Characterization of a Unique Nuclear Estrogen-binding Component in an Estrogen-responsive Mouse Leydig Cell Tumor

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ABSTRACT

An estrogen-responsive mouse Leydig cell tumor line (Tumor 124958) has been shown to contain only a low-affinity binder for estradiol in the cytosol fraction. This differed from the putative estrogen receptor in terms of its hormone-binding specificity as well as affinity. In addition, the possibility that an estrogen receptor-like molecule exists in the nuclei even without hormonal stimuli was examined using purified nuclei. Scatchard plot analyses showed that these nuclei possessed a large amount of estrogen binder having a high affinity for estradiol and diethylstilbestrol. The content of this nuclear binding component was not diminished by using molybdate, a potent inhibitor for receptor activation, and in vitro incubation of collagenase-dispersed cells with estradiol did not cause significant increase in the number of nuclear binding sites when compared with the values obtained by direct incubation of isolated nuclei with estradiol. These results support the view that this nuclear estrogen binder is not due to artificial migration of the cytosol receptor into nuclei during homogenization. The characterization of this nuclear binding component under cell-free conditions revealed that its affinity for estradiol in Mg2+-containing buffer was temperature dependent (Kd 3 nM at 30°C and 12 nM at 0°C) without significant alteration in the number of maximum binding sites. Introduction of a chelating agent (ethylenediaminetetraacetate) into the buffer system abolished the temperature effect on the affinity, resulting in high affinity for estradiol at both low and high temperatures. These Mg2+ and temperature effects were reversible. In addition, when compared with putative nuclear estrogen receptors, this nuclear binding was observed to be relatively resistant to high salt or micrococcal nuclease treatments in relation to solubilization from nuclei. However, trypsin digestion was found to result in a marked decrease in the nuclear binding sites, indicating that this unique nuclear binding component contains a protein unit(s). These results suggest the possibility that this tumor line contains a unique unoccupied nuclear estrogen binder which might be able to transmit estrogen signals to tumor cell nuclei with regard to tumor growth.

INTRODUCTION

Many human malignant tumors such as breast cancer and prostate cancer have been recognized as steroid hormone-dependent cancers (9). A significant advance in endocrine therapy of breast cancer has been the demonstration that there exists a positive correlation between the presence of ER3 proteins in cytosol preparations of these tumors and a favorable response to endocrine manipulation (10). The possibility that there is a different pathway(s) by which estrogens exert an influence on growth in malignant estrogen-sensitive cells in comparison with that in normal target cells, however, has not been fully examined. McGuire, with Geier (7) and Zava (28) proposed that the human breast cancer cell line MCF-7 contains a large amount of unoccupied nuclear receptor using crude nuclear preparation. When highly purified nuclei were used, however, almost complete disappearance of this nuclear receptor was reported (4), suggesting that the apparent unoccupied nuclear receptor is due to contamination of cytosol receptor in nuclear fraction during subcellular fractionation. These observations are still a matter of controversy (3, 8). In order to find new hormone action pathways in malignant cells, we have attempted to investigate the relationship between estrogen binding systems and estrogen-induced tumor growth in various Leydig cell tumor lines. The approach has been successfully undertaken to point out the presence of a unique cytosol estrogen-binding component in malignant cells (17, 18). In the present studies, we have investigated the nuclear estrogen binder in an estrogen-responsive tumor line. With this Leydig cell tumor system, we show that the high-affinity binder is present only in the nuclear fraction even in the absence of estrogen stimuli and suggest that the pathway of steroid response involves this nuclear binder.

MATERIALS AND METHODS

Animals and Experimental Tumors. The original Leydig cell tumors had been produced in testes of BALB/c mice by the s.c. implantation of a fused 10-mg pellet of 10% DES in cholesterol (DES pellet) (27). The tumors were serially passaged in BALB/c males by mincing the tumor tissue and introducing 1- to 2-mm cubes into the s.c. tissue with the aid of a trocar. These tumor-bearing mice and host animals were kindly supplied by Dr. R. A. Huseby and have been maintained in our laboratory.

