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Effect of Retinoic Acid on Proliferation of Estrogen-responsive Transformed Murine Leydig Cells in Serum-free Culture[1]

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ABSTRACT

B-1 F cells, one of the sublines established from mouse Leydig cell tumor, have been found to be maintained as an estrogen-responsive cell line under the serum-free culture conditions. Reported results that retinoids have action mechanisms similar to those of estrogen prompted us to examine the effect of retinoids on the proliferation of B-1 F cells.

Stimulation of B-1 F cell growth by retinoic acid in a dose-dependent manner was observed, whereas retinoic acid did not promote but inhibited the proliferation of MCF-7 cells (estrogen- and retinoic acid receptor-positive human breast cancer cells). To elucidate the mechanism of retinoic acid-dependent cell growth, simultaneous treatment with retinoic acid and estradiol was carried out. The result did not show the additive effect on B-1 F cell growth. Hydroxytamoxifen, a potent antiestrogen, inhibited not only estradiol-dependent but also retinoic acid-dependent cell growth. However, retinoic acid failed to be associated with estrogen receptor, suggesting that retinoic acid induced enhancement of B-1 cell growth through its interaction with retinoic acid receptor. Northern blot analyses of polyadenylated RNA with complementary DNA probes for human retinoic acid receptor α, β, and γ revealed the presence of transcripts encoded by retinoic acid receptor α gene in B-1 F cells.

These results would suggest that enhancement of the B-1 F cell growth is mediated through interaction of retinoic acid with retinoic acid receptor α. This stimulatory activity is inhibited by estrogen receptor complexed with hydroxytamoxifen.

INTRODUCTION

Retinoids comprise the group of analogues of vitamin A. Except for the well-known role of retinoids in the visual cycle, their other biological functions are not well characterized. One of the major problems in the field of retinoid actions has not been clarified until recently. The cloning of cDNA[3] for RAR provides us with a valuable tool to attack this important but unresolved issue (1–3). Analysis of RAR cDNA clearly indicates that RAR is classified as a member of the steroid/thyroid receptor family (4). On the other hand, estrogen-induced enhancement of cell proliferation is believed to be mediated through ER (5). These tempt us to speculate that RA can modulate the growth of estrogen-responsive transformed cells. For this purpose, a murine transformed Leydig cell line was utilized in the present study. This cell line, called B-1, has been found to be growth stimulated under serum-free culture conditions by both estrogen and androgen (6), leading us to speculate that the growth of this cell line would be regulated by various nuclear factors belonging to the steroid/thyroid hormone receptor family. In addition, early reports have shown that deprivation of RA results in the atrophy of rodent testis (7). Although the detailed molecular mechanism is still unknown, these results would raise the possibility that the function of testicular cells such as Leydig cells is regulated by RA. Thus we analyzed the effect of RA on the growth of B-1 cells under serum-free culture conditions and compared it with that of MCF-7 cells, ER- and RAR-positive human breast cancer cells (8).

MATERIALS AND METHODS

Materials. Eagle's minimum essential medium, Ham's F-12, and DME were obtained from Nissui Pharmaceutical Co., Ltd, (Tokyo, Japan). FCS was from Irvine Scientific (Santa Ana, CA). 17β-[1,2,6,7-^{3}H]Estradiol (specific activity, 95 Ci/mmol) was from New England Nuclear (Cambridge, MA). The restriction enzymes were purchased from Toyobo Co., Ltd. (Tokyo, Japan). RA, 17β-estradiol (estradiol), and diethylstilbestrol and BSA (crystallized and lyophilized) were from Sigma Chemical Co. (St. Louis, MO). OHTam was kindly supplied by ICI Pharma (Osaka, Japan). The other reagents used here were of analytical grade.

Cell Culture. The parent cell line (B-1) was established from estrogen-responsive mouse Leydig cell tumor (T 124958-R) in our laboratory as published previously (9). To completely eliminate the effects of FCS which was used to maintain B-1 cells, we tried to obtain the subline of B-1 cells which could be maintained in the serum-free medium. After 50 passages in HMB supplemented with 10^{-8} M estradiol medium (serum-free medium), the cells, called B-1 F, were isolated. It was found that B-1 F cells could be maintained in HMB medium in 95% air-5% CO_2 at 37°C. MCF-7 cells, from an estrogen-responsive human breast cancer cell line, were kindly supplied by Dr. R. L. Sutherland (Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, Australia) and maintained in DME containing 10% FCS.

