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Multiple Estrogen Binding Sites in Malignant Mouse Leydig Cells and Their Role in Cell Proliferation*

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Abstract—*Incubation of dispersed cells derived from an estrogen-responsive mouse Leydig cell tumor with* [^{3}H]*estradiol in the presence or absence of unlabeled estradiol showed the existence of two types of binding components with high* ($K_d \sim 10^{-9}$ M) *and low* ($K_d \sim 1.5 \times 10^{-8}$ M) *affinity respectively. The use of unlabeled diethylstilbestrol as a competitor, however, abolished the low-affinity binder, resulting in the demonstration of the high binding site. This diethylstilbestrol-suppressible binding site was exclusively located in the nuclear fraction, even without hormonal stimuli. A long (5-hr) exposure of these cells to estrogen caused the decrease in the number of nuclear estrogen binding sites, which was similar to so-called 'processing' of putative nuclear estrogen receptor. The direct stimulative effect of estrogen on the proliferation of these cells was demonstrated by measuring cell numbers as well as thymidine incorporation into DNA in the cultured condition. These results would indicate that estrogen directly exerts its effects on this malignant Leydig cell through this unique nuclear binding site.*

INTRODUCTION

THE PRESENCE of estrogen receptor (ER) in the malignant cell cytosol has been considered to be a primary requirement for successful endocrine manipulation [1]. This central dogma has been doubted since a human breast cancer cell line (MCF-7) was reported to possess an unoccupied nuclear receptor [2]. The concomitant presence of putative cytosol ER in MCF-7 cells, however, has complicated the biological role of this nuclear binding component. In addition, the presence of an unoccupied nuclear receptor in MCF-7 cells has not been completely accepted [3]. Identification of estrogen-responsive cells containing only the unoccupied nuclear receptor without cytoplasmic ER would be able to lead to the clarification of its significance in transmitting estrogen signals. These cells, if present, might also provide us with a powerful tool for understanding the translocation mechanism of ER from the cytosol to the nuclei, usually occurring in many target cells.

We reported that one of the mouse Leydig cell tumor lines contains the high-affinity unoccupied nuclear estrogen-binding component when analyzed under cell-free conditions [4]. In addition, this tumor line was found to lack a putative cytosolic ER. The aims of the present study were to confirm our previous results using intact dispersed cells and to demonstrate the direct effects of estrogen on cultured malignant Leydig cells.

MATERIALS AND METHODS

Chemicals

17β[2,4,6,7-^{3}H]Estradiol (E_2) (115 Ci/mmol) and [methyl-^{3}H]thymidine ([^{3}H]TdR, 46 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Hormonal steroids and diethylstilbestrol (DES) were from Steraloid, Inc. (Wilton, NH). Collagenase (type IV) and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO) and Worthington Biochemical Corp.

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(Freehold, NJ) respectively. Calf fetal serum was from Collaborative Research (Waltham, MA). The other reagents used here were of analytical grade.

Experimental tumors

The original mouse Leydig cell tumors and their host mice (BALB/c) were kindly supplied by Dr R. A. Huseby, Henry Ford Hospital, Detroit, MI. One of the estrogen-responsive tumors, designated as tumor code 124958 [4], was used (30-34th generation). Its tumor growth was accelerated by estrogenization of host mice. In the present experiments, however, tumors slowly grown in non-estrogenized mice were subjected to *in vitro* analyses.