Three different tumor lines developed from different origins were used in this study. Tumor 124958 had been classified as "estrogen independent" during early passages from 10 to 18 generations, since its tumor growth had not been affected by estrogenization (17). However, later transfers (23 to 29 generations) were found to grow more rapidly in hosts estrogenized with DES pellet than in nonestrogenized mice (see Chart 1). Tumor 22137, which had been transplanted for 116 to 125 generations, was a stable tumor line and grew in castrated hosts but regressed in estrogenized mice. Tumor 134486, which had been transplanted for 17 to 20 generations, grew only in estrogenized hosts, but its tumor growth recently slowed down (1 g in 12 to 14 weeks) when compared with that of 6 to 8 generations (1 g in 6 to 8 weeks).

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: ER, estrogen receptor; DES, diethylstilbestrol; TEM buffer, 10 mM Tris:1.5 mM EDTA:2 mM mercaptoethanol, pH 7.4, at 20°C; Buffer 1, 0.32 M sucrose:5 mM MgCl2:10 mM Tris:0.2 mM mercaptoethanol, pH 7.4, at 20°C; Hank's buffer, Hank's salt solution:5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid:0.1% (w/v) bovine serum albumin, pH 7.4, at 37°C; CB-154, ergocryptine.
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Chart 1. DES-induced growth enhancement of Tumor 124958. In A, tumor pieces were transplanted into DES pellet-implanted and castrated (■), castrated only (○), or untreated (▲) mice. Ten mice were used for each group. Tumor sizes were measured as a rule. In B, all mice were castrated 3 days before transplantation of tumor pieces. On the day of transplantation, 2 groups of mice were implanted with DES pellet. CB-154 (50 μg/mouse in 0.5 ml sesame oil) injection was also initiated followed by every-other-day treatment during the entire experiment period (CB+/■). Mice not treated with CB-154 (CB−/▲) were given injections of a vehicle. Ten mice were used for each group. DES pellet implanted only; CB-154 injected only; A, CB-154 noninjected. Points, means; bars, S.E. The tumor size measurements were discontinued when more than 50% of host animals died.

Tissue Preparations. All procedures were done at 0–4°C unless specified otherwise. The host mice were sacrificed by cervical dislocation. In the case of Tumor 134486, DES pellets were removed from the host animals 3 days prior to sacrifice. For the in vitro steroid-binding studies of Tumors 124958 and 22137, the explants in nonestrogenized but androgensized homogenates were used. After removal of surrounding connective tissue and areas of necrosis, the tumors were homogenized in 4 volumes of TEM buffer with a Teflon:glass homogenizer. Each homogenate was centrifuged at 800 × g for 10 min. The supernatant was subjected to further centrifugation at 105,000 × g for 60 min to obtain the cytosol. The pellet from 800 × g centrifugation was washed 4 times with TEM buffer and then resuspended in appropriate volumes (3 to 5 volumes) of TEM buffer (crude nuclei). When the purified nuclei were prepared, the tumors were homogenized in 4 volumes of Buffer 1 with a Dounce homogenizer (10 strokes with the loosely fitting pestle and 5 strokes with the tightly fitting pestle), followed by further homogenization in a loosely fitting Teflon:glass homogenizer and 5 strokes at 1300 rpm, each stroke followed by a 30-sec pause; then the preparation was filtered through 2 layers of nylon gauze (200 mesh) as reported previously (17) with minor modifications. These homogenization conditions were found to be suitable to obtain intact isolated nuclei with a minimum of contamination by cytoplasmic tags as judged by crystal violet and fast orange staining methods. The filtrate was centrifuged at 800 × g for 10 min. The supernatant was centrifuged at 105,000 × g for 60 min to obtain the cytosol. The pellet from centrifugation at 800 × g was washed once with Buffer 1 and then with Buffer 1 supplemented with 0.25% Triton X-100. The washed pellet was resuspended in 4 volumes of Buffer 1. The aliquots (2 ml) of this suspension were layered on 3 ml of heavy sucrose [1.5 m sucrose:0.1% (w/v) bovine serum albumin:5 mM MgCl2:25 mM KCl:10 mM Tris:2 mM mercaptoethanol, pH 7.4, at 20°C], followed by centrifugation at 20,000 × g for 30 min. The pellet was resuspended in an appropriate volume (usually 4 volumes) of Buffer 1 and used as purified isolated nuclei. In some experiments, Na2MoO4, which has been known as an inhibitor of steroid receptor activation (14), was included in all buffers for preparing purified nuclei at a final concentration of 10 mM. The recovery of DNA into isolated nuclei was 40 to 60%.