Cell Growth Experiments. B-1 F cells or MCF-7 cells were plated onto three replicate 35-mm culture dishes at an initial cell density of 2 $\times 10^4$/dish in HMB medium or DME containing 5% FCS, respectively. On the following day (day 1), the medium was changed into HMB medium supplemented with various test compounds. To completely remove the residual FCS, the dishes in which MCF-7 cells were seeded were washed with phosphate-buffered saline twice. The final concentration of ethanol was always less than 0.02% which was found not to affect the growth of B-1 F cells and MCF-7 cells. The solvent alone was added to control cells. These media were changed every 2 days. At the indicated times, the viable cells were counted as described previously (10).

ER Assay. The whole cell binding assay was used to analyze ER in B-1 F cells (11). Briefly, B-1 F cells grown in HMB medium were harvested and washed twice with Eagle's minimum essential medium containing 0.1% (w/v) BSA. The aliquots (10^6 cells/tube) of the cell suspension were incubated with the increasing concentrations of competitors in the presence of 4 nM [^{3}H]estradiol in a final volume of 0.25 ml. After incubation at 37°C for 1 h in 95% air-5% CO_2, the amount of [^{3}H]estradiol specifically bound to B-1 F cells was quantitated.

Northern Blot Analysis for RAR. Total cellular RNA was isolated from B-1 F cells or MCF-7 cells by extraction with guanidium chloride and ultracentrifugation through 5.7 M cesium chloride (12). A polyadenylate-rich fraction was obtained by passing through oligodeoxy thymidylate-cellulose column (Pharmacia, Uppsala, Sweden). Polyadenylate-rich RNA (4 μg/lane) was separated in 1% (w/v) agarose gel containing 2.2 M formaldehyde and transferred onto a nitrocellulose filter. The complete human RAR-α (3), $-\beta$ (13), and $-\gamma$ (14) cDNAs, which were kindly supplied by Dr. P. Chambon (INSERM, Strasbourg,

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[3] The abbreviations used are: cDNA, complementary DNA; RAR, retinoic acid receptor; ER, estrogen receptor; RA, retinoic acid; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BSA, bovine serum albumin; OHTam, 4-hydroxytamoxifen; HMB, Ham's F-12:MEM (1:1) containing 0.1% (w/v) BSA; SDS, sodium dodecyl sulfate.

France), were used as a ^{32}P-labeled probes. Hybridization was then carried out in 50% [v/v] formamide; 5 × 0.18 M NaCl-10 mM NaH_2PO_4-1 mM EDTA, pH 7.4, at 20°C; 1 × Denhardt's solution [0.02% (w/v) Ficoll-0.02% (w/v) polyvinylpyrrolidone-0.02% (w/v) BSA], 0.1 (w/v) SDS, 100 μg/ml salmon sperm DNA, and 10% (w/v) dextran sulfate at 42°C for 18 h. Then, the filter was washed with 2 × 0.18 M NaCl-0.015 M sodium citrate, pH 7.0, at 20°C containing 0.1% (w/v) SDS at 65°C twice for 30 min and then once with 0.5 × 0.18 M NaCl-0.015 M sodium citrate, pH 7.0, at 20°C containing 0.1% (w/v) SDS at 65°C for 15 min. Autoradiography was carried out at −70°C with Kodak X-Omat AR film with intensifying screens.

Statistical Analysis. Paired Student's *t* test was used to discuss the significant difference. The data presented here were expressed as mean ± SE.

RESULTS

Effects of RA on Estradiol-dependent Growth of B-1 F Cells. Since B-1 F cells were subcloned from an estrogen-dependent cell line (B-1) (6), the possibility was addressed that the growth of B-1 F cells was also stimulated by estradiol. We found this was the case (Fig. 1). The growth of B-1 F cells was markedly enhanced by adding estradiol in HMB medium, with a maximum effect at 10^{-8} M. Further evaluation of estradiol to 10^{-7}–10^{-6} M resulted in a decreased cell multiplication rate. Although the molecular mechanism of some inhibitory effects elicited by unphysiological high concentrations of estradiol remained to be obscure, these results clearly demonstrated that B-1 F cells are able to respond to estradiol in relation to their proliferation rate. The simultaneous exposure of 10^{-8} M RA to estradiol-stimulated B-1 F cells resulted in enhanced cell growth at low concentrations (0–10^{-9} M) of estradiol. The significant difference could not be observed at the high concentrations (10^{-8}–10^{-6} M) of estradiol. These results indicated that RA alone could growth stimulate B-1 F cells but that RA did not show the additive effect on cells stimulated with the high concentrations of estradiol.

