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Glucocorticoids and androgens up-regulate the Zn- α_2-glycoprotein messenger RNA in human breast cancer cells.

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Summary

We have studied the hormonal regulation of the gene encoding Zn-α_2-glycoprotein (Zn-α_2-gp), a human protein with a high degree of amino acid sequence similarity to class I histocompatibility antigens that is produced by a specific subset of breast carcinomas. Northern blot analysis revealed that dexamethasone and 5α-dihydrotestosterone strongly induced the accumulation of Zn-α_2-gp mRNA in T-47D human breast cancer cells. Furthermore, the effect of these two hormones was shown to be additive, since the combination of both hormones produced a stimulation of Zn- α_2-gp mRNA of at least 3-fold over that produced by either hormone alone. By contrast, the addition of 5β-dihydrotestosterone, 17β-estradiol, or progesterone failed to induce the expression of Zn-α_2-gp. The stimulatory effect of glucocorticoids and androgens on Zn-α_2-gp expression was produced in a time and dose dependent manner, without significantly affecting the cell proliferation rate. A time-course study demonstrated that the induction of Zn-α_2-gp mRNA by androgens and glucocorticoids reached a level of 4 or 3.2-fold over the untreated control after seven days of incubation in the presence of a 10^{-7} M concentration of 5α-dihydrotestosterone or dexamethasone, respectively. A dose-response study showed that as little as 10^{-11} M of 5α-dihydrotestosterone or dexamethasone produced an accumulation of Zn-α_2-gp mRNA of 2.4 or 2.1-fold over the control, respectively. On the basis of these results, we propose that Zn-α_2-gp may be useful as a biochemical marker of breast carcinomas with a specific pattern of hormone responsiveness in whose development glucocorticoids and/or androgens may play a significant role.

Introduction

Breast cancer is the most common malignant tumor in the female population and represents a major cause of death in women from Western countries [1]. According to many experimental and epidemiological studies, breast carcinomas differ widely in their

biological and clinical behavior, so that the management of the disease could be improved by the finding of specific markers for predicting its natural course or monitoring the response to hormonal therapy. In recent years, among the increasing number of substances which may be useful for this purpose, proteins accumulated in cyst fluid from women with gross cystic disease of the breast have attracted considerable interest due to their potential usefulness as biochemical markers of breast carcinomas with specific patterns of hormone responsiveness [2].

A large number of studies on the role of cystic proteins as tumor markers have focused on GCDFP-24, the major protein component in this pathological fluid, recently identified in our laboratory as apolipoprotein D (apo D) [3]. This major intracystic protein binds pregnenolone, progesterone, and bilirubin [4-7], and has been proposed as a marker of steroid action in human breast cancer cells [8, 9]. Thus, estrogenic stimulation of ZR-75-1 and MCF-7 breast cancer cell proliferation is accompanied by a decrease in the secretion of apo D. Furthermore, exposure of ZR-75-1 cells to androgens stimulates apo D release with a simultaneous decrease in cell proliferation. Similar studies have demonstrated that the production and secretion of the intracystic protein termed GCDFP-15 are also enhanced by androgens and inhibited by estrogens [10-13]. However, at present, much less information is available on the hormonal regulation of Zn-α_2-glycoprotein (Zn-α_2-gp), the remaining major protein found in breast cysts.

This glycoprotein consists of a single polypeptide chain with a molecular mass of about 40 kD and was first identified in human plasma by Bürgi and Schmid [14]. It is also present in sweat, saliva, cerebrospinal fluid, breast secretions, urine, and seminal fluid [15-18]. In addition, several studies have demonstrated the existence of a certain subtype of breast carcinomas which produce significant amounts of Zn-α_2-gp [18-22]. The biological role of this protein remains unknown, but recent elucidation of its amino acid sequence has revealed an extensive structural similarity to antigens of the human major histocompatibility complex, pointing to a possible role of Zn-α_2-gp in the immune response as a soluble HLA [23-25]. However, despite the putative relevance of its biological function and its potential value as tumor marker in breast cancer, the lack of appropriate molecular probes has hampered studies directed to investigate the regulation of Zn-α_2-gp expression. Thus, at present, the only available data are limited to the finding that, as previously demonstrated for apo D and GCDFP-15, androgens are able to enhance its secretion into the culture medium of human breast cancer cell lines [26, 27].

