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Selective Increase in Type II Estrogen-binding Sites in the Dysplastic Dorsolateral Prostates of Noble Rats[1]

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ABSTRACT

We demonstrated previously that simultaneous treatment of intact Noble (NBL) rats with testosterone and estradiol-17β (E_2) for 16 weeks consistently induced a putative precancerous lesion, termed dysplasia, in the dorsolateral prostate (DLP) of all animals. Since treatment of rats with androgen alone did not elicit the same response, we concluded that estrogen played a critical role in the genesis of this proliferative lesion. In the present study, using radioligand binding assays, we investigated the properties and distributions of nuclear estrogen-binding sites in the two major prostatic lobes (DLP and ventral prostate) of the rat gland and examined the kinetics of alterations in estrogen-binding site levels following treatment of NBL rats with testosterone plus E_2. Saturation analyses revealed two distinct types of nuclear [^{3}H]E_2-binding sites in the rat prostate. The high-affinity species or type I sites bound [^{3}H]E_2 with high affinity (K_D 4–5 nM) and low capacity (0.4–0.6 pmol/mg DNA) and had a ligand specificity similar to that described for the classical estrogen receptor. The second estrogen-binding species or type II sites bound [^{3}H]E_2 with moderate affinity (K_D 25–30 nM) and higher capacity (2–4 pmol/mg DNA) and had characteristics similar to those of type II estrogen-binding sites found in the rat uterus. Type I sites were found in the nuclei of both ventral prostate and DLP, and their levels in the two prostatic lobes did not change following testosterone plus E_2 treatment of NBL rats. In contrast, type II sites were present exclusively in the nuclei of DLP. Treatment of NBL rats with testosterone plus E_2 for a period of 16 weeks induced a gradual increase in the levels of DLP nuclear type II sites, which was accompanied by parallel increases in DLP wet weight and total DNA content. Since nuclear type II sites have been implicated as a proliferation regulator, our findings suggest that (*a*) the lobe-specific localization of type II sites in rat DLP may confer unique estrogenic susceptibility on this tissue and (*b*) elevation of nuclear type II sites in rat DLP following testosterone plus E_2 stimulation may be the underlying cause of enhancement of cell proliferation and dysplasia induction in this prostatic lobe.

INTRODUCTION

Evidence has accumulated to suggest a contributory role for estrogen in the pathophysiology of the prostate. Past findings have demonstrated that estrogens alone can induce focal proliferation in the prostate (1–5) and that estrogens, in synergism with an androgen, are involved in the development of aberrant growth of the gland (1, 3, 6–9). Studies in NBL[3] rats clearly indicate that estrogens play a determining role in the genesis of carcinomas of the prostate. Long-term (over 52 weeks) treatment of NBL rats with testosterone induces a low incidence (18%) of adenocarcinoma in DLP of the rat gland (8), whereas treatment of rats with testosterone and an estrogen dramatically increases the incidence of carcinoma (9, 10) and shortens the latency period for cancer development (9).

More recently, we demonstrated the development of a proliferative lesion, termed dysplasia, arising exclusively in the DLP of NBL rats treated simultaneously with testosterone and E_2 for 16 weeks (11, 12). The lesion closely resembled a putative, precancerous lesion, termed intraductal dysplasia (13) or prostatic intraepithelial neoplasia (14), reported in the human prostate. Since exposure of NBL rats to testosterone or the nonaromatizable androgen, 5α-dihydrotestosterone, alone for 16 weeks did not produce prostatic dysplasia (11) we concluded that the action of an estrogen was necessary for the induction of this early preneoplastic lesion which might eventually develop into carcinoma in rat DLP.

Although little is known about the mechanism of action of estrogen in the prostate it is widely accepted that this steroid exerts its action via intracellular receptors (15). Using ligand-binding assays we and others have demonstrated that multiple forms of estrogen-binding species exist in the prostates of the rat (16–19) and of other mammalian species (20–29). The classical estrogen receptor or type I ER, which binds estrogen with high affinity and low capacity, has been demonstrated in all prostatic tissues studied (16–29). Type I ER is present in very low concentrations in adult prostatic tissues and principally resides in the fibromuscular stroma (24, 25). The biological significance of prostatic type I ER is unclear, although it has been suggested that it may mediate the suspected action of estrogen in nodular hyperplasia, which involves stromal proliferation (25).

