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THE INTERACTION OF UTERINE ESTROGEN RECEPTORS WITH DNA*

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SUMMARY

Recent studies indicate that estrogen-receptor complexes in the uterus migrate to the cell nucleus and attach to chromatin 'acceptor' sites. As an initial attempt to describe this event, the association of estrogen receptors with purified DNA was investigated. Receptor complexes extracted from both rat and calf uteri were reacted with DNA and analyzed by sucrose gradient centrifugation. Both cytoplasmic and nuclear receptors can bind to DNA with an affinity sufficient to withstand centrifugation through sucrose gradients in the presence of 0·1 M KCl. However, the complex is dissociated with higher salt concentrations. Since several types of DNA are effective, this interaction does not completely explain the properties expected of chromatin acceptor sites. However, these results suggest that a receptor–DNA interaction may contribute to the chromatin acceptor site or may function in events which follow the attachment of receptor to chromatin.

INTRODUCTION

THE BINDING of estrogen to specific proteins in the uterus is now well established. In uterine cells, [^{3}H]-estradiol is localized in both cytoplasmic and nuclear compartments[1, 2], and specific estrogen binding proteins have been identified in both subcellular fractions (see [3] and [4] for review). The significance of these binding components has been defined through the temporal analysis of nuclear and cytoplasmic receptors following the administration of estradiol, either *in vivo* [5, 6] or during tissue incubation[7–9]. These studies indicate that, after its formation in the cell, the cytoplasmic hormone-receptor complex is transferred to the nucleus where it is associated with chromatin material. Comparable results have now been reported for the receptors of progesterone in the chick oviduct[10] and for androgens in the ventral prostate[11]. That the chromatin of target tissues contains specific acceptor sites for the attachment of receptors is indicated from the cell-free binding of receptors to isolated nuclei or chromatin[10, 12–15].

Little is known about the mode of interaction between estrogen receptors and chromatin or about the molecular components that are involved in this process. As an initial attempt to describe the mechanism of chromatin attachment, the interaction of estrogen receptors with purified DNA was investigated. In this report, the binding of both nuclear and cytoplasmic receptor proteins to DNA is demonstrated, and the possible involvement of this interaction in the attachment of receptors to chromatin is discussed.

EXPERIMENTAL

Cytosol fractions

Uteri from immature, 20–23 day old Holtzman rats were rinsed in 0·9% NaCl and homogenized (Polytron #PT-10, Brinkman) in 0·05 M Tris-HCl pH 7·5, 0·01 M thioglycerol and 1 mM EDTA (buffer A), 5 uteri/ml. The homogenate was

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centrifuged for 10 min at 15,000 *g* and then for 1 h at 114,000 *g* to obtain the high-speed supernatant or cytosol fraction.

Fresh calf uteri (Neuhoff Packing Co., Nashville) were minced, rinsed in 0·9% NaCl and homogenized in 4 volumes of buffer A containing 0·01 M KCl, using first a Waring blender and then a Polytron homogenizer. The cytosol was obtained by centrifugation, as given above.

Cytosol fractionation with ammonium sulfate

Two methods of fractionation were utilized based on the procedures of DeSombre *et al.* [16, 26]. To obtain stable 4S receptor, 30 ml of calf uterine cytosol containing 5×10^{-8} M [^{3}H]-estradiol (New England Nuclear, 40 Ci/mmol), 4 mM $CaCl_2$ and 1·0 M KCl was incubated for 1 h at 4°C. An ammonium sulfate solution, pH 7·5, saturated at 4°C, was then added slowly with stirring to produce a fractional saturation of 20 per cent. After 30 min, the precipitate was recovered by centrifugation (10 min, 15,000 *g*) and dissolved in 3 ml of solution containing 0·1 M Tris-HCl, pH 7·5, 1 mM $CaCl_2$, 0·01 M thioglycerol and 0·4 M KCl.

