake assay, nuclei were prepared from 0.5 g of DES-treated chick oviducts. The tissue was homogenized in 1.0 ml of TESH-0.15 M KCl (Polytron PT-10, Rheostat setting 3) by six 5-sec bursts with 1-min cooling periods between bursts, centrifuged 5,000 × g for 5 min, and the nuclear pellet washed twice with 2.0 ml of TESH-0.15 M KCl by resuspension and centrifugation. The nuclear pellet was suspended in a 2.0-ml sample of binding protein preparation abled with $[^3]H$progesterone (30 ng) or resuspended in a blank of TESH-0.15 M KCl containing $[^3]H$progesterone (10 ng) equiv-

alent to the free steroid concentration in the test experiments. After a 20-min incubation at 0°, 30 µg of unlabelled progesterone were added to reduce the nonspecific adsorption of $[^3]H$progesterone. After 10 min the nuclei were centrifuged 5,000 × g for 5 min, washed twice with 3 ml of TESH by centrifugation, and then extracted with 1.0 ml of TESH-0.4 M KCl for 40 min. The

$^1$The abbreviations used are: cytosol, cytoplasmic soluble fraction; DES, diethylstilbestrol; $[^3]H$progesterone, [1,2-$^3$H]progesterone.

$^2$C. A. Strott, unpublished procedure.
preparation was centrifuged at 20,000 × g for 20 min, and the supernatant fraction was saved for sucrose gradient analysis.

Binding of Components to Crude Nuclear Chromatin—After incubation of nuclei with binding proteins, some samples were washed and extracted with TESH containing 0.4 M KCl as described above. Other equivalent nuclear preparations were resuspended after washing in 2 ml of TESH-0.15 M KCl and homogenized with 10 strokes of a teflon-glass homogenizer to break the nuclei. The homogenate was then layered over 3 ml of 1.75 M KCl. The supernatant fraction containing unbroken nuclei and debris was decanted, extracted with dichloromethane, and counted for ³H. The chromatin pellets were resuspended in 1.0 ml of TESH-0.15 M KCl, extracted with dioxane-methanol, and counted for ³H also.

Interaction of Binding Protein with DNA—The progesterone-binding proteins were assayed for their ability to interact with purified DNA by ultracentrifugation of binding protein-DNA mixtures on 5 to 20% sucrose gradients. Since DNA is a macromolecule which rapidly sediments into the lower part of the gradient, interaction between binding proteins and DNA was detected by measuring the disappearance of the normal 4 S binding protein radioactivity peak when DNA (=14 S) was present. Either crude cytosol or binding Components A or B containing 20,000 to 40,000 cpm of bound [³H]progesterone were incubated for 1 hour at 4°C with 100 µg of purified native calf thymus DNA (Sigma) or native highly purified chick DNA in TESH containing 0.1 M KCl. The final reaction volume was 0.5 ml. After incubation, 0.2-ml samples were layered on 5 to 20% sucrose gradients in TESH-0.15 M KCl. The gradients were then centrifuged and handled as described above.

Interaction of Binding Proteins with Chromatin—Binding component preparations or crude cytosol were assayed for their capacity to interact with chromatin by two methods which have been described in detail elsewhere (20, 21). The first method used low speed centrifugation to collect and wash the chromatin after binding. Binding protein preparations (0 to 200 µl) containing up to 10,000 cpm of [³H]progesterone were added to 50 µg (as DNA) of ovotracheal or spleen chromatin in TESH-0.15 M KCl. The reaction was done in plastic tubes with 0.5 mg of bovine serum albumin added. This procedure prevented excess adsorption of purified receptors to the cell walls. The final volume was 0.5 ml. After 1 hour at 0°C the chromatin was pelleted by centrifugation at 2,000 rpm for 5 min in a swinging bucket rotor. The chromatin pellets were resuspended, washed, and recentrifuged first in 0.01 M Tris-HCl-0.15 M NaCl-0.01 M MgCl₂, pH 7.0 (TNM buffer). The chromatin was then collected on Millipore filters (HAWP) previously washed in TNM. Each tube was rinsed twice with TNM and finally each filter was washed with 15 to 20 ml of TNM. The filters were dried in air and counted in 5 ml of Spectrafluor-toluene counting solution. After scintillation counting, each filter was removed from the scintillation cocktail, dried, the DNA hydrolyzed with HClO₄, and DNA assayed by the diphenylamine method. Binding of [³H]progesterone was normalized per mg of DNA and was plotted as a function of the amount of binding protein preparation added.

