The binding of [^3H]oestradiol–receptor complexes to calf uterine chromatin

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Various aspects of the interaction of oestrogen–receptor complexes with calf uterine chromatin covalently coupled to cellulose were analysed. Partially purified [^3H]oestradiol–receptor complexes were bound to intact, or partially deproteinized, chromatin resins. Proteins were removed from the chromatin–cellulose resins by extraction with high molarities of salt, including NaCl/urea, guanidine hydrochloride and guanidine thiocyanate. After extensive washing to remove the salt, [^3H]oestradiol–receptor–complex solutions were added to the resins and the degree of binding was determined. The extent of [^3H]oestradiol–receptor–complex binding to chromatin was enhanced by extraction of chromosomal proteins. By varying the molarity of the salt, and consequently the extent of protein removal, it was possible to resolve [^3H]oestradiol–receptor–complex binding to guanidine thiocyanate-extracted chromatin into two components. Similarly, [^3H]oestradiol–receptor–complex binding to guanidine hydrochloride-treated chromatin included three regions of enhanced binding capacity. The [^3H]oestradiol–receptor–chromatin interaction was saturable with respect to both intact and salt-extracted resins. Thus uterine chromatin may contain three or more specific classes of acceptors for the oestrogen–receptor complex.

For many years the hypothesis that steroid-hormone receptors interact specifically with a component of the nucleus has been included in the schematic representation of steroid-hormone action (Liao & Fang, 1969; O'Malley & Means, 1974; Gorski & Gannon, 1976). Early data favoured this general concept; however, the precise nature of the acceptor region has been difficult to determine. More recent efforts probing the nuclear site of action of steroid hormones have suggested the acidic-protein fraction of chromatin as being important in steroid-hormone–receptor interactions (Spelsberg *et al.*, 1979).

The initial binding by oestrogen to its receptor is characterized by specificity, high affinity and saturability; presumably binding to specific regions in the nucleus is regulated by similar parameters. Thus the nucleus represents a second level of specificity. O'Malley *et al.* (1972) reported that progesterone–receptor complexes interact specifically with an acidic protein fraction of chick oviduct chromatin, suggesting that non-histone proteins constitute the nuclear site of action of steroid-hormone–receptor complexes. Subsequently, the binding activity of this

non-histone protein fraction was dissociated from isolated chromatin by 7M-GuHCl (Thrall *et al.*, 1978). When these chromosomal proteins were separated by isoelectrofocusing and selectively re-annealed to DNA, the ability of the progesterone receptor to bind was not limited to a single protein. Rather, three distinct sets of acidic protein–DNA complexes exhibited acceptor activity. These reports from cell-free systems are consistent with other studies which suggest the presence of at least two distinct nuclear binding sites for steroid-hormone receptors (Ruh *et al.*, 1979; Clark *et al.*, 1976; Markaverich & Clark, 1979).

The most extensive work on nuclear acceptors has been performed by Spelsberg and co-workers (Spelsberg *et al.*, 1975; Webster *et al.*, 1976; Pikler *et al.*, 1976; Spelsberg *et al.*, 1979), using the chick progesterone receptor. Other steroid-hormone systems are currently under investigation (Klyzsejko-Stefanowicz *et al.*, 1976; Hamana & Iwai, 1978; Perry & Lopez, 1978; Weinberger & Veneziale, 1980; Taylor *et al.*, 1980). Therefore our studies were initiated to explore the following possibilities: (1) acidic non-histone proteins may serve as nuclear acceptors for the oestrogen–receptor complex in a mammalian species; (2) the interaction of the oestrogen receptor and chromatin may be specific;

Abbreviations used: GuHCl, guanidine hydrochloride; GuSCN, guanidine thiocyanate.

and (3) more than one species of oestrogen-receptor acceptor site may exist. Our approach utilized a cell-free system which allowed direct analysis of oestrogen-receptor-chromatin binding.