To obtain 8S receptor, 30 ml of cytosol containing 5×10^{-8} M [^{3}H]-estradiol and 4 mM $CaCl_2$ was incubated at 4°C for 1 h. Saturated ammonium sulfate solution was then added slowly to produce a fractional saturation of 30 per cent. After 30 min, the precipitate was recovered by centrifugation and dissolved in 3 ml of solution containing 0·1 M Tris-HCl, pH 7·5, 0·01 M KCl, 0·01 M thioglycerol and 1 mM EDTA.

Nuclear receptor

Following the methods of Harris [17], rat uteri were incubated in basal Eagle's medium containing [^{3}H]-estradiol (0·05 μg/2 ml) at 4°C for 5 min. The uteri were then transferred to medium without estradiol and incubated for 20 min at 37°C in an atmosphere of 95% O_2, 5% CO_2. The uteri were then rinsed in saline and homogenized in 0·01 M Tris-HCl, pH 7·5, 10% glycerol and 0·02 M thioglycerol (5 uteri/ml). The nuclear-myofibrillar fraction was obtained by centrifugation (800 *g*, 10 min) and the pellet was resuspended and centrifuged twice in the same buffer containing 20% glycerol. The final pellet was suspended in 0·01 M Tris-HCl buffer, pH 7·5 containing 0·1 M $(NH_4)_2$ SO_4, 20% glycerol, 0·03 M thioglycerol plus pancreatic deoxyribonuclease (DNase) (20 μg/ml, Worthington, electrophoretically pure). After incubation for 30 min at 4°C, 1/100 volume of 0·1 M EDTA was added to inactivate the DNase and the particulate material was removed by centrifugation (15,000 *g*, 10 min). The supernatant fraction containing approximately 60 per cent of the nuclear-bound estradiol was then used to determine DNA binding.

DNA

Highly purified rat spleen and salmon sperm DNA were prepared by the methods of Marmur [18] with additional ribonuclease and pronase treatments to give preparations that contained less than 0·5 per cent contamination with RNA or protein. Calf thymus DNA (Sigma, type 1) was used without further purification.

DNA binding assay

Specific techniques are listed in figure legends. In general, cytosol fractions (0·4–0·5 ml) containing 100–200 μg DNA were incubated for 1 h at 4°C. Ali-

quots (0·2 ml) were layered onto 5–20% sucrose gradients containing 0·01 M Tris, pH 7·5, 1 mM EDTA, 0·01 M thioglycerol (buffer B), with or without the addition of KCl. After centrifugation (16 h, 40,000 rev./min, Spinco SW 50.1 rotor), 20 fractions (0·25 ml) were collected by piercing the tube bottom. The tube bottom containing the DNA pellet was cut off and placed in scintillation counting fluid to measure bound [^{3}H]-estradiol. The 8S region indicated in the figures was estimated using cytosol 8S receptor or bovine serum albumin as sedimentation standards. All radioactivity was measured by liquid scintillation using a 5 ml cocktail containing toluene: Triton X-100: spectrafluor-PPO:POPOP (Amersham–Searle): H_2O in proportions of 24 : 12 : 1 : 3·6. Counting efficiency was 35 per cent.

RESULTS

The binding of the '8S' estrogen receptor to purified DNA was detected by analyzing the sedimentation of bound [^{3}H]-estradiol during high speed centrifugation on sucrose gradients. Figure 1 illustrates the effect of purified rat spleen DNA on the [^{3}H]-estradiol bound in rat uterine cytosol. In the presence of the added DNA, the 8S receptor complex is carried to the gradient bottom, apparently bound to the DNA pellet.