The method above took advantage of the fact that chromatin in high salt and Mg²⁺ can be sedimented at low speeds in a centrifuge. In order to do the same type of experiments with purified DNA, however, it was necessary to centrifuge the reaction mixture in an ultracentrifuge at 100,000 × g for 18 hours. The DNA or chromatin pellets obtained in this fashion were washed gently with TNM and the buffer discarded. Then the pellets were resuspended in TNM and spot-dried on Millipore filters.

RESULTS

Metabolic Activity in Receptor Component Preparations—Numerous enzymes have been described which metabolize progesterone in reproductive tissues (29–32). The possibility therefore existed that either of the receptor components might be important not as a "receptor" but merely as a metabolic enzyme. To study this, binding protein preparations labeled with [³H]progesterone were assayed for their capacity to metabolize progesterone in vitro, as described under "Methods." Results of these experiments are shown in Table I. As expected these data show that progesterone was metabolized extensively in the postmitochondrial supernatant fraction during preparation of the cytosol at 0°C. Substantial amounts of monols and diols were produced. Further metabolism occurred upon incubation of the cytosol at 37°C (the addition of NADPH was without effect on metabolism; there were sufficient cofactors in the preparations to sustain the reactions).

When receptor Components A and B were assayed after incubation at 0°C, both showed substantially less metabolic breakdown of progesterone than was seen in cytosol, and the two thin layer chromatographic patterns were virtually identical. Over 90% of the tritium bound to the receptors chromatographed as progesterone or its metabolite, 5α-pregnane-3,20-dione although only 70% of the steroid in the cytosol was progesterone. Furthermore, the 37°C incubation of either Component A or B failed to alter the pattern of metabolites seen at 0°C. Thus neither A nor B had detectable enzymatic activity for metabolizing progesterone in vitro. In fact, the receptors may have protected bound hormone from further metabolism.

Isolation of Binding Protein Components Labeled in Vivo
Medium at 0" for 5 min in 10-* M [3H]progesterone. The oviducts of DES-primed chicks incubated in vitro in Basal Eagle's Medium, then transferred to fresh BEM for 30 min at indicated temperature. Total radioactivity in 0.1 M KCl nuclear extract and resultant amounts in A and B component peaks after chromatography of diluted extract on DEAE-cellulose was chromatographed on DEAE-cellulose by stepwise KCl elution, the results of Fig. 1A were obtained.

The appearance of A and B components in nuclei was studied by incubating oviducts at three different temperatures with the results shown in Table II. This table shows that the amount of tritium bound in nuclei after 30 min increased with temperature. In addition, Table II shows that both Components A and B appeared at all three temperatures; the amount of each component increased with increasing temperature.

The first peak (Fractions 2 to 5) contained progesterone not bound to macromolecules. Fractions 11 to 14, eluted with 0.15 M KCl in TESH, contained radioactivity bound to macromolecules identified as binding Component A as described previously (2). At 0.3 M KCl, the peak from Fractions 21 to 25 eluted, corresponding to binding Component B. As was the case for cytosol labeled in vitro, equal amounts of radioactivity were eluted in the A and B peaks, suggesting that they were present in equal amounts.