Materials and methods

Receptor preparation

Calf uteri were collected fresh at a local slaughterhouse and immediately placed in ice-cold Tris/HCl buffer (10 mM, pH 7.5). All procedures were performed at 4°C unless otherwise indicated. After vessels and connective tissue had been dissected free, the tissue was minced in a meat grinder and gently homogenized in a blender at 4°C in 2 vol. of TED buffer (50 mM-Tris/HCl/1.5 mM- Na_2EDTA /1 mM-dithiothreitol/5% glycerol), pH 7.5. The homogenate was centrifuged at 130 000 g (Beckman SW 27 rotor) for 60 min to yield a receptor-containing supernatant. Cytosol subsequently was diluted with TED buffer to 10 mg of protein/ml. At this time cytosol was charged with [^3H]oestradiol (46–50 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.) to a final concentration of 20 nM during a 60 min incubation at 4°C. Charged cytosol was then either precipitated with $(\text{NH}_4)_2\text{SO}_4$, or more extensively purified by heparin-Sepharose affinity binding before $(\text{NH}_4)_2\text{SO}_4$ precipitation. To precipitate the receptor complexes, finely powdered $(\text{NH}_4)_2\text{SO}_4$ was slowly added to give 25% saturation while the pH was maintained at 7.5. After incubation for 60 min at 4°C, cytosol was centrifuged at 27 000 g (DuPont Instruments-Sorvall HB-4 rotor) for 30 min. Receptor pellets were stored at –70°C. In the more extensive receptor-purification step, heparin-Sepharose, which had been prepared by CNBr activation (Cuatrecasas, 1970), was incubated with charged cytosol (150 mg of resin/ml of cytosol) for 60 min at 4°C (Sica & Bresciani, 1979). Four washings with TED buffer (5 vol. each) on a sintered-glass Buchner funnel eliminated free protein and unbound radiolabelled ligand. The heparin-Sepharose was incubated with 5 vol. of TED buffer/0.4 M-KCl for 30 min to extract non-receptor proteins. This slurry was centrifuged at 1000 g for 5 min and the heparin-Sepharose pellet was resuspended in 5 vol. of TED buffer/0.4 M-KCl for 10 min and centrifuged again. The heparin-Sepharose pellet was resuspended in 5 vol. of TED buffer/0.9 M-KCl for 60 min to extract the oestrogen receptor (Molinari *et al.*, 1977). The extract obtained after centrifugation was treated with $(\text{NH}_4)_2\text{SO}_4$ as described above; pellets were stored at –70°C until the day of the experiment. Several hours before an experiment, receptor pellets were redissolved in TESH buffer (10 mM-Tris/HCl/1 mM- Na_2EDTA /12 mM-monothioglycerol), pH 7.5. Receptor solutions were dialysed against a minimum of 100 vol.

of TESH buffer at 4°C for 3 h; dialysed solutions were centrifuged at 100 000 g (HB-4 rotor) for 10 min. These supernatants were assayed for protein content and radioactivity. The receptor solution was diluted with TE buffer (2 mM-Tris/HCl/0.1 mM- Na_2EDTA), pH 7.5, which contained KCl to give the desired incubation concentrations of proteins and salt.

Chromatin-cellulose preparation

The procedures for purifying nuclei and chromatin and preparing chromatin-cellulose follow the general method of Spelsberg *et al.* (1978). Calf uteri, or spleens as a non-target tissue, were minced in a meat grinder and gently homogenized in a blender in 2 vol. of 0.5 M-sucrose/TKM buffer (50 mM-Tris/HCl/25 mM-KCl/2 mM- MgCl_2), pH 7.5. This initial homogenate was diluted with 0.5 M-sucrose/TKM to a buffer/tissue ratio of 3:1 (v/w) before homogenization in a Glenco glass/Teflon homogenizer. After being strained through dampened cheesecloth, the homogenate was centrifuged at 27 000 g (GSA rotor, DuPont Instruments-Sorvall) for 5 min. Pellets were pooled, homogenized in 9 vol. of sucrose/TKM to a final concentration of 2.3 M-sucrose (2.2 M-sucrose for spleen pellets) and re-centrifuged at 27 000 g for 60 min. The pellets were resuspended in the respective sucrose/TKM solution and re-centrifuged at 27 000 g for 60 min to yield a nuclear pellet. Nuclei were resuspended with a glass/Teflon homogenizer in TKM buffer containing 0.2% Triton X-100. After filtration through organza cloth, the nuclear solution was centrifuged at 27 000 g for 5 min.