To assure that these observations represent an interaction with DNA, the effect of deoxyribonuclease treatment was studied. DNA which had been hydolyzed with deoxyribonuclease did not alter the sedimentation of bound estra-

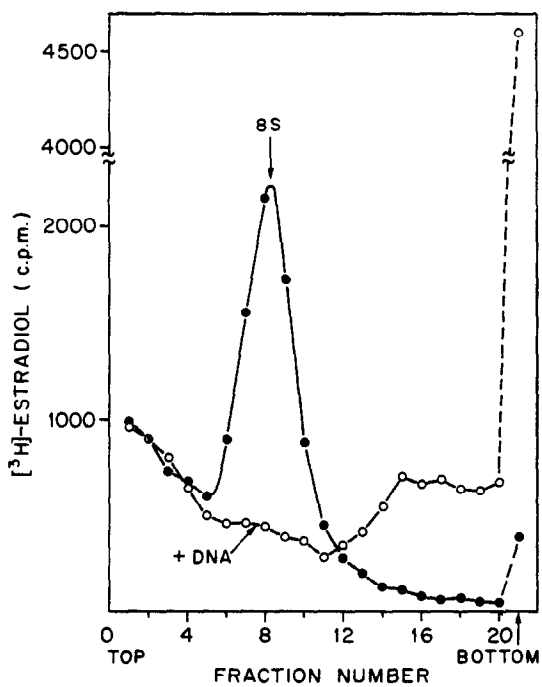

Fig. 1. The effect of DNA on the sedimentation of bound [^{3}H]-estradiol in sucrose gradients. Rat uterine cytosol containing 10^{-8} M [^{3}H]-estradiol was incubated in ice for 1 h with (open circles) or without (closed circles) the addition of rat spleen DNA (340 μg/ml cytosol). Samples (0·2 ml) were layered on 4·8 ml gradients of 5–20 per cent sucrose in buffer B plus 0·05 M KCl. These were centrifuged for 16 h at 114,000 *g*.

diol, as illustrated in Fig. 2. Also evident from this figure is the binding of rat uterine receptor to calf thymus DNA. Comparable results were obtained using purified DNA from salmon sperm, indicating that, with present methods, preference of the receptor for the homologous DNA is not apparent.

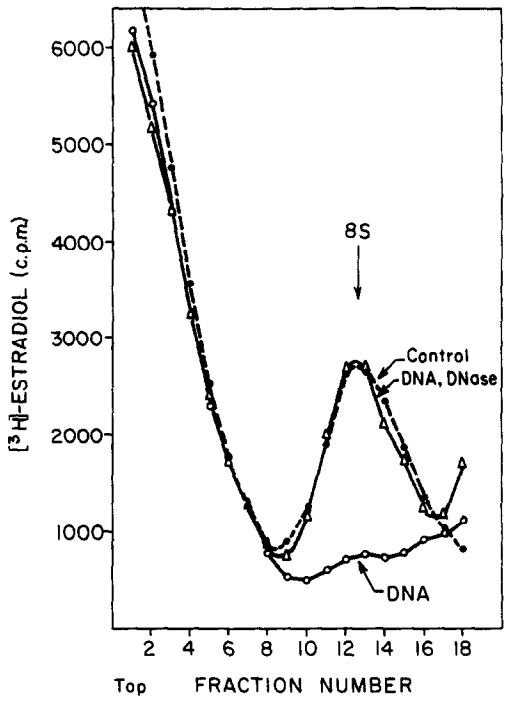

Fig. 2. The effect of DNase treatment of the binding of estrogen receptor to DNA. Aliquots (0·3 ml) of rat uterine cytosol containing [^{3}H]-estradiol were incubated in ice for 1 h following the addition of 0·1 ml of H_2O (broken line), calf thymus DNA (100 μg, open circles) or the same amount of DNA which had been previously treated with DNase (25 μg/ml for 30 min at 37°C) (triangles). Samples (0·2 ml) were layered on 5–20 per cent sucrose gradients in buffer B and were centrifuged for 16 h at 149,000 g.