Effects of RA on B-1 F Cell Growth. Next, an experiment was conducted to examine the effects of RA on B-1 F cell growth rate. As shown in Fig. 2*a*, the addition of 10^{-8} M RA into HMB medium resulted in 3-fold increase in cell yield on days 5–9. The calculated doubling times of B-1 F cells stimulated and unstimulated with 10^{-8} M RA were 43 and 94 h, respectively. The degree of RA stimulation varied from experiment to experiment. However, RA was found to consistently

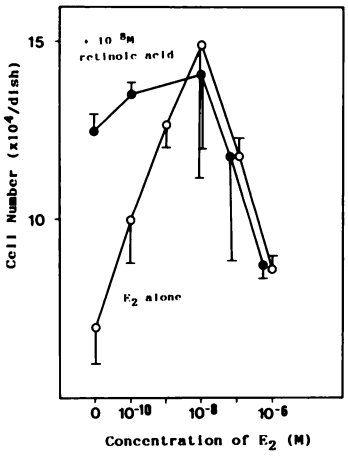

Fig. 1. Proliferative effects of various concentrations of estradiol (E_2) alone or in combination with 10^{-8} M RA on B-1 F cells. B-1 F cells were plated and cultured in HMB medium supplemented with various concentrations of estradiol alone or in combination with 10^{-8} M RA as described in "Materials and Methods." The viable cells were counted on day 7.

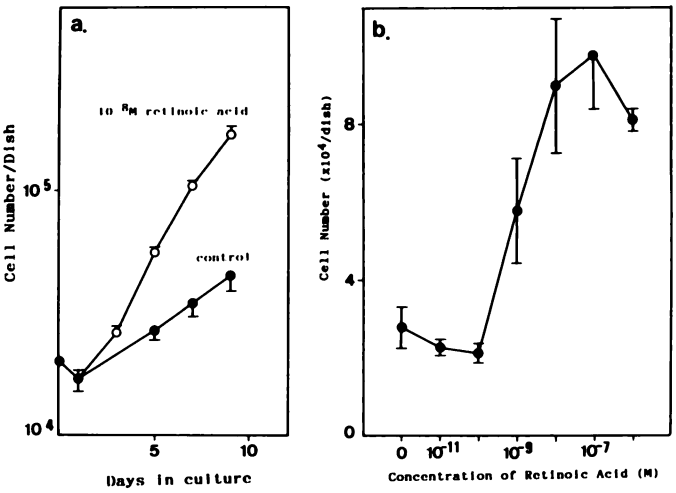

Fig. 2. Growth-stimulatory effect of RA on B-1 F cells. B-1 F cells were plated in HMB medium. RA treatment was initiated on day 1. In *a*, to calculate the growth rate, the viable cells stimulated with 10^{-8} M RA or unstimulated were counted for the indicated periods of time. In *b*, effects of increasing concentrations of RA were also examined by counting the viable cells on day 7.

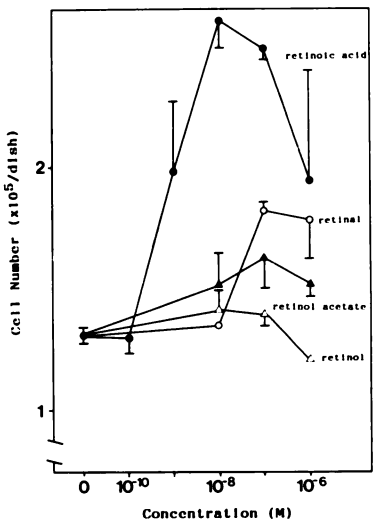

Fig. 3. Effects of various retinoids on B-1 F cell growth. B-1 F cells were plated in HMB medium and then exposed to increasing concentrations of various retinoids from day 1 to day 7. The viable cells were counted on day 7.