[3H]Estradiol Binding to Cytosol or Nuclei. The cytosol in TEM buffer or Buffer 1 was incubated with various concentrations of [3H]estradiol at 0°C for 2 hr (total reaction volume, 250 μl). Parallel incubations with [3H]estradiol were performed in the presence of a 100-fold molar excess of radioinert estradiol to obtain the nonspecific binding values. After incubations, 100 μl of dextran-coated charcoal solution [1% (w/v) charcoal and 0.01% (w/v) dextran T-70 in TEM buffer] were added to each tube, followed by incubation at 0°C for 10 min with intermittent vortexing. The tubes were centrifuged at 1200 × g for 5 min, and the aliquots (175 μl) of the supernatants were counted.

When the nuclear estrogen-binding component was studied, crude or purified nuclear suspensions (150 μl) were incubated with [3H]estradiol in the presence or the absence of a 100-fold molar excess of radioinert competitors at 0°C for 2 hr or at 30°C for 1 hr unless noted otherwise. After the incubation periods, 2 ml of the corresponding buffer (TEM buffer or Buffer 1) were added to all tubes, and the nuclei were pelleted down at 800 × g for 10 min, followed by washing twice with the buffer. The radioactivity associated with these washed nuclei was extracted with liquid scintillation fluid as described below.

Effects of Enzyme Digestion or KCI Extraction on Nuclear Estrogen Binder. The purified nuclei from Tumor 124958 were digested with micrococcal nuclease (20 absorbance units/ml at 0°C for 30 min), pancreatic RNase (100 μg/ml at 25°C for 30 min), or soybean trypsin (100 μg/ml at 0°C for 30 min). In the case of micrococcal nuclease digestion, nuclei were treated in Buffer 1 supplemented with 1 mM CaCl2, followed by centrifugation at 800 × g for 10 min. The pelleted nuclei were allowed to be swollen in TEM buffer at 0°C for 30 min. These nuclei treated with various enzymes were pelleted down at 800 × g for 10 min and washed twice with Buffer 1. These washed pellets were incubated with 10 nM [3H]estradiol and 1 μM unlabelled estradiol at 0°C for 2 hr. After incubation, nuclear suspensions were processed as described above to measure the specific binding.

To investigate the degree of extraction of nuclear binding components with KCI solution, purified nuclei from Tumor 124958 were labeled with 10 nM [3H]estradiol ± a 100-fold molar excess of unlabelled estradiol at 0°C for 2 hr and washed as noted previously. The aliquots (approximately 100 μg DNA per tube) of these washed nuclei were exposed to various concentrations of KCI in TEM buffer at 0°C for 1 hr (final volume, 0.5 ml), and then centrifuged at 1500 × g for 10 min. The radioactivities in the supernatant and the pellets were measured. Comparative studies were performed using purified nuclei from Tumor 22137 which had been preincubated at 0°C for 1 hr with heat-activated (25°C for 30 min) ER prelabeled with [3H]estradiol and washed twice with Buffer 1. These nuclei of Tumor 22137 labeled with [3H]estradiol:ER complexes under cell-free conditions were treated with various concentrations of KCI as described above.