In addition to type I ER a second estrogen-binding species, referred to as type II estrogen-binding sites (type II sites), has been reported in the prostates of the rat (16–19) and the human (20). Type II sites bind estrogen with moderate affinity and high capacity. Studies in the rat uterus (30, 31) and in a variety of cancer tissues (32, 33) have implicated this estrogen-binding species as an important regulator of proliferation. In an earlier study we partially characterized type II sites in the prostates of SD rats and showed that this estrogen binder is localized exclusively in the DLPs of SD rats (19). We also obtained preliminary data to demonstrate an increase in type II sites in DLPs of NBL rats harboring dysplasia (11). In the present study we have reconfirmed the lobe-specific localization of type II sites in the DLPs of NBL rats. Furthermore, we have demonstrated a selective increase in type II sites, attended by a parallel increase in tissue wet weights and total DNA contents, in the DLP following treatment of the animal with testosterone plus E_2. Since the increases in DLP type II sites and tissue wet weights and DNA contents occurred prior to the appearance of dysplasia in this prostatic lobe, we contend that type II site-mediated cell proliferation is causally linked to dysplasia induction in this tissue.

MATERIALS AND METHODS

Animals and Sex Steroid Treatments. Sexually mature NBL rats (280 g) were surgically implanted with two 2-cm Silastic capsules (no. 602-205; 1.0 mm inner diameter x 2.2 mm outer diameter; Dow-Corning Corporation, Corning, NY) filled with testosterone (Sigma, St. Louis, MO) and one 1-cm capsule filled with E_2 (Sigma). Rats treated for 16 weeks were used in all studies except for the time study experiment, in which rats were treated for 1, 4, 8, and 16 weeks. Controls were age-matched untreated animals.

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[3] The abbreviations used are: NBL, Noble; DLP, dorsolateral prostate; VP, ventral prostate; E_2, estradiol-17β; SD, Sprague-Dawley; DES, diethylstilbestrol; TEG buffer, 10 mM Tris-HCl, 1.5 mM Na_2 EDTA, 10% glycerol, pH 7.4; MeHPLA, methyl-*p*-hydroxylphenyllactate; DHBA, 4,4′-dihydroxylbenzylidene acetophenone; ER, estrogen receptor.

Steroids. Steroids used to fill Silastic capsules were purchased from Sigma (St. Louis, MO), while those for biochemical studies were from either Steraloids (Pawling, NY) or Sigma. [2,4,6,7-^{3}H(N)]E_2 (90–100 Ci/mmol) was purchased from Research Products International (Mount Prospect, IL). 16α-[^{125}I]-iodo-E_2 (~200 Ci/mmol) was purchased from NEN Research Products (Boston, MA). All stock solutions were prepared in 1000× concentrations in absolute ethanol and stored in brown bottles under nitrogen at −20°C. MeHPLA was synthesized by Sean Collins in Dr. Edward Brush's laboratory of the Chemistry Department at Tufts University. DHBA was the generous gift of Dr. Barry M. Markaverich of the Center for Biotechnology at the Baylor College of Medicine.

Buffers and Solutions. The TEG buffer contained 10 mM Tris-HCl, 1.5 mM Na_2 EDTA (Sigma), and 10% glycerol (EM Science, Cherry Hill, NJ) (pH 7.4). Nuclei wash buffer contained 10 mM Tris-HCl (Sigma), 0.25 M sucrose (Sigma), and 5 mM $MgCl_2$ (Fisher, Pittsburgh, PA) (pH 7.5). Culture medium (Gibco, Grand Island, NY) was Eagle's minimal essential medium with Earle's salts, minus bicarbonate and glutamine, and with 0.25% bovine serum albumin (Sigma) (pH 7.5). Scintillation fluid was Bio-Safe NA from Research Products International Corp.