Preparation of dispersed tumor cells and their interaction with [^{3}H]E_2

The detailed method to obtain the dispersed tumor cells with collagenase–trypsin digestion has been published previously [4]. The aliquots (0.27 ml) of these cells suspended in modified Eagle's medium (usually 3–5 × 10^6/ml) were incubated with [^{3}H]E_2 in the presence or absence of a large excess of unlabeled competitors in a final volume of 0.3 ml in 95% O_2:5% CO_2. After incubation these cells were washed three times with ice-cold phosphate-buffered saline (PBS), pH 7.4. When the subcellular distribution of estrogen-binding components was investigated, the washed cells were homogenized in 1 ml of 0.32 M sucrose–5 mM $MgCl_2$–10 mM Tris–2 mM mercaptoethanol, pH 7.4, at 20°C (buffer 1) using a Teflon-glass homogenizer. This homogenate was subjected to centrifugation at 800 *g* for 10 min. The pellet was washed once with buffer 1, with buffer 1 supplemented with 0.25% Triton X-100 and then once more with buffer 1. The procedure used here provided us with relatively clean nuclei contained with few cytoplasmic tags, judged by an aceto-orcein fast green staining method. In addition, further purification of nuclei using a discontinuous sucrose density gradient did not affect the values of nuclear estrogen binding sites on the basis of per DNA (data not shown). Therefore, a time-consuming sucrose density gradient procedure was omitted in order to handle many samples. The difference in [^{3}H]E_2 associated with washed nuclei or cells with and without a competitor was considered as the specific binding. The supernatant from initial centrifugation at 800 *g* was employed to measure the content of the cytoplasmic estrogen binder with the hydroxylapatite method when specified [5]. The subcellular fractionation of tumor tissues was performed using the method described previously [4]. Aliquots (0.15 ml) of the cytosol or nuclear suspension were incubated with increased concentrations of [^{3}H]E_2 ± 4 μM unlabeled E_2 (see Fig. 4, legend).

Identification of E_2 metabolites

The cells incubated with [^{3}H]E_2 as described above were extracted twice with 15 vols of CH_2Cl_2. Radioactive steroids in the aliquots of the extracts were separated by thin-layer chromatography on 5 × 20-cm Merck F-254 silica gel plates using $CHCl_3$–CH_3OH (95:5, vol/vol) [6]. After scanning the radioactivity on the plates, the regions co-migrated with estrone, estradiol and estriol were scraped off, and the radioactivity in these regions ws estimated.

Cell growth experiments

Cells prepared as described above were plated at the cell density of ~2 × 10^5 in a 35-mm^2 dish (Falcon Plastics Co., Los Angeles, CA) and grown in 2 ml of improved minimal essential medium (MEM) supplemented with 2 mM glutamine, kanamycin (60 mg/l) and 10% fetal calf serum, which had been pretreated with 1% charcoal–0.01% dextran at 40°C for 30 min. This treatment lowered the endogenous estradiol concentration in the serum to less than 10^{-14} M, measured by radioimmunoassay. Cells were cultured in the presence or the absence of various concentration of E_2 and fed every 48 hr. At specified intervals, cells were harvested in PBS containing 0.2% EDTA and 0.05% trypsin, and counted with a hemocytometer. The viability (more than 85%) and identification (more than 90% in the cultured cells) of malignant Leydig cells were confirmed by the trypan blue dye exclusion and nitroblue tetrazolium staining methods respectively [7]. To carry out [^{3}H]TdR incorporation experiments, cells precultured in 10^{-8} M E_2–10% charcoal-treated fetal calf serum in MEM were subcultured at a cell density of 2.5 × 10^5/ml in Costar 96-well tissue culture clusters (Bellco Glass Inc., Vineland, NJ). E_2 was added to the wells at a 10-fold concentration in a volume of 0.02 ml to give the final concentrations indicated. Cells were cultured for 15 days as described above. [^{3}H]TdR (1 μCi/well) was added to each well 6 hr before cells were harvested. The aliquots of harvested cells were used for cell number counting. Another aliquot was mixed with an equal volume of ice-cold 0.4 N perchloric acid (PCA) followed by centrifugation at 800 *g* for 10 min. The precipitated materials were washed twice with 0.2 N PCA and then subjected to measurement of the radioactivity as previously published [8].