In order to further study the mechanisms controlling the expression of Zn-α_2-gp in breast cancer cells as well as to determine whether, as in the case of other cystic proteins, there is an antagonism between cell proliferation and inducible production of Zn-α_2-gp, studies were undertaken to examine the effects of different hormones on the expression of this glycoprotein. In this work, and by using a cDNA probe coding for Zn-α_2-gp and specific antibodies against the purified protein from breast cyst fluid, we show that glucocorticoids and androgens up-regulate Zn-α_2-gp mRNA levels and secretion in T-47D human breast cancer cells. In addition, we present evidence that these stimulatory

effects on Zn-α_2-gp expression and secretion are not accompanied by a significant inhibitory effect on cell proliferation in this human breast cancer cell line.

Materials and methods

Materials

All media and supplements for cell culture were obtained from Sigma Chemical Co. (St. Louis, MO), except for fetal bovine serum (FBS), which was from Boehringer Mannheim (Mannheim, Germany). The hormones 17β-estradiol (E_2), 5α-dihydrotestosterone (DHT), 5β-dihydrotestosterone, and progesterone (PROG) were also from Sigma Chemical Co. Flutamide was purchased from Schering Corp. (Kenilworth, NJ). [α-^{32}P] deoxycytidine 5'-triphosphate (dCTP) (3000 Ci/mmol) and the nylon membranes for RNA blots were from Amersham International (Amersham, UK). The random priming labelling kit was from Boehringer Mannheim.

Cell cultures, hormonal treatments, and cell proliferation studies

The T-47D cell line (HTB 133) was obtained from the American Type Culture Collection and routinely grown in RPMI-1640 medium containing phenol red and supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The T-47D cells were subcultured weekly in Costar (Cambridge, MA) 75 cm^2-flasks containing 12 ml of medium. Cell cultures were maintained at 37 ° C in a humidified atmosphere of 5% CO_2.

For the hormone induction experiments, T-47D cells were grown previously for 8 to 10 days in phenol red-free RPMI-1640 medium supplemented with 5% dextran-coated charcoal-treated FBS. T-47D cells were plated out in 6-well plates at a density of 2 x 104 cells/well and allowed to adhere to substrate for 48 h. Thereafter, medium was replaced with fresh medium containing the indicated concentrations of steroids, with medium changes every 48 h. Hormones were added to the growth medium in alcohol solutions so that the final concentration of ethanol was 0.1% in both control and hormone-treated cultures. Cells were cultured in parallel with the same hormone treatments for determination of cell number. At the end of the incubation period, T-47D cells were harvested with 0.05% trypsin-0.02% EDTA and counted at the appropriate dilution in a hemocytometer chamber.

Zn-α_2-glycoprotein purification and antiserum production

Zn-α_2-gp was purified from cyst fluid from women with gross cystic disease of the breast by size-exclusion HPLC, according to the procedure described previously [18]. Antiserum against the purified protein was raised in New Zealand white rabbits following the method described by Vaitukaitis [27]. The immunized rabbits were bled 6 weeks after protein injection and the obtained serum was dialyzed for 24 h at 4 ° C against 20 mM

phosphate buffer, pH 7.2. Then, the dialyzed material was chromatographed in a column of DEAE-cellulose equilibrated and eluted in the same phosphate buffer, and finally, the IgC containing fractions were collected and stored at - 20 ° C until used.

Immunocytochemical staining

T-47D cells were plated on Thermanox Coverslips (Nunc, Inc) and grown for 7 days in phenol red-free RPMI-1640 medium supplemented with 5 % treated FBS in the presence of the indicated hormones at a concentration of 10 .7 M. The cells were fixed with 10% formaldehyde for 10 min and then washed for 5 min with distilled water. Endogenous peroxidase and non-specific binding were blocked by sequential incubation of the cells in 3 % hydrogen peroxide solution and in normal serum. Incubation with specific antibodies against Zn-α_2-gp at a 1:500 dilution was carried out overnight at room temperature. The immunoreactivity was detected by using the avidinbiotin-peroxidase complex [29]. Peroxidase activity was demonstrated by incubating the cells with 0.05 % diaminobenzidine in 50 mM Tris buffer (pH 7.5) containing 0.05% hydrogen peroxide. Finally, the cells were counterstained with a modification of the formaldehyde-thionine method [30], dehydrated, cleared in eucalyptol, and mounted with Eukitt.