Purified nuclei were suspended in 80 mM-NaCl/20 mM- Na_2EDTA , pH 6.3, with a glass/Teflon homogenizer before homogenization in a Dounce glass/glass homogenizer. Pellets were collected after a 5 min centrifugation at 27 000 g (HB-4 rotor) and resuspended in 0.35 M-NaCl. A second 5 min centrifugation (27 000 g) yielded pellets which subsequently were placed in TE buffer and strained through organza cloth. This filtrate was left on ice for 15 min before being centrifuged at 27 000 g for 10 min. After this spin, the chromatin pellets were combined, rehomogenized in TE buffer, and stored at –70°C. Yields of chromatin were calculated as mg of DNA determined by the diphenylamine procedure of Burton (1956). Before and after coupling to cellulose, protein/DNA ratios were ascertained for each batch of chromatin. Total protein was determined by hydrolysis for 30 min in 1 M-NaOH at 22°C. Histones were removed with 0.2 M- H_2SO_4 for 30 min at 4°C; non-histone proteins were stripped with 0.1 M-NaOH at 22°C for 30 min (Spelsberg *et al.*, 1978). Protein was determined with the Bio-Rad Protein Assay (Bradford, 1976).

Cellex 410 (Bio-Rad Laboratories, Richmond,

CA, U.S.A.) was washed three times with boiling ethanol and once with each of the following: 0.1M-NaOH, 1mM- Na_2EDTA and 10mM-HCl (Spelsberg *et al.*, 1978). After extensive washes with distilled water the cellulose was air-dried. Washed cellulose was hydrated at 4°C for 45min in TE buffer (25g/100ml). Resuspended chromatin was added to the cellulose solution and swirled intermittently for 2h. The chromatin-cellulose slurry was adjusted to 0.1M-KCl and centrifuged at 7500g (HB-4 rotor) for 10min. Chromatin-cellulose was incubated in ethanol for 15min followed by two washes with ethanol, with centrifugation of 1000g for 10min each time. The chromatin-cellulose slurry was exposed to 0.6J/cm² (6×10^6 ergs/cm²) u.v. light (254 nm) from a Mineralight Short Wave Lamp (Ultra-Violet Products, San Gabriel, CA, U.S.A.) (Spelsberg *et al.*, 1978) and then centrifuged at 1000g. Coupled chromatin-cellulose was rehydrated with TE buffer for 15min at 4°C, washed twice with TE buffer by centrifugation at 1000g, filtered to dryness on a sintered-glass Buchner funnel and stored dry.

Assay for receptor-complex-binding activity

Chromatin-cellulose resin equivalent to 25–50 µg of DNA was either weighed into 1.5 ml microfuge tubes or dispensed as a slurry in extraction buffer [1mM-Tris/HCl (pH 8.5)/5mM- Na_2SO_3 /0.1M-mercaptoethanol]. The pellets were extracted with 1 ml of the appropriate agent (GuHCl, NaCl/urea or GuSCN) at 22°C for 30min. After the extraction, chromatin-cellulose pellets were washed three times by centrifugation at 7800g for 15s (Beckman Microfuge B) with cold 2mM-Tris/HCl/1mM-EDTA, pH 7.5.

Specific quantities of protein from prepared [³H]oestradiol-receptor solutions containing 0.15M-KCl were incubated with the extracted resins for 90min at 4°C (Webster *et al.*, 1976). Afterwards the chromatin-cellulose pellets were washed with TE buffer to remove unbound receptor complex by three centrifugations (7800g for 15s). The labelled ligand was then extracted in 1 ml of ethanol. A sample of the extract (0.5 ml) was added to 4 ml of scintillation fluid (0.4% Omnifluor in xylene) and counted for radioactivity in a liquid-scintillation spectrometer. DNA was hydrolysed from the chromatin-cellulose pellets by incubation at 90°C for 30min in 0.5M- HClO_4 . The hydrolysate was assayed for DNA by the diphenylamine method (Burton, 1956). The binding of [³H]oestradiol-receptor complexes to washed cellulose was determined routinely and subtracted from binding to chromatin-cellulose.