Preparation of Nuclei for Estrogen Receptor Assay. Different prostate lobes were excised, minced, and washed once in culture medium and once in TEG buffer. They were then homogenized in 5 volumes (per gram tissue wet weight) of TEG buffer with Tissumizer (Tekmar, Cincinnati, OH) using eight 7-s bursts at a rheostat setting of 8 with 30 s of cooling between bursts. The tissue homogenate was centrifuged at 800 × *g* for 20 min. The supernatant was discarded, and the nuclear pellet was resuspended in 10 volumes of nuclei wash buffer, filtered once through double-layered cheesecloth, and washed twice with 10 volumes of buffer by resuspension and centrifugation at 800 × *g* for 15 min. The washed nuclear pellet was resuspended in 5 volumes (for original weight of tissue) of TEG buffer with a Teflon glass homogenizer. An aliquot was taken for DNA determination, and the rest of the sample was used immediately in an [^{3}H]E_2 binding assay. Assay conditions optimized for [^{3}H]E_2 binding to prostatic type II sites had previously been reported by Yu *et al.* (19) and were used in all assays.

[^{3}H]E_2 binding in each sample was analyzed by saturation analysis. Aliquots of a sample were incubated with [^{3}H]E_2 over a range of 0.1 to 40 nM. The incubation was carried out for 30 min at 35°C in the presence or absence of a 300-fold molar excess of DES. At the end of the incubation period hydroxylapatite (Bio-Rad, Richmond, CA) suspension (30% hydroxylapatite in TEG buffer) was added to each incubate, and the bound and free steroids were separated according to the procedure described by Ho *et al.* (34), except that the hydroxylapatite pellet was washed with TEG buffer instead of a phosphate buffer. Specifically bound [^{3}H]E_2 at each [^{3}H]E_2 concentration was calculated by subtracting the nonspecifically bound (radioactivity determined in the presence of DES) from the total bound (radioactivity determined in the absence of DES). DNA contents of the nuclear suspensions were determined by the diphenylamine procedure (35). The amount of bound [^{3}H]E_2 radioactivity was expressed as fmol or pmol/mg DNA in the samples. In a limited number of experiments [^{125}I]E_2 was used instead of [^{3}H]E_2 for the receptor binding assay.

Statistical Methods. Data points are group mean values. *n* indicates the number of nuclei preparations used to obtain a group mean value. One-way analysis of variance was used to analyze whether there was a significant difference among the various group means, and a multiple range test using the Tukey-B procedure was used to compare the individual group means.

RESULTS

Presence of a Single Class of Estrogen-binding Sites in the VPs of NBL Rats. Radioligand binding assays revealed only a single class of [^{3}H]E_2-binding sites in the VP nuclei of NBL rats. It binds estrogen with high affinity (K_D 4–6 nM) and low capacity (0.6–0.8 pmol/mg DNA). It has characteristics of the classical estrogen receptor (15) and is identical to the type I ER found in the VP nuclei of SD rats (19). Since the characteristics of prostatic type I estrogen-binding sites in SD rats have previously been reported (19) we have not included a detailed description of this receptor species in the current paper. Nevertheless, it is of interest to note that the levels of type I estrogen-binding sites in the VPs of NBL rats were not altered following testosterone plus E_2 treatment of the animals.

Heterogeneity of Estrogen-binding Sites in the DLPs of NBL Rats and Effects of Testosterone plus E_2 Treatment on Their Binding Characteristics. Fig. 1 shows a representative saturation analysis of specific binding of [^{3}H]E_2 to crude nuclei isolated from the DLPs of an untreated (Fig. 1*A*) and a testosterone plus E_2-treated (Fig. 1*B*) rat. The saturation curves are biphasic in nature, and each can be resolved into two separate components (type I and type II) by a graphic method previously described (36). Each of these components has been subjected to Scatchard analyses (37), and plots of the data are as shown in Fig. 2*A*.

The apparent dissociation constant (K_D) and the capacity (*c*) of type I estrogen-binding component were estimated from the Scatchard plots (Fig. 2*A*). The K_Ds for type I estrogen-binding component in the DLPs of untreated and testosterone plus E_2-treated rats were 4.8 ± 0.6 (mean ± SEM, n = 3) and 5.2 ± 0.7 nM (n = 3), respectively. The capacity of type I estrogen-binding component in the DLPs of untreated and testosterone plus E_2-treated rats was 0.42 ± 0.05 (n = 9) and 0.60 ± 0.10 pmol/mg DNA (n = 9), respectively. The values from untreated and treated animals were not statistically different from each other ($P > 0.05$).