RESULTS

Time course studies on the binding of [^{3}H]E_2 *to dispersed malignant Leydig cells*

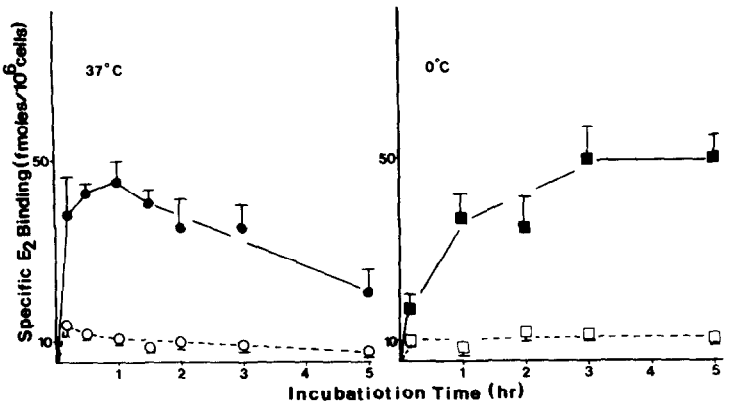

Fig. 1. Effects of incubation periods and temperatures on association of [^{3}H]E_2 to transformed Leydig cells. The well-dispersed cells were incubated with 10 nM [^{3}H]E_2 ± 1 μM unlabeled E_2 at 37 (left panel) or 0°C (right panel) for the indicated periods of time. After incubation cells were washed and subjected to a subcellular fractionation between nuclei (●——●) and cytoplasm (○——○) as described in Materials and Methods. The hydroxylapatite slurry (50 μl) was added to the cytoplasmic fraction. In this experiment the radioactivity associated with washed hydroxylapatite was considered to be the amount of the cytoplasmic binder. The data (mean ± S.E.) on the specific binding were obtained from four separate experiments.

Well-dispersed cells derived from one of the mouse Leydig cell tumor lines (T-124958) were incubated with 10 nM [^{3}H]E_2 ± 1 μM unlabeled E_2 for the indicated periods of time at 37 or 0°C (Fig. 1). Exposure to [^{3}H]E_2 at 37°C resulted in the rapid association of [^{3}H]E_2 with the nuclear fraction and the maximum number of the specific E_2-binding sites was found at approximately 1 hr, with a gradual decrease thereafter, reaching half of the maximum values of 5 hr. On the other hand, a more delayed nuclear binding of [^{3}H]E_2 was observed when these cells were incubated at 0°C. In both low- and high-temperature conditions it is noteworthy that the cytoplasmic fraction did not contain a significant amount of ER when measured by the hydroxylapatite method. In addition, a 5-hr incubation at 37°C caused neither a conversion of E_2 to its metabolites nor a decrease in cell viability (Table 1).

Saturation analysis of estrogen-binding components in malignant Leydig cells

To 270-μl aliquots of cell suspension were added various concentrations of [^{3}H]E_2 in the presence (non-specific) or absence (total) of 4 μM

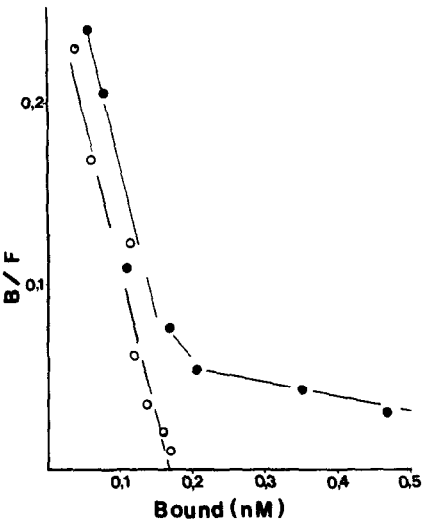

Fig. 2. Saturation analysis of the estrogen-binding component in intact transformed Leydig cells. The dispersed cells were incubated with various concentrations of [^{3}H]E_2 in the absence or presence of 4 μM unlabeled E_2 (●——●) or DES (○——○) at 37°C for 1 hr. After being washed, the specific binding associated with these cells was analyzed according to the Scatchard method.