Preparation of Dispersed Tumor Cells with Collagenase:Trypsin Digestion and Their Interaction with [3H]Estradiol. The tumor tissue, which had been finely minced with scissors and placed in a 50-ml Erlenmeyer flask, was briefly shaken in Hanks’ buffer (1 g/10 ml). The flask was placed in an ice bath for 3 min to settle the minced tissue. The supernatant which contained cell debris and dead cells was aspirated off with Pasteur pipet. This procedure was repeated once more. The washed tissue was incubated with collagenase (0.5 mg per ml) and soybean trypsin (0.1 mg per ml) in Hanks’ buffer (1 g tissue per 20 ml) at 37°C for 30 min in 95% O2:5% CO2 with gentle shaking (20 times per min). After initial incubation, the flask was kept in an ice bath for 3 min. Cells released into the medium were taken out into the centrifugation tube. Undigested tissue was allowed to incubate for 2 additional 30-min periods with collagenase and trypsin in fresh Hanks’ buffer. At the end of each incubation, the cell suspension was filtered through 2 layers of nylon gauze (350 mesh), and the cells collected were centrifuged at 150 × g for 5 min and resuspended in Hanks’ buffer (5 ml/g original tissue) containing 200 μg of trypsin inhibitor. After standing in an ice bath for 5 min, cells were pelleted down at 150 × g for 5 min and washed 3 times with Hanks’ buffer. Cells were resuspended in an appropriate volume (3 to 5 × 10⁶/ml) of modified Eagle’s medium containing 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4 at 37°C) by gentle vortexing. The cell suspension (150 μl) was incubated with various concentrations of [3H]estradiol in the presence or the absence of a 100-fold molar excess of radioinert estradiol at a final volume of 250 μl for 1
hr at 37° in 95% O2:5% CO2. After incubation, cells were pelleted down at 150 \times g for 5 min and washed 3 times with 2 ml of Hanks’ buffer. The washed cells were resuspended in 2 ml of Buffer 1 containing 0.25% Triton X-100 and kept at 0° for 10 min with intermittent vortexing. This treatment killed more than 95% of the cells and released nuclei with a minimum of contamination by cytoplasmic tags in the buffer. The nuclei were centrifuged at 800 \times g for 10 min and washed twice with Buffer 1. The radioactivity associated with washed nuclei was counted. Cell counts were performed with a hemocytometer, and the viability of the cells was determined by the trypan blue dye exclusion method and was found to be 80 to 95%.

Radioimmunoassay Procedures. Serum prolactin concentrations were determined with a double antibody radioimmunoassay using materials and protocols supplied by the National Institute of Arthritis, Metabolism, and Digestive Diseases Pituitary Hormone Distribution Program (Bethesda, Md.).

Statistical Analyses. All values presented in the text charts are mean ± S.E. The paired or unpaired Student t test was used to discuss the significant difference.

Miscellaneous Assays. The protein concentrations were determined by the method of Lowry et al. (12). DNA was measured by the method of Burton (1). Orinol reaction was used for RNA determination (11). Radioactivity was determined in a Nuclear Chicago Isocap/300 liquid scintillation spectrometer with 5 ml toluene-Triton X-100 scintillation cocktail as reported before (19). Counting efficiency for 3H was 50 to 55%.

Chemicals. 17β-[2,4,6,7-3H]Estradiol (100 to 115 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). The purity of [3H]-estradiol was periodically checked by thin-layer chromatography, using an appropriate solvent (chloroform:methanol, 95:5) system. Nonradioactive steroids and DES were from Steraloid, Inc. (Wilton, N. H.). Acid-washed charcoal (Norit A), collagenase (type IV), fatty acid-free bovine serum albumin, and pancreatic RNase were obtained from Sigma Chemical Co. (St. Louis, Mo.). Micrococcal nuclease, soybean trypsin, and ligma bean trypsin inhibitor were from Worthington Biochemical Corp. (Freehold, N. J.). The other chemicals used here were of analytical grade. CB-154 was kindly supplied by Sandoz Pharmaceuticals (East Hanover, N. J.).

RESULTS

Enhanced Growth in Tumor 124958 by Estrogenization. In order to establish estrogen-dependent tumor growth, the tumor was transplanted in BALB/c males estrogenized by s.c. implantation of a DES pellet. As shown in Chart 1A, estrogenization resulted in enhancement of tumor growth. Also, the average life span of the tumor-bearing mice was shortened by estrogenization (data not shown). Thus, Tumor 124958 can be classified as an estrogen-responsive tumor at this stage, although this tumor is transplantable into nonestrogenized BALB/c mice.