Results

Extraction characteristics of chromatin-cellulose

Our laboratory has been interested in characterizing the binding of oestrogen-receptor complexes to uterine chromatin. Chromatin isolated from immature calf uteri was analysed for protein and DNA content. The mean protein/DNA ratios were: total proteins, 2.1; histones, 1.1; non-histone proteins, 0.7.

Fig. 1 indicates the efficiency of extracting chromatin-bound proteins from calf uterine chromatin-cellulose resins with increasing concentrations of chaotropic agents. Three different agents, GuHCl, NaCl/urea and GuSCN, were used in various concentrations until their limits of solubility were reached. In Fig. 1(a) essentially all of the histones were extracted by 2–3M-GuHCl,

Fig. 1. Effect of various chaotropic agents on the extraction of histone and non-histone proteins (NHP) from calf uterine chromatin-cellulose resins

Each assay tube contained 100mg of DNA as chromatin-cellulose. Each point represents the mean of three determinations performed in duplicate. For full details see the Materials and methods section.

whereas only 75% of the non-histone proteins were extracted by 2M-GuHCl. An additional 13% of the non-histone proteins were removed from the chromatin by 3–8M-GuHCl. In contrast with the complete removal of the histones, GuHCl was unable to extract all of the non-histone proteins. With the use of 1M-NaCl/5M-urea (Fig. 1b), approx. 95% of the histones and 60% of the non-histone proteins were removed. Increasing the NaCl concentration to 4M in the extraction media surprisingly caused a decreased dissociation of non-histone proteins from the chromatin resin while completely removing the histone fraction. However, the chaotropic agent GuSCN produced an extraction profile of histones and non-histone proteins only slightly different from the profile with GuHCl; GuSCN appeared to be more efficient at extracting histones and slightly less efficient at extracting non-histone proteins.

Binding characteristics of NaCl/urea- and GuSCN-extracted chromatin

After extraction of chromatin–cellulose with chaotropic agents, [^3H]oestradiol–receptor complexes bound more extensively to the chromatin (Fig. 2). [^3H]Oestradiol–receptor binding to cellulose was subtracted in all cases. Urea alone, although dissociating histones, did not dissociate acidic proteins, and urea extraction did not facilitate [^3H]oestradiol–receptor complex binding to chromatin. However, NaCl/urea combinations did dissociate proteins selectively and influenced the binding capacity of the chromatin–cellulose resin

(Fig. 2a). When 20 μg of protein from a [^3H]oestradiol–receptor complex solution was added to chromatin–cellulose extracted with 1–4 M-NaCl in 5M-urea, the extent of binding was slightly enhanced with respect to intact chromatin (Fig. 2a). Increasing the quantity of [^3H]oestradiol–receptor complex (60 or 200 μg of protein) present in the binding assay demonstrated enhanced acceptor activity after unmasking of binding sites. Although deproteinization unmasked acceptor activity in these experiments, there was no clear indication that acceptor activity could be eluted from the chromatin–cellulose. At maximal [^3H]oestradiol–receptor complex binding to chromatin–cellulose, histones were completely removed from the resin, implicating the acidic non-histone proteins in the binding interaction. [^3H]Oestradiol–receptor complex bound less to chromatin–cellulose extracted with 1M-NaCl than to chromatin–resin extracted with 3M- or 4M-NaCl (always with 5M-urea).