Isolation of RNA and Northern-blot analysis

Total RNA from the T-47D cells was isolated according to Chomczynski and Sacchi [31]. Samples of 10 µg were separated by electrophoresis in 1% agarose-formaldehyde denaturing gels and blotted onto Hybond Nylon filters (Amersham International). The integrity of the RNA was ascertained by direct visualization of the stained gel and the nylon membrane under UV light. Filters were prehybridized for 2 h at 42 ° C in 50% formamide, 5 x SSC (1 x = 150 mM NaC1, 15 mM sodium citrate, pH 7), 2 x Denhardt's solution (1 x = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 0.1% SDS, and 0.1 mg/ml of denatured herring sperm DNA, and then hybridized for 48 h under the same conditions, using the cDNA coding for Zn-α_2-gp as a probe [25]. Filters were washed in 1 x SSC, 0.1% SDS at 60 ° C for 2 h and autoradiographed. X-ray films were scanned in a Chromoscan 3 (Joyce Loebl) densitometer.

Results

In order to study the possible hormonal stimuli involved in the production of Zn-α_2-gp by breast tumors, T-47D human breast cancer cells were first treated with different steroid hormones and synthetic glucocorticoids and the expression of Zn-α_2-gp examined by Northern-blot analysis. For this purpose, cancer cells were incubated for a period of seven days in the presence of 10 -7 M concentration of DEX, DHT, E2, PROG, and 5β-dihydrotestostetone, and total cellular RNAs were purified and analyzed by Northern blot using an specific Zn-α_2-gp cDNA probe. As illustrated in Fig. 1, the addition

of DHT or DEX to the culture medium strongly induced the accumulation of the 1.3 kb Zn-α_2-gp mRNA. By contrast, the addition of 5β-dihydrotestosterone failed to induce Zn-α_2-gp expression. Furthermore, when both DEX and DHT were added simultaneously a clear additive effect was observed. Thus, the combination of both hormones produced an accumulation of Zn-α_2-gp mRNA of at least 3-fold over that produced by either hormone alone. In addition, it is worthwhile mentioning that the stimulatory action of DHT on Zn-α_2-gp expression was extensively blocked by addition of the pure antiandrogen Flutamide (FLU) at the same concentration (10^{-7} M) (Fig. 1), although higher concentrations of FLU (10^{-5} M) were required to completely abolish the effect of DHT. According to these data, we suggest that the action of DHT is mediated via an androgen receptor mechanism.

We also examined the putative effect of estrogens and progestagens on Zn-α_2-gp expression by breast cancer cells. However, as shown in Fig. 1, after treatment with E_2 there was not a detectable increase in the expression of Zn-α_2-gp. In addition, E_2 did not reverse the stimulatory effect of DHT on expression Zn-α_2-gp. Similarly, there was no increase in Zn-α_2-gp mRNA detectable levels in T-47D cells grown in the presence of either 10^{-7} (Fig. 1) or 10^{-5} M PROG (data not shown). It may be interesting to note that in our conditions the control culture usually showed little or no expression of the Zn-α_2-gp although some variability was observed, probably pertaining to the batch of serum being used in the different experiments.

After these results showing that androgens and glucocorticoids, but not estrogens or progesterone, are able to induce the production of Zn-α_2-gp by breast cancer cells, we tried to examine the possibility that these hormone-mediated actions would be related to the respective effects of the different hormonal treatments on cell proliferation. In order to do that, we studied the growth rate of the cells in the presence of a 10^{-7} M concentration of the different hormones. As shown in Fig. 2, after a seven-day incubation in the presence of DHT an overall positive effect on cell proliferation was observed, with an increase of 27% over the number of cells in the control. A similar effect was observed for DEX and E_2, with a total increase of 16% and 30% respectively over control values. By contrast, exposure of the T-47D cells to PROG inhibited cell growth by approximately 40% (Fig. 2).