Since the administration of estrogens into animals has been reported to cause an increase in prolactin secretion from the pituitary gland (5), we investigated whether prolactin is involved in the DES-induced enhancement of tumor growth. We injected CB-154 (200 μg/mouse in sesame oil) every other day immediately following inoculation of the tumor seed; serum was obtained by cardiac puncture 60 days after transplantation. Estrogenization with a DES pellet was found to elevate the serum prolactin level to 17.5 ± 3.4 ng/ml (n = 6), while nonestrogenized control mice showed a lower level (6.6 ± 2.4 ng/ml; n = 10). The simultaneous administration of CB-154 to estrogenized mice lowered the serum prolactin value to 6.9 ± 2.1 ng/ml (n = 7). The lowest value (1.5 ± 0.15 ng/ml; n = 11) was observed in mice treated with CB-154 alone. On the other hand, the administration of this potent dopamine agonist had no effect on DES-dependent or -independent tumor growth, a result consistent with the idea that growth enhancement by DES is not elicited by indirect influence of estrogen on prolactin-producing cells in the pituitary gland (Chart 18).

Characterization of Estrogen-binding Component in the Cytosol. We have previously reported that Tumor 124958 contained a unique estrogen-binding component in the cytosol which was very weakly associated with estradiol, but not with DES (17, 18). First, we examined the possibility that the conversion from this unique binder to a putative ER did occur during serial transplantsations, since this conversion was shown to be achieved by simple in vitro manipulations such as a removal of small molecules from the cytosol (18). When the difference in counts between samples incubated with and without a 100-fold molar excess of unlabeled estradiol was considered to be the concentration of specifically bound [3H]estradiol, the high-affinity binding sites for estradiol were not identified in this experiment or 5 other experiments at the range of 0.3 to 20 nm [3H]estradiol. Application of the same procedure to cytosol preparations of 2 other tumor lines (Tumor 22137 and 134486) easily demonstrated the high-affinity sites (Kd 10^10 to 10^-9 M). The use of TEM buffer did not modify the results on these binding studies (Chart 2A). The experiment on binding specificity revealed that the presence of unlabeled estradiol in the reaction mixture caused some inhibition of the binding of [3H]estradiol to the macromolecular component in the cytosol from Tumor 124958, while radioinert DES did not affect [3H]estradiol-macromolecular interaction at the concentration used in this experiment (Chart 2B); this result is similar to our previous observations (17, 18). However, we should mention that modest inhibition of [3H]-estradiol binding by radioinert estradiol revealed the presence of a low-affinity but saturable binding site for estradiol in this cytosol. Scatchard (21) plot analyses of the values of total binding depicted on Chart 2B showed the presence of the saturable binding site with a Kd of approximately 10^-7 M. Because of this low affinity for estradiol, no saturable binding sites could be demonstrated at the range of 0.3 to 20 nm [3H]estradiol, as described above. In addition, the possibility was also tested that this unusual cytosol binder might be transformed to an authentic receptor-like protein by exposure to relatively high temperatures. Incubation of the cytosol of Tumor 124958 with [3H]estradiol at 25° for 30 min, however, did not cause the appearance of receptor-like high-affinity binder irrespective of the presence of Na2MoO4 (data not shown).

Presence of Unoccupied Nuclear Estrogen-binding Component. To gain an insight into the mechanism of the growth response to DES, we have investigated whether or not there is a high-affinity binder for estradiol as well as DES in the other subcellular fractions. We prepared the purified nuclei of Tumor 124958 from nonestrogenized host animals. Exposure of these nuclei to 4 nm [3H]estradiol ± 0.4 μM unlabeled estradiol at 0° or 30° resulted in the demonstration of the presence of a specific binding site for estradiol in nuclei as shown in Chart 3. In this experiment, much higher capacity was observed at 30° than at 0°. This temperature-dependent difference in binding capacity could be due either to the presence of a binder already occupied with estrogens or to a temperature-induced alteration in affinity for estradiol. To test which of these possibilities is the case, we incubated these nuclei with an increasing concentration of [3H]-estradiol in the presence or the absence of a 100-fold molar
Chart 2. Binding characteristics of the cytosol components of various tumor lines. In A, the cytosol from Tumor 124958 (●), 22137 (○), or 134486 (△) which had been prepared in Buffer 1 was incubated with the increasing concentrations of [3H]estradiol (E2) in the presence or the absence of a 100-fold molar excess of radioinert estradiol at 0° for 2 hr, followed by dextran-coated charcoal assay. The cytosol in TEM buffer from Tumor 124958 was also provided (×). In B, the cytosol in TEM buffer from Tumor 124958 was incubated with 10 nM [3H]estradiol in the presence of various concentrations of unlabeled estradiol or DES at 0° for 2 hr, followed by dextran-coated charcoal assay. Values are percentage binding, taking the value in the absence of unlabeled estrogens as 100%. Points, means of 4 experiments; bars, S.E.