Figure 3 demonstrates the dependency of DNA binding upon ionic strength. In this experiment, aliquots of rat uterine cytosol containing [^{3}H]-estradiol were incubated with various concentrations of KCl in the presence of rat spleen DNA. Samples were then analyzed on sucrose gradients having the corresponding KCl concentration. DNA binding was determined by the reduction of [^{3}H]-estradiol in the 8S region. As shown in Fig. 3, the DNA-receptor complex is stable at lower ionic strengths and is actually facilitated somewhat by increasing the KCl concentration to 0·1 M. This increase may represent a higher affinity of the receptor for DNA in 0·1 M KCl or it may be caused by a decrease in less specific, but interfering, interactions such as the absorption of other cytosol protein to DNA, or the association of receptors to polyribonucleotides in the cytosol. In any event, this stimulatory effect of ionic strength is not apparent when partially purified receptor preparations are used (for example, see Fig. 5, A and B). KCl concentrations above 0·1 M decreased the binding of receptor to DNA with a concomitant appearance of bound estradiol in the 4S region of sucrose gradients. It is interesting to note that salt concentrations which dissociate the DNA-receptor

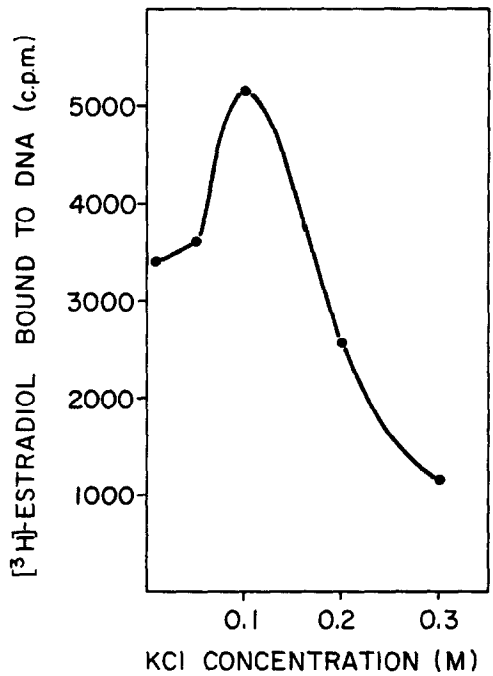

Fig. 3. The effect of ionic strength on the binding of 8S receptor to DNA. Fractions of rat uterine cytosol containing [³H]-estradiol, rat spleen DNA (240 μg/ml cytosol) and KCl as indicated were incubated for 1 h in ice. Samples (0·2 ml) were layered on 5–20 per cent sucrose gradients in buffer B containing the corresponding KCl concentration and were centrifuged for 16 h at 149,000 *g*. The binding of receptor to DNA was determined by comparing the amount of [³H]-estradiol bound in the 8S or 4S region to a control gradient of cytosol containing no DNA and 0·01 M KCl. In the control gradient, 70 per cent of the [³H]-estradiol was bound in the 8S region and the remaining radioactivity was unbound.

complex are comparable to concentrations which convert the 8S receptor to a 4S form [19–21] and are also comparable to concentrations needed to extract bound estrogen from nuclei [22, 23].

Since the nuclear receptor is the form which attaches to chromatin in the cell, an attempt was made to test the binding of this component to purified DNA. Recent studies indicate that the cytosol receptor undergoes slight structural modification upon entering the nucleus to a form which sediments somewhat faster in sucrose gradients containing 0·3 M KCl (≃ 5S or 6S, depending upon experimental conditions [6, 24]). The binding of the '5S' receptor to DNA has been difficult to test since this form tends to aggregate under conditions of low ionic strength. A recent method described by Harris [17] has therefore been used. After uteri were incubated with [³H]-estradiol, nuclei were isolated and then incubated with deoxyribonuclease under proper ionic conditions. A hormone-receptor complex was released which then sedimented at ≃ 8S in sucrose gradients without KCl. This nuclear receptor preparation was tested for DNA binding activity after DNase had been inactivated by EDTA. As illustrated in Fig. 4, this receptor form readily binds to DNA.