To further characterize the potent stimulatory action of androgens and glucocorticoids on the expression of Zn-α_2-gp, we next studied the effect of different concentrations of DHT and DEX on Zn-α_2-gp mRNA accumulation by using Northern blot analysis. As illustrated in Fig. 3, the incubation of T-47D cells for 7 days in the presence of increasing concentrations of DHT produced an accumulation of Zn-α_2-gp mRNA, with a maximal 4.5-fold stimulation at a 10 -7 M concentration of DHT. The addition to the growth medium of lower concentrations of the hormone, 10^{-9} and 10^{-11} M, produced increments in the amount of Zn-α_2-gp mRNA detectable in the cells of 2.7 and 2.4-fold, respectively. Comparable effects were observed in the presence of the synthetic glucocorticoid DEX. As shown in Fig. 3, a 7-day incubation with 10-7M DEX increased the Zn-α_2-gp mRNA content of the cells 3.5-fold over the control untreated cells. Concentrations of 10^{-9} and

10^{-11} M of DEX produced an increment of the Zn-α_2-gp mRNA levels of 3.2 and 2.1-fold, respectively. Although the maximal stimulation of Zn-α_2-gp mRNA accumulation was observed for a 10^{-7} M concentration of DHT and DEX, it is interesting to note that both hormones were effective at concentrations closer to the physiological range, suggesting that the observed effects are not pharmacological artifacts.

We next investigated the time course of the effect of the treatment with DHT and DEX on the expression of Zn-α_2-gp. As shown in Fig. 4, incubation with 10 -7 M concentration of DHT for 1, 3, 5, or 7 days increased Zn-α_2-gp mRNA expression by 1.5, 3, 3.7, and 4-fold, respectively. An analogous effect was observed for DEX, which, at a concentration of 10^{-7} M, produced a 1.8, 2.7, and 3.2-fold accumulation of Zn-α_2-gp mRNA after 3, 5, or 7 days of incubation of the T-47D cells in the presence of the hormone (Fig. 5).

Finally, we tried to determine if the effect of DHT and DEX causing a time and dose-dependent increment in the amount of Zn-α_2-gp mRNA was in fact reflected at the protein level in the intact cell. To examine the intracellular accumulation of Zn-α_2-gp protein in response to these hormones, T-47D cells were treated with either 10 .7 M DHT or DEX for a seven-day period and then stained for Zn-α_2-gp immunoreactivity, using specific antibodies raised against the purified protein from breast cyst fluid. As shown in Fig. 6, there was a remarkable staining in secretory granules present in T-47D cells treated with DHT. A similar effect was observed after treatment of these breast cancer cells with glucocorticoids (data not shown). By contrast, little or no staining was visible in the control cells (Fig. 6). It is also noticeable that in the different experiments performed there was a certain degree of variability in the staining of the control cells, as we had also observed at the mRNA level. In the cultures that were treated with E_2 no positive staining for Zn-α_2-gp was observed (data not shown). Finally, and with regard to the sensitivity of this semiquantitative method used to detect Zn-α_2-gp, it should be mentioned that according to parallel data from enzyme immunoassay and Western blot analysis, the immunocytochemical staining procedure is able to detect about 1 ng of Zn-α_2-gp in T-47D breast cancer cells.

Discussion

In the present study we show that expression of Zn-α_2-gp, a major breast cyst fluid protein belonging to the immunoglobulin superfamily and secreted by some breast carcinomas, is strongly induced by glucocorticoids and androgens in T-47D breast cancer cells. This is the first report showing that hormonal treatments alter the steady state levels of the mRNA coding for Zn-α_2-gp, and provides a basis to explain previous observations indicating that androgens stimulate secretion of this protein into the culture medium of breast cancer cells [26, 27]. In addition, we show that the effect of glucocorticoids and androgens is additive and is not abolished by estrogens. The time course and dose-dependence analysis performed in this work indicate that the sensitivity of the response of Zn-α_2-gp to DHT and DEX is similar to that found for the remaining major intracystic proteins (apo D and GCDFP-15) in breast cancer cells [8-13].

Finally, we show that the treatment of T-47D cells with DHT or DEX strongly induces the accumulation of anti- Zn-α_2-gp reacting material within the cells, in agreement with the results obtained by Haagensen et al. [27] in the conditioned media of hormone-treated T-47D cells.

However, and in spite of these similarities, the results presented in this work indicate that in contrast to data previously reported for apo D and GCDFP-15, there is not an antagonism between inducible expression of Zn-α_2-gp and cell growth. Thus, although it has been well established that the stimulation of secretion of these cystic proteins is accompanied by inhibition of proliferation of ZR-75-1 breast cancer cells [8-11], we have found that the stimulatory effect of glucocorticoids and androgens on Zn-α_2-gp expression does not correlate with any inhibitory effect of these hormones on T-47D proliferation. Even more, both DHT and DEX had a slight stimulatory effect on the growth rate of these breast cancer cells. In this regard, it is worthwhile mentioning that Chalbos et al. [32], Horwitz and Freidenberg [33], and Haagensen et al. [27] have shown that, in agreement with data presented herein, the addition of androgens and estrogens to T-47D cells displays minimal alterations in the cellular growth rate.