Chart 3. Association kinetics of the nuclear binding component in Tumor 124958 at 0° or 30°. The isolated nuclei in Buffer 1 were incubated with 4 nM [3H]estradiol in the presence or the absence of a 100-fold molar excess of unlabeled estradiol for the indicated periods of time at 0° or 30°. After incubations, the nuclei were pelleted down and washed as described in "Materials and Methods." Scatchard plot analyses of these data revealed that incubation at 30° caused a marked increase in affinity for estradiol (Kd 3.1 ± 0.9 nM; n = 7) without significantly altering the number of maximum binding sites (2000 ± 260 fmol/mg DNA; n = 7) in comparison with those (Kd 11.6 ± 2.7; number of maximum binding sites, 1500 ± 227 fmol/mg DNA; n = 7) obtained by incubation at 0° (Chart 4A). Therefore, temperature-dependent alterations in binding parameters appear to be due not to an exchange of endogenous estrogens with [3H]estradiol at the nuclear binding sites but to a temperature-induced change of Kd.

To verify that this nuclear estrogen-binding component was not due to an artificial migration into nuclei during subcellular fractionation, we examined the estradiol-binding ability of nuclei of 2 other tumor lines (Tumors 22137 and 134486) containing putative ER in their cytosol fractions under the identical experimental conditions (see Chart 2A). As shown in Chart 4B, only Tumor 124958 was found to possess a large amount of nuclear binding sites when these tumors were nonestrogenized. In Tumor 134486, a high-affinity estrogen binder was exclusively localized in the cytosol while the presence of some nuclear binding sites (19.6 ± 10% of total cellular binding sites; n = 4) was observed in Tumor 22137. We also investigated the effect of Na2MoO4 [a potent inhibitor of receptor activation in all steroid hormone receptors examined (14)] on the subcellular localization of estrogen binders. Introducing this agent into all buffers did not

Chart 4. Saturation analyses of nuclear binding components of various tumor lines. In A, the aliquots of purified nuclear suspension in Buffer 1 prepared from Tumor 124958 were incubated with the increasing concentrations (0.3 to 20 nM) of [3H]estradiol ± a 100-fold molar excess of unlabeled estradiol or DES at 0° for 1 hr, followed by dextran-coated charcoal assay. Values are percentage binding, taking the value in the absence of unlabeled estrogens as 100%. Points, means of 4 experiments; bars, S.E.
modify the characteristics of nuclear and cytosol binder in Tumor 124958 (Chart 5). Moreover, incubation with \(^{3}H\)estradiol at 37° of intact dispersed cells of Tumor 124958 prepared by collagenase:trypsin digestion method revealed a number similar (1470 ± 200 fmol/mg DNA; \(n = 4\)) to that obtained by direct incubation of isolated nuclei (Chart 6). These results might support the conclusion that Tumor 124958 contains the unoccupied nuclear estrogen-binding site having a high-affinity for estradiol.

Some Characteristics of Nuclear Binding Site. Binding specificity of this nuclear component seems to be quite important, since tumor growth is enhanced by DES and DES does not suppress \(^{3}H\)estradiol binding to the macromolecular component in the cytosol. As shown in Chart 7, DES could well compete with \(^{3}H\)estradiol for the nuclear binding component, while other steroids had little or no influence. The results on hormone specificity of this nuclear binding component are identical to those of putative cytosol ER, but contrast with those of the cytosol estradiol binder of this tumor line as presented here and in our previous papers (17, 18).

In addition to the temperature effect on \(K_d\) as described above, the buffer composition could affect the affinity of this nuclear component. Resuspension of isolated nuclei in TEM buffer almost completely abolished the temperature effect on \(K_d\) (3.1 ± 1.2 nM at 0° versus 2.2 ± 0.8 nM at 30°; \(n = 4\)) with some decrease in binding sites of nuclei exposed to 30° (1,284 ± 488 fmol/mg DNA at 0° versus 861 ± 393 fmol/mg DNA at 30°; \(n = 4\)) (Chart 8). When crude nuclei in TEM buffer were reisolated and suspended in Buffer 1, temperature dependency of \(K_d\) (10.5 ± 1.8 nM at 0° versus 2.5 ± 1.1 nM at 30°; \(n = 4\)) reappeared, indicating that the effect of the buffer (probably divalent ion effect) is reversible.