Table 1. Influences of a 5-hr incubation at 37°C on cell viability and [^{3}H]E_2 metabolism

Incubation periods (hr)	Cell viability (%)	% conversion of [^{3}H]E_2 into metabolites		
		Estrone	E_2 (non-metabolized)	Estriol
0	86 ± 2	0	98 ± 1	0
5	84 ± 5	1.0 ± 0.5	89 ± 5	2.1 ± 1.4

The cell viability and [^{3}H]E_2 metabolism were examined before and after the incubation of these transformed Leydig cells with 10 nM [^{3}H]E_2 as described in the legend of Fig. 1. The data presented here are mean ± S.E., obtained by four separate experiments.

unlabeled E_2, and incubations was carried out for 1 hr at 37°C. Replotting the binding data for specifically bound [^{3}H]E_2 according to Scatchard's method [9] resulted in the demonstration of the existence of two binding components in the malignant cells; apparent dissociation constants (K_d) of these two sites were ~10^{-9} and ~1.5 × ~10^{-8} M respectively (Fig. 2). The use of unlabeled DES to obtain the value for non-specific binding, however, revealed a single class of binding site. Its K_d and the maximum number of binding sites were similar to those of the high-affinity site obtained by using radioinert E_2 as a competitor. In the following section the estrogen binding site whose association with [^{3}H]E_2 was inhibited by DES is designated as the DES-suppressible site and the binder whose association with [^{3}H]E_2 was blocked by a large excess of unlabeled E_2 is called the E_2-suppressible site. The data on E_2 - and DES-suppressible binding sites are summarized in Table 2.

Subcellular localization of [^{3}H]E_2*-binding sites*

After these cells were incubated with 10 nM [^{3}H]E_2 in the presence or absence of 1 μM radioinert E_2 or DES at 37°C for 1 hr, the subcellular fractionation was performed as described in Materials and Methods. The data depicted in Fig. 3 clearly show that the DES-suppressible site is exclusively present in the nuclear fraction. On the other hand, approximately one-half of the E_2-suppressible sites are not localized in nuclei. As described in Fig. 1, the hydroxylapatite method failed to demonstrate the presence of the cytoplasmic E_2-suppressible site, indicating the lack of interaction of this cytoplasmic binder with hydroxylapatite in this condition. In addition, the number of nuclear E_2-suppressible sites was almost equal to that of DES-suppressible sites. These results observed in intact cells were compared with the findings under cell-free conditions. The cytosol and isolated nuclei prepared from tumor 124958 were

Table 2. Summarized data on the dissociation constant and number of the maximum binding sites

	High-affinity site		Low-affinity site	
	K_d (nM)	MBS (fmol/10^6 cells)	K_d (nM)	MBS (fmol/10^6 cells)
E_2-suppressible site	0.90 ± 0.15	58.0 ± 19.7	16.2 ± 5.6	246 ± 54
DES-suppressible site	1.00 ± 0.27	35.3 ± 6.2	not detectable	

The malignant Leydig cells were incubated with the various concentrations of [^{3}H]E_2 in the absence or the presence of 4 μM radioinert E_2 (E_2-suppressible site) or DES (DES-suppressible site) at 37°C for 1 hr to obtain the dissociation constant (K_d) and the maximum number of binding sites (MBS). The data (mean ± S.E.) were obtained from 3~5 separate experiments.

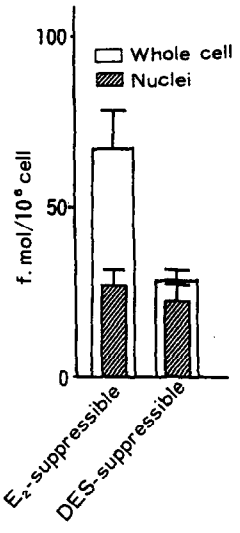

Fig. 3. Subcellular distribution of E_2- or DES-suppressible binding sites. Malignant Leydig cells were incubated with 10 nM [^{3}H]E_2 ± 1 μM *unlabeled E_2 or DES at* 37°C *for* 1 hr. *After incubation cells were washed three times with PBS. The aliquots of washed cells were used to calculate the amount of specific binding of* [^{3}H]E_2 *to intact cells. The remaining cells were homogenized in buffer* 1 *and centrifuged at* 800 *g for* 10 min. *The pellets were washed as described in Materials and Methods and the radioactivity associated with washed nuclei was measured.*

incubated with increasing concentrations of [^{3}H]E_2 ± 1 μM unlabeled E_2 at 0°C for 2 hr and at 30°C for 1 hr respectively [4]. Scatchard plot analysis [9] of the binding data showed the existence of high-affinity nuclear estrogen-binding sites without the concomitant presence of high-affinity cytosolic E_2-binding (DES-suppressible) sites (Fig. 4). These results were compatible with those previously reported from our laboratory [4].