The binding of the [^3H]oestradiol–receptor complex to calf uterine chromatin resins previously extracted with GuSCN is shown in Fig. 2(b). When each assay tube received 20 μg of protein from the [^3H]oestradiol–receptor complex solution, acceptor activity was unmasked by 1M- and 5M-GuSCN and diminished by 3M- and 6M-GuSCN, respectively. If larger quantities of [^3H]oestradiol–receptor complex (60 μg of protein) were employed in the binding assay, two regions of greater binding capacity (1M- and 5M-GuSCN-extracted chromatin) were more apparent. As the amount of [^3H]oestradiol–receptor complex was increased to 200 μg of protein, the

Fig. 2. Effect of varying the protein concentration of the hormone–receptor preparation on binding of [^3H]oestradiol–receptor complexes to chromatin–cellulose previously extracted with various concentrations of NaCl/urea (a) or GuSCN (b)

Protein concentration is expressed as μg per assay tube. Each assay tube contained 50 μg of DNA as chromatin–cellulose. Each point represents the mean $\pm$ S.E.M. for two or three determinations performed in triplicate. For full details see the Materials and methods section.

double-peaked profile of enhanced binding became obscured by elevated extents of binding to all chromatin fractions. At 1400 $\mu\text{g}/\text{ml}$ binding was increased, but no binding peaks were present. These changes in the binding profile might be attributed to more non-specific binding and trapping in the chromatin-cellulose matrix at the higher protein concentrations. Thus the assay was more discriminating at lower protein values.

GuHCl-extracted chromatin-cellulose

Experiments similar to those described above were conducted with GuHCl as the extracting salt (Fig. 3). As observed in the experiments with GuSCN-extracted chromatin, the lower quantities of proteins present during the binding assay improved the resolution of the assay. With 20 μg of protein in the binding assay, a peak of [^3H]oestradiol-receptor-complex binding to chromatin-cellulose was obtained with chromatin extracted with 1 M-GuHCl. Presumably this elevated binding capacity was a consequence of the removal of masking proteins. It was possible to remove some acceptor activity by extracting the chromatin-cellulose resin with 2–3 M-GuHCl. A more significant finding was the appearance at an intermediate protein value (60 μg) of a second region of enhanced binding capacity. These data suggest the possibility of multiple chromatin sites for [^3H]oestradiol-receptor-complex binding.

In order to test the necessity for an intact hormone-receptor complex, the following experi-

Fig. 3. Effect of varying the protein concentration of the hormone-receptor preparation on binding of [^3H]oestradiol-receptor complexes to chromatin-cellulose previously extracted with various concentrations of GuHCl

Conditions of assay are as described in Fig. 2.

ments were performed. Chromatin-cellulose resins extracted with 1–8 M-GuHCl were incubated with free [^3H]oestradiol or denatured [^3H]oestradiol-receptor complexes obtained by previous heating of the receptor solution to 40°C for 60 min. In the former situation, [^3H]oestradiol was added to a concentration which approximated the radioactivity content of the [^3H]oestradiol-receptor-complex solution. The denatured [^3H]oestradiol-receptor complex was added at the same protein concentration as the intact [^3H]oestradiol-receptor complex. Free [^3H]oestradiol did not bind to the chromatin resin above background binding to cellulose (Fig. 4). Denatured [^3H]oestradiol-receptor complex interacts non-specifically with the chromatin-cellulose resin; binding to intact and extracted chromatin was consistently negligible. Thus the integrity of the receptor complex is a requirement for binding to both intact and partially deproteinized uterine chromatin.

To determine target-tissue specificity, spleen chromatin resins were prepared and partially

Fig. 4. Effect of [^3H]oestradiol-receptor-complex binding to GuHCl-extracted uterine and spleen chromatin-cellulose

[^3H]Oestradiol-receptor complexes were incubated with uterine-chromatin-cellulose resin (●). As controls, [^3H]oestradiol-receptor complexes were denatured (40°C for 60 min) before incubation with uterine chromatin-cellulose resin (○), or free [^3H]oestradiol (▲) was used in the incubation. [^3H]oestradiol-receptor complexes were also incubated with spleen-chromatin-cellulose resin (□). Protein concentration was 60 $\mu\text{g}/\text{assay tube}$. Each point represents the mean $\pm$ s.e.m. for three determinations performed in triplicate.

deproteinized by 1–8 M-GuHCl. This non-target-tissue chromatin–cellulose was then used in the [^3H]oestradiol–receptor–complex binding assay. As shown in Fig. 4, there was an increased binding of [^3H]oestradiol–receptor complexes to 1 M-GuHCl-extracted spleen chromatin. This binding remained constant with 1–8 M-GuHCl-extracted spleen chromatin resins. There was no indication of binding peaks as was seen with the uterine chromatin–cellulose. The binding to spleen chromatin that was observed was consistently lower than with uterine chromatin.