Chart 7. Hormone specificity of the nuclear binder in Tumor 124958. Purified nuclei resuspended in Buffer 1 were incubated with 10 nM \(^{3}H\)estradiol (E, ± 1 μM concentrations of unlabeled competitors at 0° for 2 hr. After incubations, nuclei were pelleted down and washed with Buffer 1 as described in “Materials and Methods.” The values in the absence of the competitor were considered as 100% binding. DHT, dihydrotestosterone; DEX, dexamethasone; PROG, progesterone. Columns, means of 4 experiments; bars, S.E.

Chart 8. Effects of buffer compositions on \(K_d\) and the maximum binding sites of the nuclear estrogen binders in Tumor 124958. Tumor tissues were homogenized in TEM buffer and centrifuged at 800 x g for 10 min. The pellets were washed 4 times with TEM buffer and resuspended in TEM buffer or Buffer 1 (A, B). These crude nuclei were incubated with \(^{3}H\)estradiol at 0° for 2 hr (A) or at 30° for 1 hr. (---). After incubations, the specific binding was obtained as indicated in “Materials and Methods.” \(\bullet, O\), molybdate buffer; \(\Delta, \triangle\), conventional buffer.

Chart 5. Lack of molybdate effects on the characteristics of the estrogen binder in Tumor 124958. The tumor was minced and divided into 2 parts. One was subjected to purification of nuclei in conventional sucrose buffers, and another was used for preparing nuclei in molybdate-containing buffers. The aliquots of these nuclei were incubated with \(^{3}H\)estradiol at 0° for 2 hr (---) or at 30° for 1 hr. (—). After incubations, the specific binding was obtained as indicated in “Materials and Methods.” \(\bullet, O\), molybdate buffer; \(\Delta, \triangle\), conventional buffer.

Chart 6. Determination of the number of the nuclear estrogen-binding sites using collagenase:trypsin-dispersed cell. The dispersed cells of Tumor 124958 obtained from the nonestrogenized host animals were incubated with \(^{3}H\)estradiol ranging from 0.3 to 20 nM in the presence or the absence of a 100-fold molar excess of unlabelled estradiol as described in “Materials and Methods.” The specific binding after being treated with Triton X-100 was analyzed according to a Scatchard plot method.

Unoccupied Nuclear D receptor and Tumor Growth
To further elucidate the nature of this binding component, effects of various enzymes as well as salt extractability were examined as shown in Chart 9. Treatment with RNase or micrococcal nuclease showed little or no decrease in the binding sites. Under these experimental conditions, acid-purified RNA and DNA were diminished to levels of 40 and 30% of nondigested values, respectively. On the other hand, trypsin had profound effects, resulting in a disappearance of more than 80% of the nuclear estrogen binder (Chart 9A). An attempt was also made to extract this unique binder from nuclei by high concentrations of KCl. The nuclear binder identified in Tumor 124958 was relatively resistant to this treatment, when compared with that of nuclear ERs in Tumor 22137 produced by incubation of isolated nuclei with heat-activated (25° for 30 min) cytosol [3H]estradiol:ER complexes (Chart 9B).

**DISCUSSION**

Steroid hormone receptors have generally been considered to be restrained in the cytoplasm of their target cells, waiting for the arrival of a specific hormonal steroid. Formation of the hormone:receptor complex presumably renders the receptor more nucophile, resulting in translocation of this complex into nuclei. Findings which show the interaction of hormonal steroids with membrane components (6, 15) have cast doubt on this central dogma of the action mechanism of steroid hormones. In view of the results reported by McGuire et al. (4, 7, 28), we must cautiously discuss the presence of unoccupied nuclear receptor in target cells. We concluded that the unoccupied estrogen binder exists in the nuclei of Tumor 124958 through the following observations: (a) the ability of the cytosol preparation to bind estrogens, especially DES, with high affinity could not be demonstrated; (b) purified nuclei were used in the present study; (c) Na2MoO4, a well-known inhibitor for receptor activation, did not affect subcellular localization of high-affinity estrogen binder; and (d) the incubation of intact cells with [3H]estradiol at 37° did not increase the number of nuclear binding sites. Based upon these experimental results, it could be speculated that the high-affinity estrogen binder is exclusively localized in the nuclei even in the absence of estrogen stimuli.