The action of progestins on the growth of T-47D cells in culture seems to have also generated some discrepancies, and it has been described as stimulatory or inhibitory [33-37]. In our case, PROG clearly behaved inhibiting the growth rate of T-47D cells, showing an inhibition of approximately 40% by day seven of the experiment. However, PROG failed to induce any stimulation of the expression of Zn-α_2-gp, in contrast with what has been described at the protein level using specific antibodies to detect the protein secreted into the culture medium [27].

Taken together, our data on Zn-α_2-gp mRNA expression support previous data suggesting that mechanisms controlling cell proliferation and secretion of cyst fluid proteins in T-47D breast cancer cells are not as directly coordinated as in ZR-75-1 [27]. The reason for these discrepancies is not clear, but it is tempting to speculate that they can be attributed to differences in the growth rate and hormone responsiveness of T-47D and ZR-75-1 breast cancer cell lines. These differences may be due to the occurrence of hormone receptor variants with altered functions in the diverse cell lines, as has been already demonstrated in the case of estrogen receptors in T-47D cells [38, 39] or in breast cancer tissues [40]. It should be also considered that high levels of progesterone receptor are constitutively expressed in this cell line, thus introducing additional problems in the interpretation of data related to this hormone. However, alternative possibilities including variation in the experimental conditions used or altered growth signaling pathways, cannot be definitively ruled out. In relation to this, it is also worthwhile mentioning that preliminary studies performed in our laboratory with ZR-75-1 cells appear to indicate that androgens and glucocorticoids are also able to induce the expression of Zn-α_2-gp in these cells, suggesting that the effect of at least these two hormones in Zn-α_2-gp levels is not limited to a single breast cancer cell line.

Regardless of these discrepancies in growth-rate responsiveness to hormonal treatments, the finding that the gene encoding Zn-α_2-gp is strongly induced by

glucocorticoids and androgens makes this protein interesting as a marker of hormone action in breast cancer cells. At present, it is widely assumed that breast cancer may result from an hormonal imbalance, with estrogens playing a major stimulatory role in the proliferation of breast cancer cells [41]. Consequently, in recent years a number of estrogen-induced proteins in breast cancer cells have been isolated and characterized [42-46]. However, the wide variability of clinical and biological behavior of breast carcinomas points to the involvement of multiple factors in their development, including hormonal stimuli other than estrogens. Therefore, the finding of proteins like Zn-α_2-gp which are indicative of a specific pattern of hormone responsiveness opens the possibility to study the mechanisms by which these hormones could contribute to the development and progression of a certain subtype of breast tumors. In relation to this, it should be mentioned that we have recently reported that about 40% of a series of 104 breast tumors have the ability to produce and secrete significant amounts of Zn-α_2-gp [22]. These results, together with those presented herein, point to a role for glucocorticoids and/or androgens in the development of this specific subset of breast carcinomas, although the potential induction of Zn-α_2-gp by other factors cannot be definitively ruled out. In addition, identification of these putative androgen/glucocorticoid dependent tumors could contribute to the selection of a subgroup of patients who, instead the widely used antiestrogen therapy, could benefit from alternative forms of endocrine therapy [8, 9, 47, 48].

Finally, it is remarkable that despite the striking structural similarity between Zn-α_2-gp and class-I major histocompatibility complex (MHC) antigens [23-25], the mechanisms controlling their expression appear to be completely divergent. Thus, according with data presented in this work, expression of Zn-α_2-gp gene is markedly dependent on specific hormonal stimuli including glucocorticoids and androgens. By contrast, these hormonal treatments did not show any significant effect on expression of class-I MHC genes, as demonstrated by Northern-blot analysis with specific probes (data not shown). These data are consistent with recent results from our laboratory indicating a wide divergence in the 5'-flanking region of the Zn-α_2-gp gene when compared with the corresponding region in class-I MHC genes [49]. Functional analysis of this region of the Zn-α_2-gp gene, which is now in progress, will be useful to better understand the molecular mechanisms by which glucocorticoids and androgens induce its expression in human breast cancer cells.