Hormone specificity of estrogen binders in malignant Leydig cells

The binding specificity of cellular estrogen binding components was looked at next. As shown in Fig. 5, estrone, estriol and E_2 could well compete with [^{3}H]E_2 for both cytoplasmic and nuclear binding components. Compared with these estrogens, DES less effectively suppressed [^{3}H]E_2 binding to whole cells. When the data in Fig. 3 were taken into consideration, the suppression (70%) of [^{3}H]E_2 binding to whole cells by a large excess of unlabeled E_2 corresponded to approximately 65 fmol/10^6 cells. The presence of

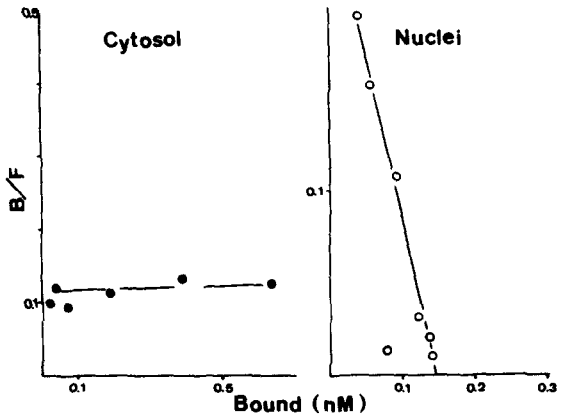

Fig. 4. Saturation analyses of estrogen-binding components in cytoplasmic and nuclear fractions under cell-free conditions. The tumor tissue was homogenized in 4 vol of Buffer 1 supplemented with 10 mM Na_2MoO_4 and centrifuged at 800 g for 10 min. The supernatant was subjected to further centrifugation at 105,000 g for 60 min to obtain the cytosol. The pellet from initial low-speed centrifugation was washed and recentrifuged using a discontinuous sucrose density gradient as previously reported [4] to obtain the purified nuclei. The aliquots (0.15 ml) of the cytosol (panel A) or nuclear suspensions (panel B) were incubated with increased concentrations of [^{3}H]E_2 in the presence or absence of 4 μM unlabeled E_2 at 0°C for 2 hr (●——●) or at 30°C for 1 hr (○——○). The amount of specifically bound [^{3}H]E_2 determined as previously published [4] was analyzed according to Scatchard method.

unlabeled DES caused only a 30% suppression of [^{3}H]E_2 binding to whole cells, which should be equal to ~30 fmol/10^6 cells as calculated by 65 fmol/10^6 cells × (30/70). On the other hand, [^{3}H]E_2 binding to nuclei was diminished to 25-30% by the presence of unlabeled E_2 or DES.

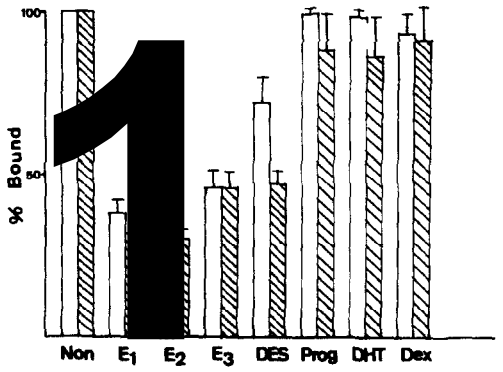

Fig. 5. Hormone specificity of estrogen binders in malignant Leydig cells. The dispersed cells were incubated with 10 nM [^{3}H]E_2 in the absence (Non) or presence of 1 μM unlabeled competitors at 37°C for 1 hr, and then washed as described in Materials and Methods. One part of the washed cells was subjected to direct measurement of their radioactivity (open bar). Another part was used to prepare the nuclei and to assay the radioactivity associated with the nuclei (hatched bar). The data (mean ± S.E. from four separate experiments) were expressed as % bound, in which the values without the competitor (Non) were considered as 100%. The following abbreviations are used in this figure: E_1, estrone; E_3, estriol; Prog, progesterone; DHT, 5α-dihydrotestosterone; Dex, dexamethasone.