No accurate estimation of the K_Ds and the capacities of prostatic type II estrogen-binding component (type II sites) can be made from the nonlinear Scatchard plots. Estimates of these parameters were obtained from the saturation curves. The K_Ds of the type II component were determined as those concentrations of [^{3}H]E_2 required for half-saturation. K_Ds of the type II estrogen-binding component in DLP nuclei obtained from untreated and testosterone plus E_2-treated NBL rats were similar; both ranged between 25 and 30 nM. Testosterone plus E_2-treatment of NBL rats significantly increased the capacity of type II estrogen-binding component in the DLP nuclei (Figs. 1 and 3*A*) from a value of 2.1 ± 0.2 pmol/mg DNA (n = 9) found in

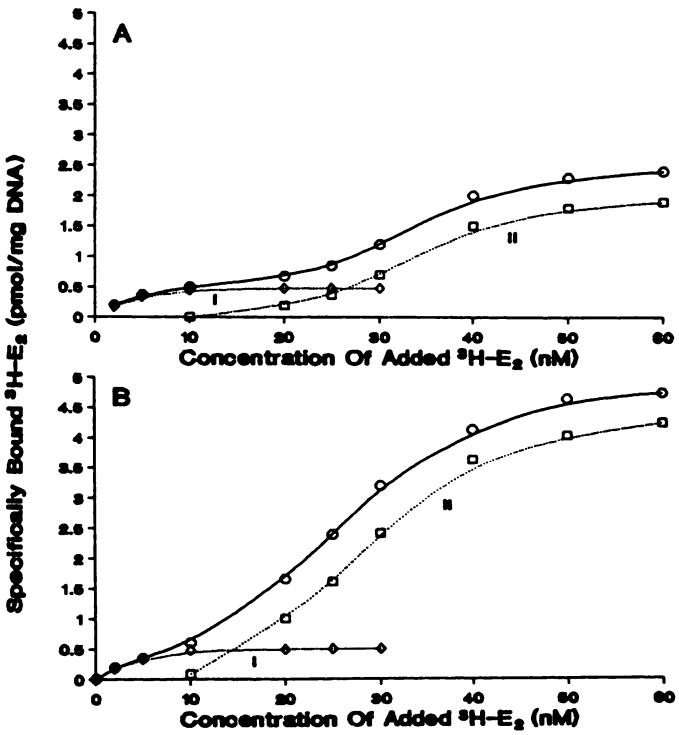

Fig. 1. Saturation analyses of [^{3}H]E_2 specifically bound to prostatic nuclei of DLPs of untreated (*A*) and 16-week testosterone plus E_2-treated (*B*) rats. ○, specific [^{3}H]E_2 binding (pmol/mg DNA); ◇, type I component; □, type II component.