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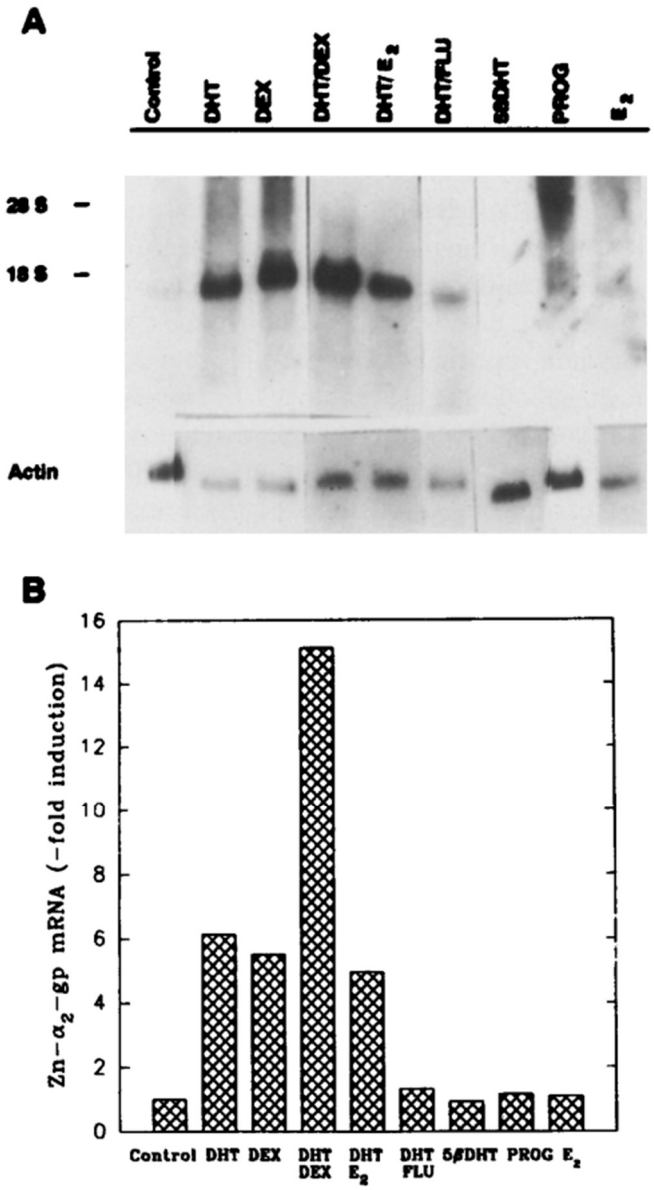

Fig. 1. Effect of steroid hormones on Zn-α_2-glycoprotein mRNA levels in T-47D human breast cancer cells. A, RNA blot analysis was performed using 10 gg of total RNA from T-47D cells incubated for seven days in the presence of a 10^{-7} M concentration of steroid hormones. RNA was electrophoretically fractionated on a 1% denaturing agarose gel and transferred to a nylon membrane. The integrity of the RNA in the different samples was ascertained by direct visualization of the stained gel and the nylon membrane under UV light. The filters were hybridized with a ^{32}P-labelled Zn-α_2-gp probe under stringent conditions. Autoradiography was a carried out for 3 days at - 70 ° C with intensifying screens. Filters were subsequently hybridized to a human actin probe in order to ascertain the differences in RNA loading among the different samples. B, Autoradiograms were scanned by using a densitometer and the signals obtained for Zn-α_2-gp correlated to the signals obtained for actin in the corresponding samples. The results are expressed as relative to the corresponding mRNA levels for Zn-α_2-gp in control cells. Three independent experiments were performed and the results here shown are representative of any of them.