Association of Receptor Components with Crude Nuclear Constituents—The purified nuclei and nuclei from tissue slices both had shown that oviduct cytoplasmic receptors rapidly became labeled when [3H]progesterone was administered in vivo (1-5). We therefore asked whether both Components A and B were labeled equally by this procedure. If so, it would support our hypothesis that both components are similar and equally important to the early hormone response.

Previous studies had shown that oviduct cytoplasmic receptors could be taken up by oviduct slices in vitro where it ultimately became localized in nuclei bound to nuclear proteins (4, 5). The time course of nuclear uptake in this system lags behind that for cytoplasmic binding, with no nuclear binding activity detectable in the absence of progesterone. Furthermore, the nuclear binding proteins are very similar to those from cytoplasm. Thus, our working hypothesis was that intranuclear binding proteins arise by translocation from the cytoplasm. The appearance of A and B components in nuclei was studied by incubating oviducts at three different temperatures with the results shown in Table II. This table shows that the amount of tritium bound in nuclei after 30 min increased with temperature. In addition, Table II shows that both Components A and B appeared at all three temperatures; the amount of each component increased with increasing temperature.

Uptake of Receptor Components by Isolated Nuclei—Since isolated, purified oviduct nuclei can take up and specifically retain binding proteins from crude cytosol (4, 5), it was of interest to study the appearance of 1000-fold purified receptor Components A and B in nuclei under cell-free conditions.

Nuclei were prepared from oviducts as described under "Methods" and were tested for their ability to take up crude or purified binding components. Fig. 2 shows results of such experiments in which binding components were reextracted from nuclei by high salt treatment and were then assayed by sucrose gradient ultracentrifugation. The figure shows that labeled crude oviduct receptor, but not [3H]progesterone in buffer alone, was taken up and retained by oviduct nuclei in a form which could be re-extracted with 0.4 M KCl.

Significantly, both Component A and Component B were retained by the nuclei. Thus the interaction of both components with intact oviduct nuclei was also seen by this test.

**TABLE II**

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>Total nuclear extract</th>
<th>Component A</th>
<th>Component B</th>
</tr>
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<tr>
<td>0°</td>
<td>6,750</td>
<td>1,371</td>
<td>2,556</td>
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<td>23</td>
<td>9,000</td>
<td>2,825</td>
<td>3,495</td>
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<tr>
<td>37</td>
<td>16,800</td>
<td>5,013</td>
<td>4,123</td>
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</tbody>
</table>

* Tissue (0.5 g each sample) incubated at 0° for 5 min in 10^-8 M [3H]progesterone in Basal Eagle's Medium, then transferred to fresh BEM for 30 min at indicated temperature.

The appearance of A and B components in nuclei was studied by incubating oviducts at three different temperatures with the results shown in Table II. This table shows that the amount of tritium bound in nuclei after 30 min increased with temperature. In addition, Table II shows that both Components A and B appeared at all three temperatures; the amount of each component increased with increasing temperature.

**Fig. 1.** DEAE-cellulose chromatography of oviduct progesterone-binding protein components. A 5.0-ml column of DEAE-cellulose (Whatman DE-52) equilibrated in TESH buffer was used. Each column received 1.0 ml of binding protein preparation (cytosol) labeled with [3H]progesterone. The column was washed first with 30 ml of TESH (Fractions 1 to 10). The column was then eluted stepwise with TESH-0.15 M KCl (Fractions 11 to 20, Component A) followed by TESH-0.3 M KCl (Fractions 21 to 30, Component B). A, column elution profile of oviduct cytosol from an estrogen-primed chick which received 200 pCi of [3H]progesterone in 0.3 ml of 0.9% NaCl 30 min before killing. The oviducts were removed and homogenized in 4 volumes of TESH and then centrifuged at 120,000 X g for 1 hour to prepare cytosol. The cytosol was chromatographed as shown. B, column elution profile of oviduct nuclear extract. The nuclei were obtained from oviducts of DES primed chick incubated in vitro in Basal Eagle's Medium at 0° for 5 min in 10^-8 M [3H]progesterone. The oviducts next were incubated in fresh medium without added steroid for 20 min at 37°. Nuclei were extracted by gentle homogenization in TESH-0.2% Triton X-100. The nuclei were washed twice in TESH and then the binding proteins were extracted with TESH-0.4 M KCl. The extracts were diluted 1: 10 with TESH for DEAE-cellulose chromatography.