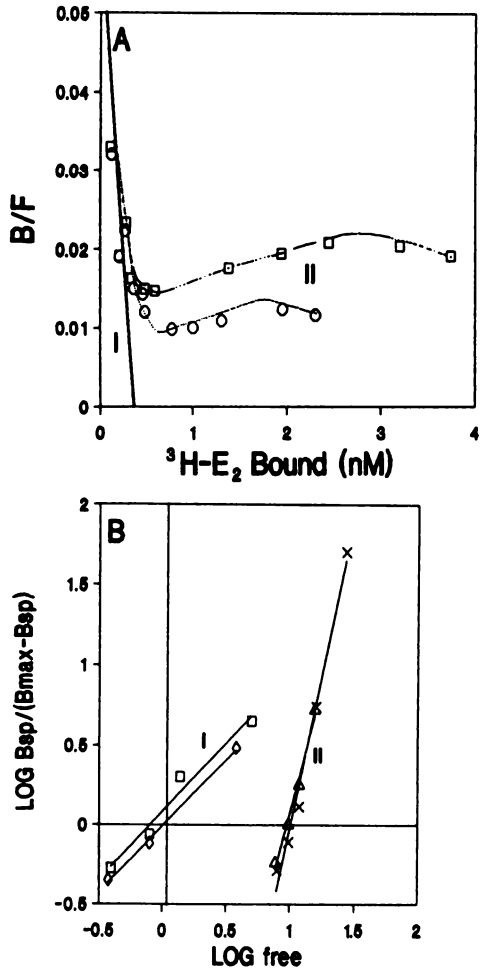

Fig. 2. *A*, Scatchard analysis of specific binding data shown in Fig. 1. ○, Scatchard plot of specific [^{3}H]E_2 binding to nuclei prepared from DLPs of untreated rats. □, Scatchard plot of specific [^{3}H]E_2 binding to nuclei obtained from DLPs of 16-week testosterone plus E_2-treated rats. *B*, Hill analyses of specific binding data shown in Fig. 1. □ and ◇, Hill plots of type I [^{3}H]E_2 binding to nuclei preparations obtained from DLPs of untreated and 16-week testosterone plus E_2-treated rats, respectively. × and ◇, Hill plots of type II [^{3}H]E_2 binding to nuclei preparations obtained from DLPs of untreated and 16-week testosterone plus E_2-treated rats, respectively.

untreated rats to a value of 4.5 ± 0.3 pmol/mg DNA (n = 9) observed in the testosterone plus E_2-treated animals ($P < 0.05$).

Hill analyses (Fig. 2*B*) of the saturation curves yielded Hill coefficients (slopes of the plots) close to 1 for the type I component and values greater than 1 (3.25 to 3.75) for the type II component. Although not conclusive, these data indicate that prostatic type II estrogen-binding component likely contains multiple estrogen-binding sites with a high degree of positive cooperativity among the binding sites, while the type I component exhibits only a single class of estrogen-binding sites with no cooperativity.

Other biochemical and binding characteristics of the type I and type II estrogen-binding components in DLP nuclei of NBL rats were found to be similar to those reported for type I and type II estrogen-binding sites in the DLPs of SD rats (19). Hence, only a brief summary of these characteristics is described here. Type I estrogen-binding component or type I ER in DLP nuclei of NBL rats has characteristics of the classical estrogen receptor (15). It binds estrogen with high affinity and low capacity; it is insensitive to sulfhydryl-reducing reagents; and it binds E_2 and DES better than E_1. Type II estrogen-binding component or type II sites in the nuclei of rat DLP binds estrogen with moderate affinity and a high capacity. It is sensitive to sulfhydryl-reducing reagents such as dithiothreitol, which completely eliminates its affinity for [^{3}H]E_2. It binds DES best, followed by E_2 and E_1 with almost equal affinity. Furthermore, MeHPLA and DHBA are effective competitors of [^{3}H]E_2 for type II estrogen-binding sites; each produces a 60% inhibition when present at 3000-fold molar excess [^{3}H]E_2 concentration. These two compounds do not bind type I ER and have been reported as specific type II site ligands in the rat uterus (32, 33).

When [^{125}I]E_2 was used as the radioligand in a limited number of receptor assays, only type I estrogen binding was observed in the DLP nuclei. This finding indicates that substitution of a bulky group at the C_{16} position drastically reduces the steroid's ability to bind to type II sites.

Changes in the Levels of Type I and Type II Estrogen-binding Component in the DLPs of NBL Rats following Testosterone plus E_2 Treatment. We examined the time course of alterations in the levels of type I and type II estrogen-binding sites in the DLP nuclei of NBL rats over a period of 16 weeks of testosterone plus E_2 treatment (Fig. 3*A*). The level of the type II sites was 2.1 ± 0.2 pmol/mg DNA (n = 17) before hormonal treatment and increased gradually over a period of 16 weeks to 4.5 ± 0.3 pmol/mg DNA (n = 9). The levels in the 16-week hormone-treated rats were statistically higher than those found in untreated animals ($P < 0.05$). In contrast, the level of type I estrogen-binding component remained unchanged throughout the entire hormonal treatment period (P = 0.124). Relative prostatic wet weight (tissue weight as a percentage of body weight) as well as