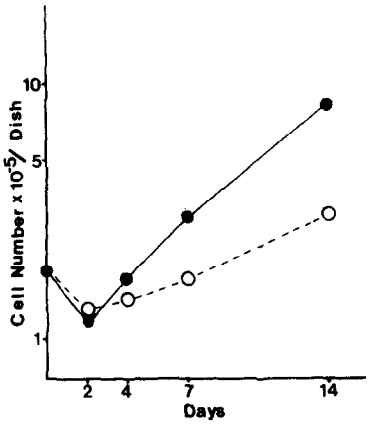

Fig. 6. Effect of E_2 on cell growth in malignant Leydig cells. The dispersed cells (1.8 × 10^5/dish) were plated in duplicate samples on day 0. Cells were grown in the presence (○——○) or absence (○——○) of 10^{-8} M E_2. On the day indicated cells were harvested and aliquots were counted. The remaining cells, stained with nitroblue tetrazolium, showed that more than 90% cells contained 3β-ol dehydrogenase. The variation in duplicates was found to be within 25%.

According to the data in Fig. 3, this 70-75% suppression could correspond to 25-30 fmol/10^6 nuclei. Combined with these results, DES-suppressible binding sites (~30 fmol/10^6 cells) were suggested to be exclusively located in nuclei, while more than half of the E_2-suppressible binding sites are present in the cytoplasmic fraction under the xperimental conditions used here.

In vitro *effects of estrogen on proliferation of malignant Leydig cells*

The primary culture of malignant Leydig cells was performed as described in Materials and Methods. We first investigated whether or not

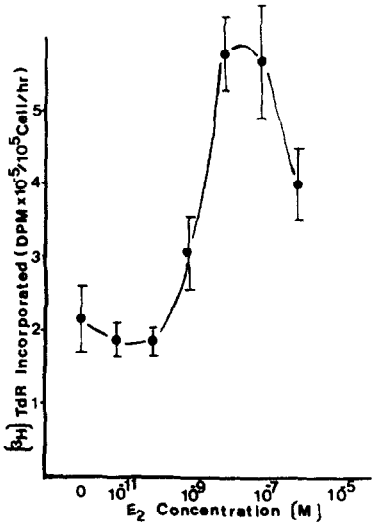

Fig. 7. Thymidine incorporation into acid-precipitable counts. Dispersed cells derived from solid tumor grew to subconfluency in the presence of 10^{-8} M E_2 for 21 days. They were then subcultured in the presence of various concentrations of E_2 for 15 days and then pulsed with [^{3}H]thymidine for 6 hr. The values shown are mean ± S.E. of triplicate samples.

estrogens could cause a comparative increase in the number of Leydig cells that can be identified by the nitroblue tetrazolium staining method. As shown in Fig. 6, E_2 at a concentration of 10^{-8} M in the culture medium did not stimulate cell proliferation until day 4, suggesting that plating efficiency of these cells would not be altered by exposure to E_2. The stimulatory effect of E_2 on cell proliferation became evident, resulting in a 3-fold increase in cell number over that seen in unstimulated controls on day 14. Next, the effect of E_2 on DNA synthesis in cultured transformed cells was determined. As depicted in Fig. 7, these cells were responsive to concentrations of E_2 as low as 10^{-9} M in relation to [^{3}H]TdR incorporation into DNA. At 10^{-8} M to 10^{-7} M the magnitude of the E_2 effect was at a maximum, with some reduction at 10^{-6} M.