**Fig. 2.** Chromatography of cytosol labeled with [3H]progesterone. The oviducts of DES-primed chicks incubated in vitro in Basal Eagle's Medium at 0° for 5 min in 10^-8 M [3H]progesterone. The column was washed first with 30 ml of TESH (Fractions 1 to 10). The column was then eluted stepwise with TESH-0.15 M KCl (Fractions 11 to 20, Component A) followed by TESH-0.3 M KCl (Fractions 21 to 30, Component B). A, column elution profile of oviduct cytosol from an estrogen-primed chick which received 200 pCi of [3H]progesterone in 0.3 ml of 0.9% NaCl 30 min before killing. The oviducts were removed and homogenized in 4 volumes of TESH and then centrifuged at 120,000 X g for 1 hour to prepare cytosol. The cytosol was chromatographed as shown. B, column elution profile of oviduct nuclear extract. The nuclei were obtained from oviducts of DES primed chick incubated in vitro in Basal Eagle's Medium at 0° for 5 min in 10^-8 M [3H]progesterone. The oviducts next were incubated in fresh medium without added steroid for 20 min at 37°. Nuclei were extracted by gentle homogenization in TESH-0.2% Triton X-100. The nuclei were washed twice in TESH and then the binding proteins were extracted with TESH-0.4 M KCl. The extracts were diluted 1: 10 with TESH for DEAE-cellulose chromatography.
from treated nuclei. Results of these experiments are shown in Table III. These data indicate that about one-half of the total radioactivity of washed nuclei remained bound to the crude chromatin during the latter's isolation. This was the case for calf thymus or chick oviduct DNA as well as many other poly-}

were washed during the isolation after hormone uptake. The rapid loss of free hormone (Fig. 2) showed that the washing procedure effectively removed any radioactivity which was weakly associated with nuclear constituents. The presence of both Components A and B in these nuclei thus pointed to the fact that both were tightly held by the nuclei. In this regard we studied the nuclear retention of both components by preparing 0.4 M KC1 extracts and also crude chromatin preparations from treated nuclei. Results of these experiments are shown in Table III. These data indicate that about one-half of the total radioactivity of washed nuclei remained bound to the crude chromatin during the latter's isolation. This was the case for both A and B, suggesting that they were both chromatin-bound in this procedure. A 0.1 M KC1 extract of similarly treated nuclei showed again the same degree of extraction for both A and B. Thus, radioactive hormone-binding protein complexes of both A and B type appeared to be tightly bound in some way to chromatin.

Interaction of Component A with DNA—The nuclear binding proteins could be extracted by treatment with high KC1 concentrations. This suggested an interaction with some intranuclear constituent. In order to test for such an interaction, the DNA binding assay described under “Methods” was used. Calf thymus or chick oviduct DNA, as well as many other polydeoxyribonucleotides, will interact with uterine estrogen receptors (22). This phenomenon can be detected by sucrose gradient ultracentrifugation in which the binding protein-hormone complex normally sediments as a sharp peak. When DNA is added to the receptor preparation before ultracentrifugation, however, the binding protein-hormone complex sediments rapidly to the bottom of the tube along with the DNA. This assay was used to measure the binding of cytoplasmic Components A and B to DNA. As shown in Fig. 3A, Component A sediments as a sharp 4 S peak on sucrose gradients containing 0.1 M KC1. When DNA was added to the preparation, the 4 S peak was eliminated and radioactivity appeared throughout the bottom half of the tube. This pattern occurred when either calf thymus or chick oviduct DNA was used, and thus was not dependent upon homologous DNA. By this criterion, then, Component A appears able to interact with DNA.