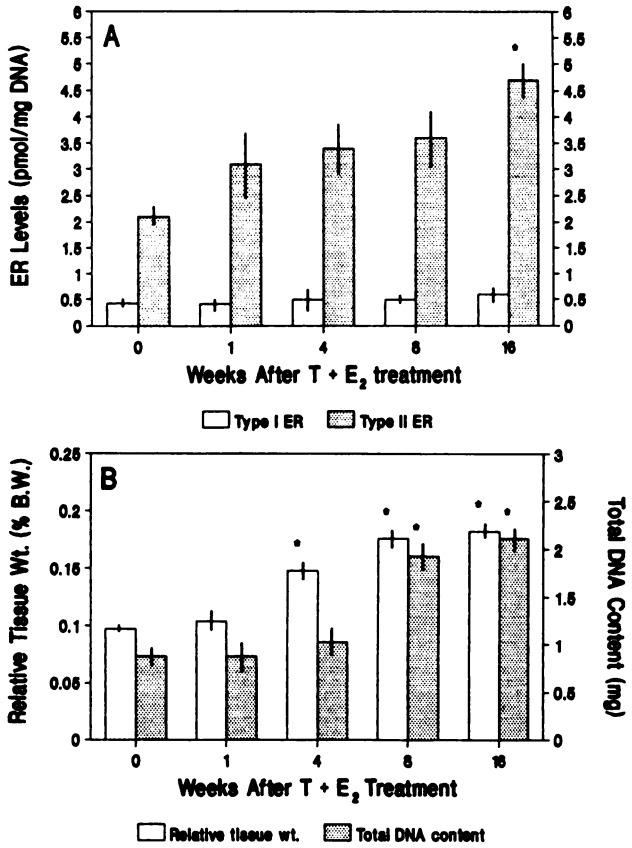

Fig. 3. Alterations in nuclear levels of type I and type II estrogen-binding sites (*ER Levels*) (*A*), in pmol/mg DNA, and changes in relative prostatic wet weights (tissue wet weight as a percentage of body weight) and total DNA contents (mg) (*B*) of rat DLP following testosterone plus E_2 treatment for 16 weeks. Each data point represents the mean ± SEM (*histograms* and *error bars*) of values found in 4–10 animals. The actual number of preparations from individuals animals used to calculate the data points was 10 for 0-week, 8 for 1-week, 4 for 4-week, 8 for 8-week, and 9 for 16-week testosterone plus E_2 treatment. *, significant difference between the mean of a testosterone plus E_2 treatment group and the untreated controls at $P < 0.05$.

total prostatic DNA content rose soon after the onset of the hormonal treatment and reached plateau levels at the eighth week of the treatment period (Fig. 3*B*). We detected DLP dysplasia histologically in 100% of the animals treated for 16 weeks (11, 12) but did not find any dysplasia in the DLPs of animals treated for only 8 weeks.[4]

DISCUSSION

We identified two estrogen-binding components in the prostatic nuclei of NBL rats. The first estrogen-binding component or type I ER exhibits a single class of $[^3H]E_2$-binding sites. It binds $[^3H]E_2$ and $[^{125}I]E_2$ with high affinity (K_D 4–5 nM) and low capacity (0.4–0.6 pmol/mg DNA), and it is insensitive to sulhydryl reagent. The second estrogen-binding component or type II site possesses multiple $[^3H]$-E_2-binding sites with positive cooperativity among them. Type II sites bind $[^3H]E_2$ with moderate affinity (K_D 25–30 nM) and large capacity (2–4 pmol/mg DNA). It exhibits no binding affinity for $[^{125}I]E_2$, and its estrogen-binding affinity is drastically reduced by exposure to sulhydryl reagent. Type II sites can also be distinguished from type I ER by virtue of its affinity for MeHPLA and DHBA, two known ligands for type II sites in the rat uterus (32, 33). These findings are in agreement with past reports that demonstrated the presence of multiple forms of estrogen-binding components in the prostates of rat (16–19), human (24), and guinea pig (38).

Prostatic type I ER possesses characteristics of the classical estrogen receptor, which has been studied in great detail in female tissues (15). Using ligand-binding assays and immunocytochemical techniques low levels of type I ER have been demonstrated in normal and disease prostatic tissues of a great variety of mammalian species (24, 26, 29, 39). Most studies have indicated a fibromuscular, stromal localization of the receptor (23, 25, 28, 39) with a few reporting focal basal epithelial residency (25, 29). Luminal epithelium of the prostate is always devoid of this receptor species (24, 28, 29). Since type I ER is principally localized in the stroma, it has been implicated to play a role in mediating nodular hyperplasia, which involves stromal proliferation. However, type I ER has not been detected in the stroma of obstructive benign prostatic hypertrophy (39) and is rarely found in prostatic carcinomas (29). Although the administration of estrogens to prostatic hypertrophy patients (26) or rhesus monkey (28) promotes nuclear association of ER and an increase in the progesterone receptor level in the prostate, the biological significance of these changes is unclear. In sum, even though type I ER is present and apparently functional in the prostate, its role in growth regulation remains speculative. In this study we detected low levels of type I ER in both the VP and the DLP of NBL rats. Since its levels remained unchanged in both prostatic lobes following treatment of NBL rats with testosterone plus E_2, we believe that this receptor species may not be a major regulator of proliferation and dysplasia development in the rat gland.