enhance the growth of B-1 F cells. To further substantiate the ability of RA to growth stimulate B-1 F cells, these cells were cultured in HMB medium supplemented with various concentrations of RA. A marked increase in the cell proliferation rate was caused by RA in a dose-dependent manner, with a half-maximum effect at 10^{-9} M and a maximum effect at 10^{-8}–10^{-7} M (Fig. 2*b*). Since retinoids are known to comprise the group of natural and synthetic analogues of vitamin A, several retinoids were examined in terms of their ability to stimulate the growth of B-1 F cells. As illustrated in Fig. 3, RA showed the most potent growth-stimulatory ability. Retinol and retinol acetate did not enhance the proliferation significantly. Retinal functioned as a weak agonist.

Effect of Antiestrogen on RA-dependent Growth of B-1 F Cells. To obtain some clue for the mechanism of RA-dependent cell proliferation, we examined the effects of a potent antiestrogen OHTam. On B-1 F cell growth, OHTam almost perfectly blocked not only estradiol-dependent but also RA-dependent cell growth (Fig. 4). OHTam alone did not significantly inhibit the growth of B-1 F cells in this experimental condition. These results would suggest that antiestrogen-ER complex impairs

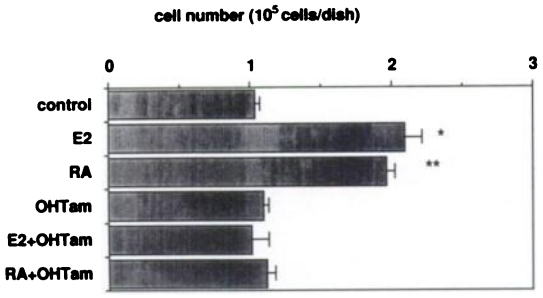

Fig. 4. Effect of antiestrogen on estradiol (E_2)- and RA-dependent growth of B-1 F cells. B-1 F cells were plated and cultured in HMB medium supplemented with 10^{-8} M estradiol or 10^{-8} M RA in the presence or absence of 10^{-7} M OHTam. The effect of OHTam (10^{-7} M) alone was also examined. The viable cells were counted on day 7. Statistical differences: *, control *versus* estradiol (E_2), E_2 + OHTam *versus* estradiol $P < 0.001$; **, control *versus* RA, RA + OHTam *versus* RA, $P < 0.005$. The difference between control and OHTam was not significant. *Bars*, SE.

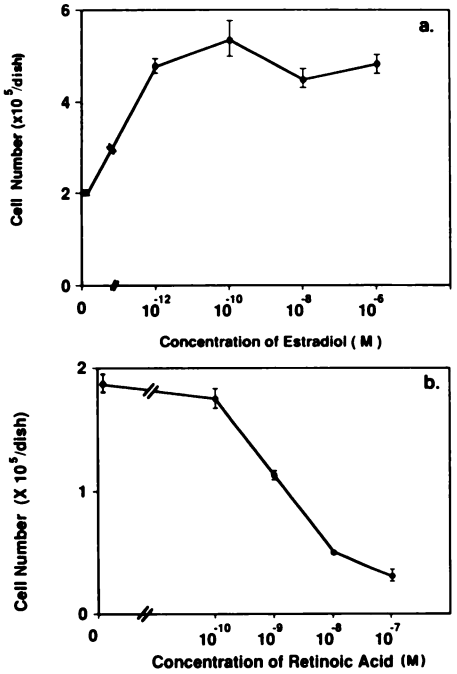

Fig. 5. Effects of estradiol and RA on MCF-7 cell growth. MCF-7 cells were plated in DME containing 5% FCS and 24 h later medium was changed to HMB medium supplemented with various concentrations of estradiol (*a*) or RA (*b*) as described in "Materials and Methods." The viable cells were counted on day 7.

the RA-dependent pathway in B-1 F cell growth.

Effect of RA on MCF-7 Cell Growth. MCF-7 cells are known as estrogen-responsive cells (8). The culture condition described here also demonstrated that estradiol stimulated MCF-7 cell growth with a half-maximum effect at less than 10^{-12} M under serum-free conditions (Fig. 5*a*). On the other hand, the cell growth was inhibited by RA in a dose-dependent manner, with a half-maximum effect at approximately 10^{-9} M (Fig. 5*b*). These data were compatible with the reported finding that RA is growth inhibitory to human breast cancer cells (15).