When binding Component B was tested in this way, the results of Fig. 3B were obtained. In this case the addition of DNA was

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Receptor-bound amount applied (X 10^-9)</th>
<th>Amount in washed nuclear pellet (X 10^-9)</th>
<th>Amount in crude chromatin (X 10^-9)</th>
<th>Amount in KC1 extract (X 10^-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.0</td>
<td>40.5</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>B</td>
<td>1.1</td>
<td>17.3</td>
<td>8.4</td>
<td>7.5</td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.9</td>
<td>4.2</td>
<td>1.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Total hormone incubated with nuclei from 0.5 g of oviduct.
* After incubation for 30 min at 0°C, two washes with TESH.
* Total hormone in chromatin pellet from broken nuclei by sedimentation of chromatin through 1.75 M sucrose.
* Total hormone extracted from nuclei by 0.4 M KC1 in parallel experiment.
was centrifuged, collected on Millipore filters, washed, and counted for 3H. The data of Fig. 4 are corrected for the slight binding by [3H]progesterone per ml of bovine serum albumin. After 1 hour at 0° the chromatin was centrifuged, collected on Millipore filters, washed, and assayed for [3H] and DNA. A, chromatin collected by low speed centrifugation from 0.5-ml reaction volume. Component A reacted with oviduct (O-O) or spleen (A-A) chromatin. Component B reacted with oviduct (● ● ) or spleen ( △ △) chromatin. B, chromatin collected by 20 hour ultracentrifugation at 120,000 χ g from 2.0-ml reaction volume. Component A reacted with oviduct ( ●-● ) or spleen ( Δ-Δ) chromatin without effect, and Component B was judged unable to interact with purified DNA in the same manner as Component A.

Interaction of Component B with Chromatin—Other studies in this laboratory had shown that the purified chromatin of target tissues contains saturable sites capable of binding receptor-hormone complexes. An in vitro cell-free assay for this binding (20) was used to study further the interaction of the binding components with oviduct chromatins. Oviduct chromatin, mixed with a binding protein preparation in 0.15 M KCl, was allowed to stand for 1 hour at 0°. Then the chromatin was centrifuged, washed free of unbound proteins, and counted for 3H. The data of Fig. 4 are corrected for the slight binding by [3H]progesterone alone, and show that Component A does not bind to purified chromatin from either oviduct or spleen, although the binding protein is a stable 4 S molecule under these assay conditions. When Component B was tested for binding to chromatin, there was substantial binding to oviduct chromatin (Fig. 4). There was much less binding of Component B to spleen chromatin.

In order to confirm this binding under conditions which approach saturation, the ultracentrifugation technique described under "Methods" and shown in Fig. 1B was used. Here Component B again bound much more extensively to oviduct chromatin than to spleen chromatin.

Thus, the tissue specificity for attachment of crude cytoplasmic binding proteins to target cell chromatin may be attributed to the B component, which binds tightly enough to remain associated with chromatin after 18 hours of centrifugation.

**DISCUSSION**

Other studies have determined the capacities of crude hormone receptor preparations to interact with nuclei (12-15, 23, 24, 33, 34), chromatin (20, 21, 28), and DNA (22, 35) and found them to be similar in several different systems. In fact, target tissue steroid-receptor systems thus far defined suggest that the steps of hormone transfer to nuclear sites in vitro may indeed be representative of the actual events of steroid action in vivo.

As further analysis of the elements of the hormone response, we recently demonstrated (2) that the crude cytoplasmic progesterone-receptor system consisted of two 4 S components, A and B. Both components are physically and chemically quite similar but are not interconvertible. Their steroid-binding characteristics and behavior during gel filtration and sucrose gradient centrifugation are identical. The major physical difference between them is the diminished stability of Component A in buffers of low ionic strength.