In the search for an alternative mechanistic basis for estrogen action in the prostate we studied the moderate-affinity estrogen-binding component or type II sites in the rat prostate. Type II sites were found to be localized exclusively in the DLP but not the VP of the rat gland. Following testosterone plus E_2 treatment of NBL rats the level of type II sites in the DLPs increased gradually over a period of 16 weeks. The increase in receptor level was accompanied by parallel increases in tissue wet weight and total DNA content in the lobe. Since dysplasia did not occur until week 16 of hormone treatment, we conclude that the sex hormone-induced increase in type II sites, attended by growth of the gland, precedes dysplasia induction in rat DLP.

Although the biological function of type II sites in rat DLP is currently unknown, studies of type II sites in the rat uterus suggest that they may play a role in proliferation regulation (30, 31). Following the administration of estrogens to female rats, nuclear type II sites in the rat uterus increased dramatically and were sustained throughout the period of cell proliferation, while the levels of nuclear type I ER in the tissue had already returned to untreated control values. Later studies have shown, however, that this protein is tightly associated with the nuclear matrix and may play a role in regulating DNA synthesis (40). The fact that type II sites are found in a variety of normal and malignant estrogen target and nontarget tissues (32, 33, 41, 42) implies a role for this protein as a ubiquitous cell proliferation regulator. In support of this view are the findings that type II sites exist in an inactive state in normal tissues while they are bound to an endogenous ligand, MeHPLA, and the receptor is "permanently stimulated" in malignant tissues such as rat, mouse, and human breast cancer tissues where MeHPLA is deficient (32, 43). In the present paper we have confirmed our earlier findings in SD rats (19) that type II sites are localized specifically to the DLP of the rat gland. The exclusive presence of this receptor species in rat DLP may confer unique estrogen sensitivity on this prostatic lobe. Furthermore, we have shown here that testosterone plus E_2 treatment induces an increase in type II sites and growth in the DLP. These data are in accord with the contention that enhanced type II site action in rat DLP may play a role in initiating and/or sustaining cell proliferation in this prostatic lobe. Ames and others (44, 45) have proposed induction of sustained cell proliferation as a critical factor in tumor formation. These authors argue that cell division *per se* increases the risk of genetic errors of various kinds and provides opportunities for pro-mutations to be converted into mutations. An accumulation of a combination of such genetic errors eventually leads to neoplastic transformation. Based on this model of cancer pathogenesis, we believe that the sex hormone-induced type II site activation leads to enhancement of cell proliferation in rat DLP, which in turn causes dysplasia development and neoplastic transformation in this tissue. The suspected role of type II sites in the DLP would certainly provide an explanation for the apparent predilection of this prostatic lobe for cancer development upon sex steroid stimulation (8–10).

The NBL rat model provides a prospect for understanding the etiology of prostatic proliferative diseases in humans. In men testosterone secretion as well as plasma testosterone levels decline with age (46–48). Plasma free estrogen levels, on the other hand, increase in male senescence because of increased conversion from androgens (47) and rising levels of the sex hormone binding protein in circulation (47, 48). These changes result in a higher ratio of free estrogen to free androgen in the circulation of elderly men, which may provide the stimulus needed to produce proliferative lesions in the prostate. In this regard it is of interest to note that Ekman *et al.* have (20) reported type II estrogen-binding sites in human prostatic tissues. Thus enhanced estrogenic action via type II site activation may well be the underlying cause of unregulated growth in the human gland, a process that, in turn, leads to tumor formation.

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[4] S-m. Ho and M. Yu, unpublished observations.

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