The binding of 8S cytosol receptor from calf uteri to DNA was found to be comparable to that of rat uteri. DeSombre, Puca and Jensen have described the isolation

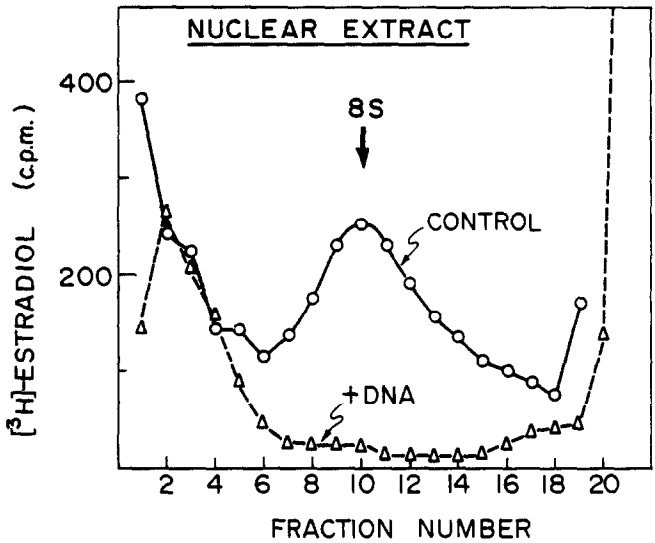

Fig. 4. The binding of nuclear '8S' receptor to DNA. [^{3}H]-estradiol-receptor complex was extracted from rat uterine nuclei by DNase treatment (see Experimental). Aliquots were incubated for 1 h in ice with or without the addition of calf thymus DNA (110 μg/ml). Samples (0·2 ml) were layered on 5–20 per cent sucrose gradients containing 10 per cent glycerol and buffer B. These were centrifuged for 15 h at 230,000 g.

and purification of both 4S and 8S receptor forms from calf uteri[16, 26]. Using their methods, two receptor forms were prepared from the cytosol using ammonium sulfate precipitation. When the 8S component was prepared by precipitation in 30 per cent ammonium sulfate, the binding of this receptor to DNA was apparent, even in the presence of 0·1 M KCl, where most of the receptor assumed a 4S form (Fig. 5, A and B).

With Ca^{2+} as a stabilizing agent during the ammonium sulfate treatment, a receptor component which sediments at 4S under both high and low ionic conditions can be prepared[16, 26]. In contrast to the foregoing results, this receptor form showed no tendency to bind DNA (Fig. 5, C and D) when tested in either 0·01 M or 0·1 M KCl.

DISCUSSION

The binding of uterine estrogen receptor to DNA has been demonstrated. While the methods employed do not readily allow quantitative measurements of the extent or affinity of this interaction, the binding is apparently quite strong, since the complex formed can withstand centrifugation through sucrose gradients under ionic conditions up to 0·1 M KCl.

Since the estrogen receptor from rat uteri can interact with DNA from calf thymus and salmon sperm, apparently DNA homology is not required. Also, while native DNA has been used throughout this report, similar receptor–DNA interactions were found using heat-denatured DNA. In recent experiments, not shown here, binding of the receptor to DNA from *Escherichia coli* and *Bacillus subtilis* and to transfer RNA from *E. coli* has been observed. However, preliminary results indicate that these latter reactions occur well only under very low ionic conditions and are much diminished in the presence of 0·05–0·1 M KCl.