The data in Fig. 4 were obtained by using 0.15 M-KCl in the receptor-binding assay. This concentration of KCl was found to be optimal for maximum binding of [^3H]oestradiol–receptor complexes to GuHCl-extracted chromatin (Fig. 5). The [^3H]oestradiol–receptor–complex binding to 1 M-GuHCl-extracted chromatin was maximal at 0.05–0.15 M-KCl and decreased at higher (0.2–0.4 M) KCl concentrations. [^3H]Oestradiol–receptor–complex binding to buffer-extracted chromatin displayed a much lower increase in receptor binding with increasing KCl concentrations. An interesting finding is that the majority of the [^3H]oestradiol–receptor–complex binding profile was resistant to 0.4 M-KCl extraction. This is in agreement with the report of Perry & Lopez (1978), who found that oestrogen receptor binding to sheep hypothalamic chromatin was largely KCl-resistant. Obviously the relationship between this KCl-resistance and that found to occur *in vivo* (Clark *et al.*, 1976; Ruh *et al.*, 1979) has yet to be determined.

Fig. 5. Effect of varying the concentration of KCl (pH 7.5) on the binding of [^3H]oestradiol–receptor complexes to chromatin–cellulose previously extracted with buffer or 1 M-GuHCl

Conditions of assay were as described in Fig. 2.

The possibility that proteolytic activity in either the chromatin–cellulose or receptor preparations may contribute to part of the [^3H]oestradiol–receptor–complex-binding profiles obtained in our experiments was investigated. Chromatin–cellulose preparations incubated for 30 min with the proteolytic inhibitor phenylmethanesulphonyl fluoride showed a [^3H]oestradiol–receptor–complex-binding profile identical with that of chromatin–cellulose not treated with the inhibitor. Furthermore, chromatin–cellulose previously extracted with 0–8 M-GuHCl demonstrated no proteinase activity towards the general proteolytic substrate Azocoll (Calbiochem–Behring Corp., San Diego, CA, U.S.A.) either at 4°C for 90 min (normal binding conditions) or at 37°C for 20 h (conditions favouring proteolysis). Similarly, oestrogen–receptor preparations exhibited no proteolytic activity under the above binding conditions. Therefore it is reasonable to assume that under the acceptor assay binding conditions the [^3H]oestradiol–receptor–complex-binding profile to chromatin was not influenced by trypsin-like proteinases.

It was possible to assess saturability of binding and to estimate K_d and number of binding sites by varying the amount of [^3H]oestradiol–receptor complex added to a constant amount of uterine chromatin–cellulose (Fig. 6). Experiments using

Fig. 6. Effect of increasing [^3H]oestradiol–receptor concentration on binding to chromatin–cellulose resins. Chromatin–cellulose had been extracted previously with 0 M-GuHCl (○), 1 M-GuHCl (△) or 4 M-GuHCl (●). [^3H]Oestradiol–receptor binding to cellulose alone is represented by □. Each assay tube contained 25 µg of DNA as chromatin–cellulose. Each point represents the mean of two to five determinations performed in quadruplicate. For further details see the Materials and methods section.

various [^3H]oestradiol–receptor complex solutions containing buffer alone, or bovine serum albumin or denatured oestrogen–receptor solutions to maintain constant protein, gave the same results. The K_d for the binding of [^3H]oestradiol–receptor complex to intact chromatin was estimated to be 0.5 nM and it contained approx. 1400 sites/pg of DNA. Chromatin–cellulose extracted with 4 M-GuHCl was characterized by a saturation curve containing two distinct plateaus. The lower plateau, saturable at lower concentrations of [^3H]oestradiol–receptor complex, corresponds to approx. 3500 sites/pg of DNA, with a K_d of 1 nM. Analysis of the second plateau indicates 5000 sites/pg of DNA and a K_d of approx. 1.2 nM. Additionally, 1 M-GuHCl-treated chromatin–cellulose is saturated at approximately the same extent and with the same affinity as the second 4 M-GuHCl-extraction plateau.