RAR and ER in B-1 Cells and MCF-7 Cells. In view of the growth-stimulatory effects of estradiol and RA, we attempted to demonstrate RAR as well as ER in B-1 F cells. To identify RAR, we utilized Northern blot analysis, since the presence of cellular retinoid-binding proteins has known to hinder the ligand binding assay for RAR (16) and there are at least three RAR subclasses, RAR-α, -β, and -γ, which are indistinguishable by ligand binding assay. The expression of mRNAs was investigated using ^{32}P-labeled human RAR-α, -β, and -γ cDNA probes under stringent conditions, showing that two mRNAs (3.8 and 2.8 kilobases, respectively) for RAR-α were found in B-1 F cells and MCF-7 cells (Fig. 6). The intensities of bands hybridized with RAR-α cDNA probe were found to be consistently more dense in MCF-7 cells than in B-1 F cells. No RAR-β mRNA could be detected in both cell lines. No RAR-γ mRNA could be detected in B-1 F cells, whereas 3.3-kilobase RAR-γ mRNA was detectable in MCF-7 cells. These results indicated that both cell lines express RAR mRNAs in quantitatively as well as qualitatively different ways.

ER mRNA expression in B-1 F cells was also confirmed by Northern blot analysis (data not shown). Thus, the ligand specificity of ER in B-1 F cells was investigated, using [^{3}H]-estradiol as a radioactive ligand (Fig. 7). ER binding was found to be specific for estrogens as well as OHTam. When the increasing concentrations of unlabeled RA were added in the binding assay mixture, RA did not compete for [^{3}H]estradiol binding to B-1 F cells even at micromolar concentrations. These results suggest that RA is unable to be associated with ER in B-1 F cells.

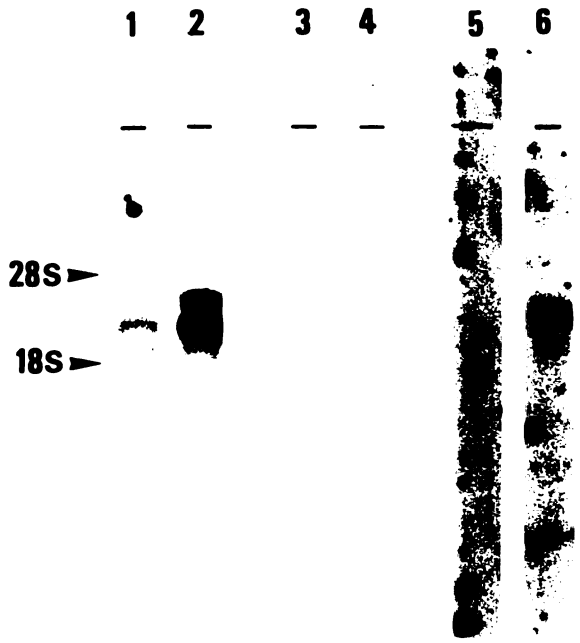

Fig. 6. Northern blot analysis of RAR in B-1 F cells and MCF-7 cells. mRNAs prepared from B-1 F cells (*Lanes 1, 3,* and *5*) or MCF-7 cells (*Lanes 2, 4,* and *6*) were analyzed with cDNA probes for human RAR-α (*Lanes 1* and *2*), -β (*Lanes 3* and *4*), and -γ (*Lanes 5* and *6*). *Arrows*, position of 18S and 28S rRNAs.

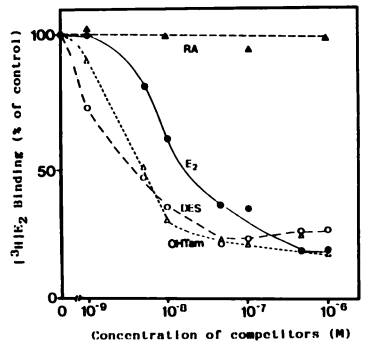

Fig. 7. Ligand binding specificity of ER in B-1 F cells. Whole cell binding assay for ER was carried out as described in "Material and Methods," and the ligand specificity of [^{3}H]estradiol (E_2) to B-1 F cells was determined. DES; diethylstilbestrol.