The existence of these components suggested that the multiplicity of receptor forms might be explained as resulting from combinations of these two components. It is unlikely that one component is a precursor of the other since they both appear in vitro and in vivo in equal amounts. However, the possibility that the two components are linked in vivo and separated during the purification procedure can be neither proven nor ruled out at this juncture.

These characteristics of the two progesterone-receptor components suggested that they both might be involved in the hormone response. It was thus of interest to determine whether the diverse capacities of the crude cytoplasmic receptors to interact with components of nuclei had also been resolved by the separation of A and B. The present study in fact shows that the separate nuclear interactions previously found with crude binding proteins can be assigned to the two distinct receptor components, A and B. Although both components appear tightly bound to chromatin in oviduct nuclei, their activities can be resolved in more purified systems. Like crude cytosol receptor, Component A binds to DNA during sucrose gradient centrifugation but Component B does not. Conversely, Component B binds to oviduct chromatin in vitro but Component A does not. Furthermore, this binding to chromatin retains the requirements for oviduct chromatin proteins and retains the tissue specificity of the crude receptor. We have recently demonstrated that the specificity for interaction of crude progesterone-receptor complex with oviduct chromatin under these conditions resides in a particular subtraction of the chromatin acidic (nonhistone) proteins (18, 19).

Since Component A binds to pure DNA (Fig. 3) and appears bound to crude chromatin from isolated nuclei (Table III), it is somewhat puzzling to find that this component does not bind to purified oviduct chromatin which contains about 4% exposed DNA template for bacterial RNA polymerase (20). One might therefore expect these regions to be accessible to Component A. We are presently unable to resolve this problem adequately. It should be pointed out, however, that the in vitro chromatin-binding assay involved vigorous washing procedures which might simply wash Component A off its chromatin-binding sites if it was bound less tightly than Component B. In addition, redistribution of nuclear proteins during chromatin isolation and purification may have obscured previously accessible Component A-binding sites. Finally it may be that Component A does indeed bind to the open DNA regions of the purified chromatins. However the amount of open DNA present in the chromatin-binding assay is 50 times less than in the DNA-binding experiments. Therefore Component A binding to purified chromatin may be simply below the limits of detectability for the assay.

Since either component can undergo a respective nuclear inter-
action in the absence of the other, the two components apparently can bind to their substrates independently. Whether such independence is sufficient for a functional hormone response in vitro or whether the A- and B-receptor subunits act in concert remains an open question. It is still conceivable that the differential affinities of the A and B components for DNA and chromatin proteins, respectively, may be resultants of their separate physicochemical properties but unrelated to the functional hormone response in vitro. The exact relationship between the purified subunits and the various crude cytoplasmic forms remains uncertain. However, the crude complexes can now be treated as combinations of two receptor components with potentially different nuclear binding functions. Furthermore, the fact that receptor components can be extracted from target tissue nuclei which are chromatographically and functionally similar to those present in cytoplasm lends further support to the concept that steroid hormones are transported into nuclei bound to proteins of cytoplasmic origin. The possibility exists that these two receptor components could play separate roles during the initial interaction of the hormone complex with chromatin-acceptor sites in the target cell. Component B may specifically bind to chromatin-acceptor proteins while Component A simultaneously or sequentially interacts with the DNA itself. At present, intact oviduct progesterone receptors or components have not yet been shown to have direct effects on chromatin transcription processes in vitro. However, recent studies from at least three laboratories (36–38), have implicated specific receptor forms in the stimulation of RNA synthesis in intact nuclei under cell-free conditions. If the hormone-receptor complex is indeed the inducer unit for steroid hormone modulation of nuclear transcription of DNA then this differential binding of receptor components to the genome may prove to be of major importance to our understanding of steroid hormone action.

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REFERENCES

3. O'Malley, B. W., and Schrader, W. T. J. Steroid Biochem. in press
19. Spelsberg, T. C., Stegglees, A. W., Chytli, F., and O'Malley, B. W. J. Biol. Chem. in press