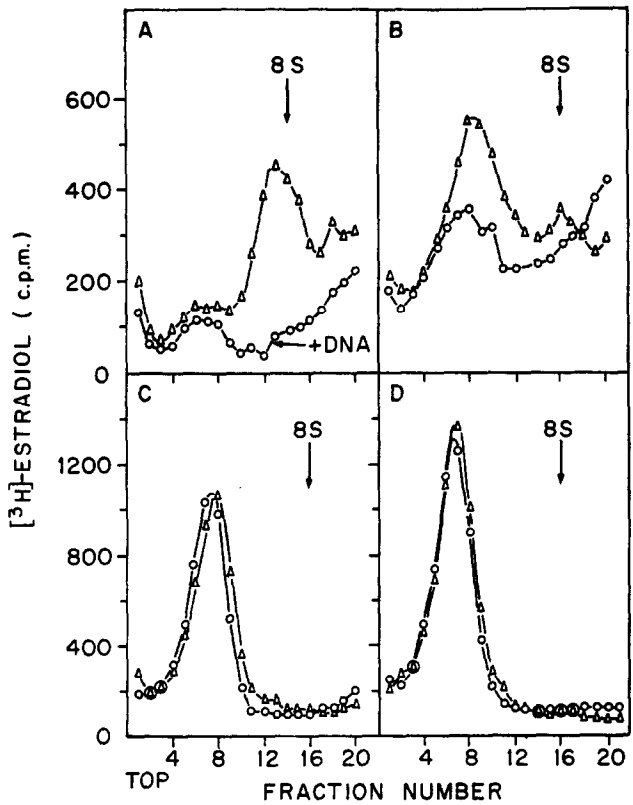

Fig. 5. The binding of receptor preparations from calf uteri to DNA. Two receptor forms (8S and 'stable' 4S) were prepared from calf uterine cytosol by ammonium sulfate precipitation (See Experimental). Aliquots of these preparations were adjusted to contain ≈ 75,000 c.p.m./ml of bound [^{3}H]-estradiol and 0·1 M KCl. These were then incubated for 1 h in ice with (triangles) or without (circles) the addition of calf thymus DNA (500 μg/ml). Samples (0·2 ml) were layered on 5–20 per cent sucrose gradients containing buffer B plus 0·01 M or 0·1 M KCl and centrifuged for 16 h at 149,000 *g*. A. 8S preparation with 0·01 M KCl. B. 8S preparation with 0·1 M KCl. C. 4S preparation with 0·01 M KCl. D. 4S preparation with 0·1 M KCl.

Attempts to quantitatively compare the acceptor capacities of various polynucleic acids are now in progress.

Both cytosol and nuclear receptor forms are able to bind DNA. However, the calcium-stabilized 4S receptor prepared by the method of DeSombre, Puca and Jensen has lost this ability. This 4S component is relatively stable and has been extensively purified and characterized[16, 26]. That this binding component is derived from the cytosol 8S estrogen receptor seems most probable. However, the inability of this substance to bind DNA suggests that some physicochemical modification has occurred, or that other factors necessary for the DNA interaction have been lost during the isolation procedure. Alternatively, DNA binding may be a capacity of a yet unidentified subunit of the 8S complex, not the estrogen-binding component. These possibilities are being investigated further and will hopefully aid in understanding the biological significance of the interaction with DNA.

It is clear that the receptor–DNA interaction reported here could not fully

explain the attachment of estrogen receptors to chromatin in the cell nucleus. Studies on the binding of receptors to uterine nuclei [12] and to isolated chromatin [15] indicate that uterine chromatin contains specific acceptor sites for the receptor which are unique to the target tissue. Similar results have been obtained with the progesterone receptor of chick oviducts [10, 14] and with the androgen receptor in ventral prostate [13, 14]. Spelsberg *et al.* [25] have studied the specific attachment of chick oviduct progesterone receptors to oviduct chromatin. Their work demonstrates the involvement of nuclear acidic proteins in determining the tissue-selective binding of receptor to chromatin. In agreement with their findings, the results presented here demonstrate a lack of tissue specificity in the binding to purified DNA. This, of course, is to be expected if one adheres to the classical concepts of tissue development from a homogeneous DNA population. On the other hand, the ready association of receptors with DNA indicates a possible involvement of this interaction either in the initial binding to chromatin or in the functional processes subsequent to chromatin attachment.

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