DISCUSSION

The present study clearly shows that RA is able to stimulate the growth of B-1 F cells in the serum-free culture. These results are unexpected, since the vast majority of studies have reported an inhibitory action of RA on cell growth of transformed as well as nontransformed cell lines *in vitro* (see Refs. 17 and 18 for reviews). Moreover, the growth of a transplantable chondrosarcoma has been observed to be inhibited by RA *in vivo* (19). The RA-dependent inhibition of cell proliferation observed *in vitro* is known to be accompanied by the reduction in saturation density and restoration of contact inhibition, suggesting that RA induces redifferentiation in some tumor cells. Actually, RA-induced differentiation has been reported in murine embryonal carcinoma cells (20) as well as rat rhabdomyosarcoma cells (21). However, B-1 F cells used here did not show RA-induced alteration in the cell morphology and saturation density (data not shown). As exceptional cases, RA has been reported to enhance the proliferation of certain eukaryotic cells *in vitro* (22–26). This RA-dependent enhancement of cell proliferation or DNA synthesis has been observed principally in cells simultaneously treated with growth-promoting agents. For instance, the growth-promoting ability of epidermal growth factor or 12-*O*-tetradecanoylphorbol-13-acetate on Swiss 3T3 (22), hamster NIL-8 (23), and NRK 536 (24) cells can be potentiated by retinoids. Although RA have been reported to enhance epidermal growth factor binding in BALB/c 3T3 cells (25), the molecular mechanism of the synergistic effects of RA on the other growth-promoting agents remains to be largely unknown. Furthermore, the serum used in many culture systems is known to complicate the growth-regulatory ability of retinoids. Bovine endothelial cells were growth-stimulated in medium containing delipidized serum by retinoids; however, retinoids inhibited the growth of these cells in medium containing the whole serum (27). These complicated results require us to use the serum-free medium in order to discuss the direct action mechanism of RA effects on the cell proliferation. To our knowledge, this is the first report showing the growth-stimulatory effect of RA in the serum-free medium without any growth-promoting agents.

The effects of RA on estrogen-responsive cells have not been extensively studied. MCF-7 cells (estrogen-responsive human breast cancer cells) were reported to be growth inhibited by retinoid treatment (Ref. 15; Fig. 5). This is in contrast with the results of B-1 F cells in the present study. Northern blot analyses indicated that the number of detectable subspecies of RARs were two (-α and -γ) in MCF-7 cells but only one (-α) in B-1 F cells. We cannot eliminate the possibility that the presence of RAR-γ in MCF-7 cells explains the difference in RA-dependent growth between two cell lines. To address this possibility, the isolation of cultured cells containing only RAR-γ is definitely required. Recently, a heterodimer formation between RAR-α and thyroid hormone receptor was reported (28, 29). The possibility that RA action in MCF-7 cells is mediated via a heterodimeric complex between RAR and ER is an interesting project to study. Alternatively, FCS used for maintaining MCF-7 cells might be related to RA-dependent inhibition of MCF-7 cell growth. These possibilities should be examined in future studies.

RA provoked the enhancement of the proliferation of B-1 F cells without the additive effect on estradiol-enhanced stimulation. These results raised the interesting possibility that B-1 F cells possess some interaction between RA and estradiol action mechanisms which would be different from those in MCF-7 cells. To address this possibility, the ability of RA to bind to ER was examined, resulting in no demonstrable binding ability. The next step of RA-induced biological events is believed to be the receptor-gene interaction. Therefore, it is possible that both estradiol-ER and RA-RAR complexes activate same genes regulating cell multiplication in B-1 F cells. If this is the case, RA-dependent enhancement of B-1 F cell growth could be antagonized by exposing cells to OHTam. Actually, OHTam was found to lower the cell growth rate to the unstimulated level. In addition, our preliminary transfection experiments revealed that RA enhances the transcription of chloramphenicol acetyltransferase reporter plasmid containing synthetic estrogen-responsive element in B-1 F cells, but not in MCF-7 cells (data not shown). These results obtained by transfecting the foreign genes should be interpreted with some reservation. The isolation and analysis of natural genes under a control of RA and/or estradiol should be carried out by the additional studies. Also, the amino acid sequence of RAR in B-1 F cells should be studied.

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