The theoretical background of the presence of this unoccupied nuclear binder seems to be worth taking into consideration. The activation process of putative ER would be mainly controlled by the dialyzable substance, a so-called low-molecular-weight inhibitor (19, 20). We have recently reported that the activation of ER is achieved even without the aid of an estrogen, causing the appearance of an unoccupied nuclear receptor under a cell-free condition (19). In addition, the cytosol of Tumor 124958 has been observed to contain a high concentration of the low-molecular-weight substance(s) rendering the estrogen-binding component in the low-affinity state for estrogen, but to contain the low concentration of the low-molecular-weight inhibitor suppressing activation of rat ER. Therefore, it seems to be plausible from these combined results that the high-affinity binder converted from the low-affinity component quickly reaches the activated state and forms the *unoccupied nuclear estrogen binder.*

Further eliciting the nature of this unique nuclear binding component, we have shown that its Kd can be altered in the temperature- and Mg2+-dependent manner. Whether these alterations reflect changes in this nuclear component itself or some other components is unclear at the present moment. However, it is of interest in this regard that these procedures have been reported to affect folding-unfolding states of chromatin architecture (13, 25). Although its nature remains unclear, this temperature effect on the affinity of nuclear estrogen binder should be kept in mind when nuclear exchange assays are performed. In addition, treatments of these nuclei with KCl or micrococcal nuclease resulted in only modest decreases in estrogen-binding sites. Under similar experimental conditions, micrococcal nuclease was found to be able to solubilize more than 60% of the nuclear ER which was created by incubating minced uterine tissue in the culture medium at 37° for 1 hr (data not illustrated). Since preferential cleavage of chromatin at certain sites such as transcriptionally active sites has been reported (26), many attempts have been made to analyze the interaction of steroid hormone receptor with chromatin by means of micrococcal nuclease (2, 13, 16, 22). Recently, this enzyme has been observed to attack nuclear ER associated at KCl extraction-resistant sites but not at sensitive sites in uterine nuclei (24). This result is not consistent with the observations presented in this paper, further suggesting the uniqueness of the nuclear binder in Tumor 124958. However, in view of the interesting observations reported by Shoenberg and Clark (23), some decrease in the nuclear binding sites by an exposure to TEM buffer, especially at 30°, might suggest that unidentified endogenous nuclease can attack this binder.

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*Unpublished observation.*
The demonstration that DES stimulates tumor growth would suggest the biological significance of this nuclear binder, since the high concentration of DES was found to have no effect on 
\[ ^{3}H \text{estradiol binding to the cytosol.} \] At early stages of transplantation (10 to 13 generations) when the tumor growth had not been influenced by estrogenization of host animals, the number of nuclear binding sites measured at 0° in the presence of 5 mM MgCl\(_2\) had been found to be approximately 100 fmol/mg DNA. The increase in the nuclear binding sites might be responsible for reacquisition of estrogen dependency in Tumor 124958, although the nuclear binding sites have unfortunately not been determined at every transplantation stage. Moreover, our recent observation has shown that estradiol is potentially equivalent to DES with regard to the enhancement of tumor growth. These results are compatible with the conclusion that this nuclear estrogen-binding component can mediate hormone action. Whether or not this unoccupied nuclear binder is able to affect cell functions without the aid of estrogens is not clear. The successful transplantation and subsequent slow growth of tumor seed in nonestrogenized host animals might be related to the presence of this unoccupied nuclear binder. However, it is quite certain that the full effect on tumor cells can be achieved by complexing with estrogen.

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Unoccupied Nuclear Receptor and Tumor Growth
Characterization of a Unique Nuclear Estrogen-binding Component in an Estrogen-responsive Mouse Leydig Cell Tumor

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