DISCUSSION

In our previous study performed in a cell-free condition, we showed that one of the Leydig cell tumor lines (T-124958) contains a unique unoccupied nuclear estrogen binder in the absence of a high-affinity cytosolic binder [4]. The simultaneous administration of CB-154 (ergocryptin) did not affect estrogen-induced tumor growth, suggesting that estrogen action on this particular tumor was directly mediated by this nuclear binder, not via pituitary factors such as prolactin [4]. In order to obtain a definite answer in this regard, however, *in vitro* analysis involving intact cells might be required. The following major points emerged from the present study: (1) this malignant Leydig cell contains a high-affinity nuclear binder similar to putative nuclear ER in the absence of the cytosol high-affinity binder; (2) this nuclear binder undergoes so-called nuclear processing in response to estrogen exposure, resulting in decreased binding capacity per cell; and (3) estrogens act directly on these cells.

The demonstration of specific E_2-binding sites in nuclei from cells pre-exposed to [^{3}H]E_2 at 0°C would support an idea that this transformed Leydig cell contains an unoccupied nuclear estrogen binder. Traish *et al.* [10] reported that incubation of uterine cells with [^{3}H]E_2 at low temperatures (0–4°C) resulted in gradual accumulation of ER in the nuclear fraction, reaching a plateau at 15 hr. Their results would imply that receptor activation can be achieved even at low temperatures in intact cells, translocating ER into nuclei. When compared with their observation, however, the relatively rapid association of [^{3}H]E_2 with nuclei at 0°C with the maximum value at 2–3 hr would favor the idea that there is an unoccupied nuclear binding site in transformed Leydig cell nuclei.

The loss of cellular ER following estrogen administration, called processing [11], has been proposed to be a prerequisite for estrogen actions. This conclusion has come from the experimental results that a rapid decrease in nuclear receptor content does occur 3–5 hr after estrogen administration while no loss of nuclear receptor is observed if ER bound to antiestrogen enters the nucleus [12]. However, exact correlation of nuclear processing of ER with biological effects has been reported as not always being observed [13]. Accordingly, it can be safely concluded that estrogen binder receiving 'nuclear processing' can be categorized as ER. In view of these considerations, it appears to be possible to say with certainty that this transformed Leydig cell contains unoccupied nuclear 'ER'.

Recent evidence has shown that the estrogen-responsive tumor growth can be modulated by pituitary factor(s). Leung and Shiu [14] showed dramatic enhancement of estrogen-dependent tumor growth of the T-47D human mammary tumor cell line in athymic mice by a co-implant of GH3/C 14 rat pituitary prolactin- and growth hormone-producing tumor cells. This observation has been extended by Sirbasku *et al.* [15], who have shown that pituitary extracts from E_2-treated ovariectomized rats have the factor(s) promoting the growth of rat mammary tumor cells (MTW9/pL) although its hormonal dependency remains obscure. Although these sophisticated studies reveal the important role of pituitary factors for the estrogen-induced tumor growth, a marked *in vitro* effect on E_2 on cell proliferation would strongly indicate that this transfomed Leydig cells used in the present experiments is influenced directly by estrogens in terms of tumor growth.

The cytoplasmic E_2-binding component might be worth taking into consideration. The use of unlabeled DES to calculate a non-specific binding site failed to demonstrate the estrogen-binding components in the cytoplasmic fraction. On the other hand, a cytoplasmic E_2-suppressible binding site was observed by the experiments on subcellular localization of the estrogen binders. No demonstrable increase in nuclear E_2-suppressible sites in response to the exposure of cells to E_2 would reveal that the cytoplasmic E_2 binder is unable to translocate into nuclei. These seem to be compatible with out previous observations [16]. Furthermore, it should be mentioned that this cytoplasmic estrogen binder was unable to interact with hydroxylapatite under the usual conditions.

The mechanism of estrogen-induced tumor growth has remained to elucidated. The require-

ment of high serum concentration in the culture medium has been recently reported to demonstrate the estrogen-induced enhancement of cell proliferation in MCF-7 human mammary cancer cells [17]. A relatively high concentration (10%) of charcoal-treated fetal calf serum was used in the present experiments. The lower concentrations (1-2%) failed to show any significant and reproducible effects of E_2 on cell proliferation (data not shown). Clearly, more research is required to clarify the molecular mechanism of estrogen-induced tumor growth. In this regard, it is desirable to establish the *in vitro* experimental system showing estrogen effects in terms of cell proliferation under serum-free conditions. Our current studies are directed toward establishing these experimental models.

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