The oestrogen receptor was more extensively purified by heparin–Sephacel affinity chromatography in an effort to minimize non-specific chromatin binding, which might obscure receptor binding to specific sites. The data from these experiments are contained in Figs. 7 and 8. Oestrogen receptors were bound to heparin–Sephacel slurries and eluted with salt (Fig. 7). KCl proved a more efficient elution agent than NaSCN; the specific radioactivity (c.p.m./mg of protein) of KCl-eluted receptor was markedly greater than that

obtained by NaSCN elution. Since KCl elution gave a receptor preparation of higher purity, it was used in later preparations. The purification resulting from elution from the heparin–Sephacel slurry and subsequent $(\text{NH}_4)_2\text{SO}_4$ precipitation was 200-fold over crude cytosol.

By using the heparin–Sephacel-purified receptor preparation, binding of [^3H]oestradiol–receptor complex in the range of 13–45 $\mu\text{g}/50\text{mg}$ of DNA to GuHCl-extracted chromatin was measured (Fig. 8). In addition to sites unmasked by 1 M- and 4 M-GuHCl, a third region of binding activity was detected with chromatin–cellulose previously extracted with 7 M-GuHCl. Chromatin, previously extracted with 1–8 M-GuHCl, bound approximately twice as much purified [^3H]oestradiol–receptor complex as did buffer-treated chromatin. [^3H]Oestradiol did not bind significantly to the chromatin resin (0–8 M-GuHCl). Therefore, under these assay conditions, it appears that multiple protein–DNA complexes may serve as acceptors for the oestrogen–receptor complex.

Discussion

The data presented here support the hypothesis that high-affinity binding sites, i.e. acceptors, for oestrogen–receptor complexes are associated with the acidic protein fraction of calf uterine chromatin.

Fig. 7. Purification of oestrogen–receptor complexes by using heparin–Sephacel

(a) Elution of proteins bound to heparin–Sephacel with stepwise salt gradient. (b) Specific radioactivity of proteins eluted from heparin–Sephacel. NaSCN elution is represented by triangles, and KCl elution is represented by circles. For further details see the Materials and methods section.

Fig. 8. Effect of varying the protein concentration of the purified receptor solution on binding to chromatin-cellulose previously extracted with various concentrations of GuHCl

Protein concentration is expressed as μg per assay tube. Each assay tube contained $50 \mu\text{g}$ of DNA as chromatin-cellulose. The 100% values for the binding of $13 \mu\text{g}$ and $45 \mu\text{g}$ of protein were 2800 c.p.m. and 19000 c.p.m. respectively. Each point is the mean $\pm$ S.E.M. for three determinations performed in triplicate. For full details see the Materials and methods section.

We have chosen to probe oestrogen-receptor acceptor sites on chromatin from a mammalian species by selectively deproteinizing chromatin with certain chaotropic agents. This has proved to serve two functions: the unmasking of acceptor sites and the extraction or removal of these same sites. The technique developed by Webster *et al.* (1976) for immobilizing chromatin on an insoluble matrix in order to unmask acceptor sites was utilized. The data from the various binding experiments suggest that considerable acceptor activity is present when the chromatin-cellulose resin has been partially deproteinized. Although increasing molarities of the various chaotropic agents appear to change only slightly the total protein content of chromatin, selected species of proteins may in fact have been removed. It would appear that these non-histone acceptor moieties on uterine chromatin apparently are masked to varying degrees by other acidic proteins. However, since a major fraction of acceptor activity is found on only partially dehistonized chromatin-cellulose, a modulating role for histone proteins cannot be excluded (Thanki *et al.*, 1979).