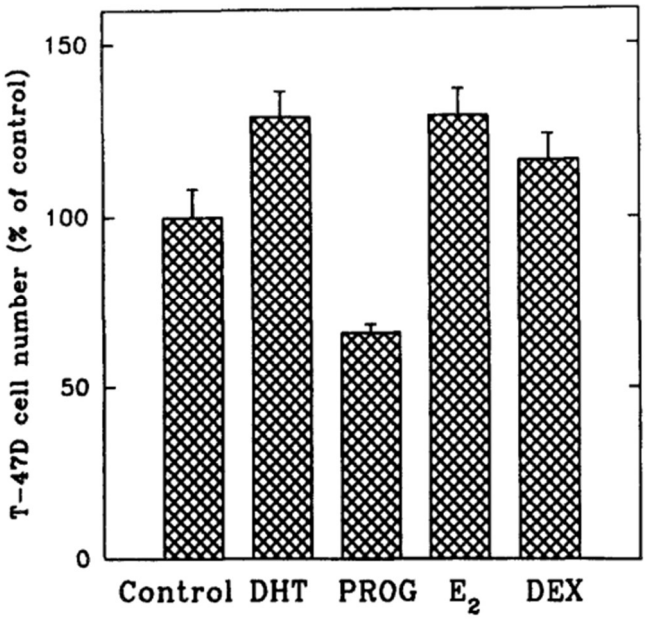

Fig. 2. Effect of steroid hormones on T-47D cell proliferation. Three days after plating, T-47D human breast cancer cells were incubated for seven days in the presence of a 10^{-7} M concentration of DHT, DEX, E_2, or PROG. Media were changed every two days. At the end of the incubation period, cell number was determined by counting the cells in a hemocytometer. The data are expressed as the mean of triplicate dishes in three independent experiments.

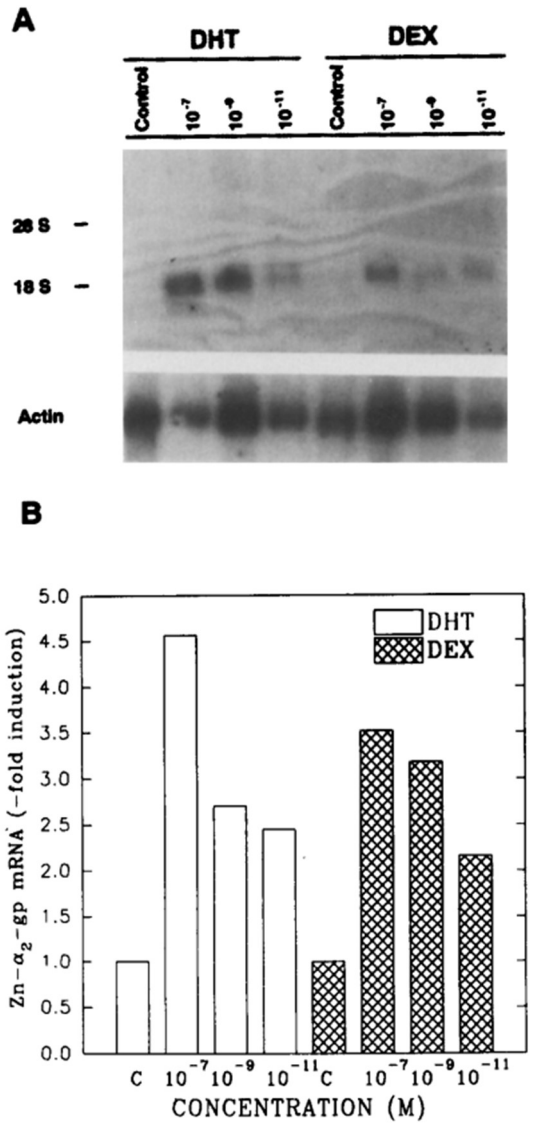

Fig. 3. Effect of increasing concentrations of dihydrotestosterone and dexamethasone on Zn-α_2-glycoprotein mRNA levels. A, T-47D cells were cultured for seven days in the presence of increasing concentrations of DHT or DEX and total RNA was analyzed by Northern blot, as described in Fig. 1. Filters were hybridized consecutively with labelled probes for Zn-α_2-gp and actin. B, Autoradiograms were scanned by densitometry and the values for Zn-α_2-gp mRNA in each sample corrected for differences of total RNA per lane. The results are expressed as relative to the values of control cultures. The experiment was performed three times and results from a representative experiment are shown.