Our findings that acidic proteins seem to con-

stitute a necessary component of acceptor activity is consistent with the related findings of Spelsberg *et al.* (1975, 1978) in the chick oviduct, Klyzsejko-Stefanowicz *et al.* (1976) in the rat prostate, Perry & Lopez (1978) in sheep hypothalamus, and Tsai *et al.* (1980) in rat Sertoli cells. Other acceptor-localization studies have considered DNA (Thanki *et al.*, 1978; Kallos & Hollander, 1978), RNA (Liao *et al.*, 1980), and the nuclear matrix (Barrack & Coffey, 1980) as nuclear acceptors. But a study of circannual rhythms and nuclear binding of the progesterone receptor demonstrated that certain protein-DNA complexes, and not pure DNA, constitute the acceptor site for steroid-hormone receptors (Spelsberg & Halberg, 1980). Klyzsejko-Stefanowicz *et al.* (1976) reported that urea/NaCl extractions of prostatic chromatin unmasked acceptor sites for the testosterone receptor. This activity resided in the acidic non-histone-protein fraction. Additionally, in the MCF-7 cell system, oestrogen-receptor complexes were found to bind to a chromatin fraction which was enriched in non-histone proteins (Scott & Frankel, 1980). The results obtained in our experiments support this acidic-protein hypothesis. It is possible that an acidic-protein-DNA combination functions as the acceptor region and provides an essential additional specificity for steroid-hormone action and that DNA alone merely contains lower-affinity, and therefore potentially less physiologically significant, binding sites.

An important aspect of acceptor studies is specificity of binding in terms of limited binding sites, high-affinity binding, the need for an intact receptor complex and a certain tissue specificity. In our system, oestrogen-receptor binding to uterine chromatin was saturable. Acceptor sites on intact chromatin as well as unmasked acceptor sites possessed limited binding capacity for oestrogen-receptor complexes. The binding of the oestrogen-receptor complex to the chromatin fractions demonstrated high affinity, with an estimated K_d of 0.5–1.4 nM. In addition, both free oestradiol and denatured oestradiol receptor had negligible binding to intact or partially deproteinized chromatin resins. Finally, neither binding peaks nor binding saturation were demonstrated with the non-target-tissue (spleen) chromatin. These results are in agreement with other reports which demonstrated specific, saturable interactions of receptor complexes with acceptors. For example, both the progesterone-receptor and the oestrogen-receptor complex interact in a saturable manner with different nuclear acceptors of chick oviduct tissue (Webster *et al.*, 1976; Kon *et al.*, 1980). Similarly, the testosterone receptor binds with high affinity and limited capacity to nuclear acceptors in epithelium of guinea-pig seminal vesicle (Weinberger & Veneziale, 1980).

Most interesting is our finding that the nuclear acceptor for oestrogen-receptor complexes may not be a single entity. It is possible that a multiplicity of acceptor classes endow the cell with more extensive regulatory ability in response to hormone stimulation. Our results using partially purified oestrogen-receptor preparations would indicate that at least two or three potential classes of acidic acceptor proteins on calf uterine chromatin may exist. By using the chromatin-cellulose system, data obtained with the progesterone receptor in the chick oviduct (Spelsberg *et al.*, 1975) showed only one area of increased acceptor activity, in contrast with our two to three areas. More recent data obtained by reconstitution techniques (Spelsberg *et al.*, 1979) indicated that the progesterone receptor may bind to three different species of acidic proteins in combination with DNA. Data from other studies support the hypothesis that binding sites with varying affinities exist (Pikler *et al.*, 1976; Webster *et al.*, 1976). Cidlowski & Munck (1980) have reported that more than one species of nuclear acceptors exist on the basis of the salt-extractability of glucocorticoid-receptor complexes from nuclei. This report is analogous to findings for nuclear binding of oestrogen-receptor complexes in the rat uterus (Clark *et al.*, 1976; Ruh & Baudendistel, 1977). Although studies of calf uterine oestrogen receptor have not demonstrated A and B subunits, as found for steroid hormones in the chick oviduct system (Vedeckis *et al.*, 1979; Schrader *et al.*, 1978), it is possible that the binding of the oestrogen receptor to multiple acceptors is affected by receptor heterogeneity and that some acceptor species may preferentially bind a receptor subunit rather than the intact receptor.

In summary, calf uterine chromatin contains saturable high-affinity binding sites for the intact oestrogen-receptor complex. Acceptor activity seems to be associated with the acidic protein fraction of chromatin and can be modulated by removal of these proteins. Additionally, multiple acceptor species appear to be involved in this interaction.

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