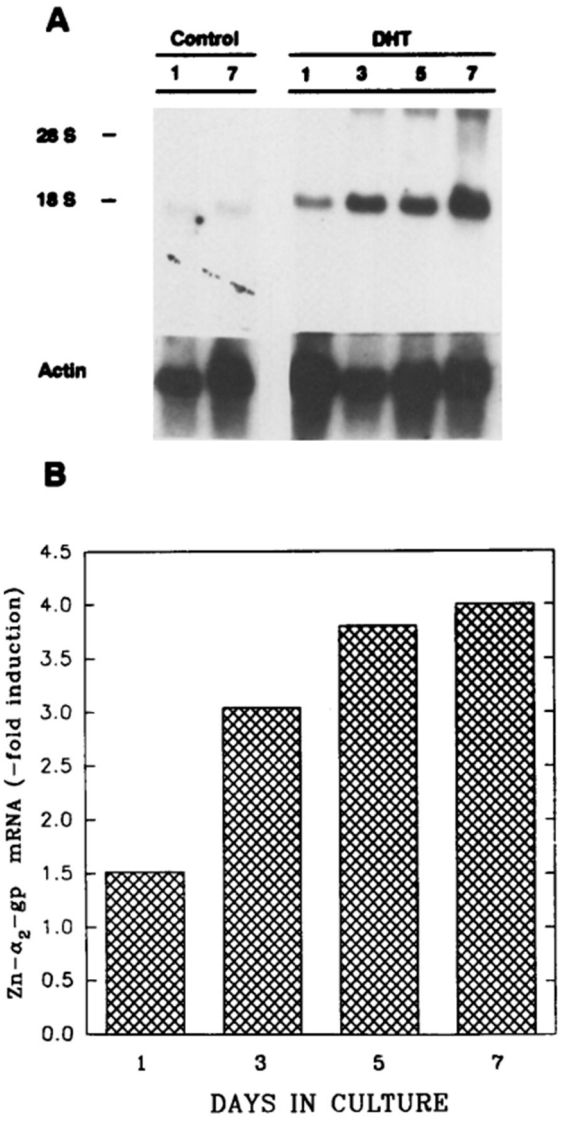

Fig. 4. Time-course of the effect of dihydrotestosterone on Zn-α_2-glycoprotein mRNA levels. A, T-47D cells were cultured in the presence of DHT 10^{-7} M for the indicated times and total RNA from each culture was isolated and analyzed as described in Fig. 1. B, The hybridization signals were scanned with a densitometer and correlated with the amount of total RNA as determined from the hybridization signal obtained with the actin probe. The values given are depicted as relative to the control values for each time point. The experiment was performed three times independently and representative results are shown.

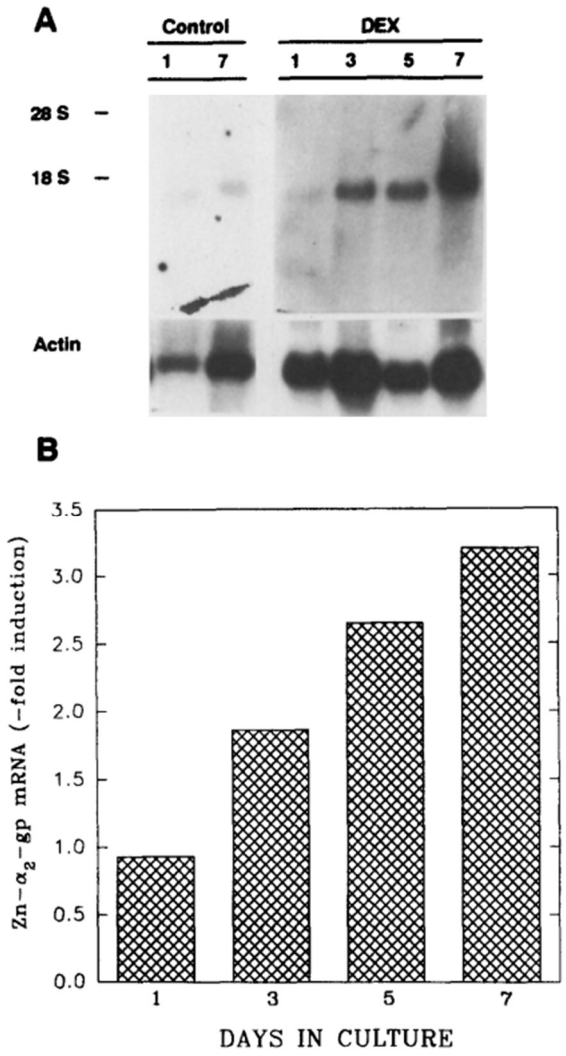

Fig. 5. Time-course of the effect of dexamethasone on Zn-α_2-glycoprotein mRNA levels. A, Total RNA from T-47D cells incubated in the presence of 10^{-7} M DEX for the times indicated was analyzed by Northern blot, as described in Fig. 1. B, Autoradiograms were scanned by densitometry and values for Zn-α_2-gp expressed as relative to control values. Three independent experiments were performed and the results here shown are representative of any of them.

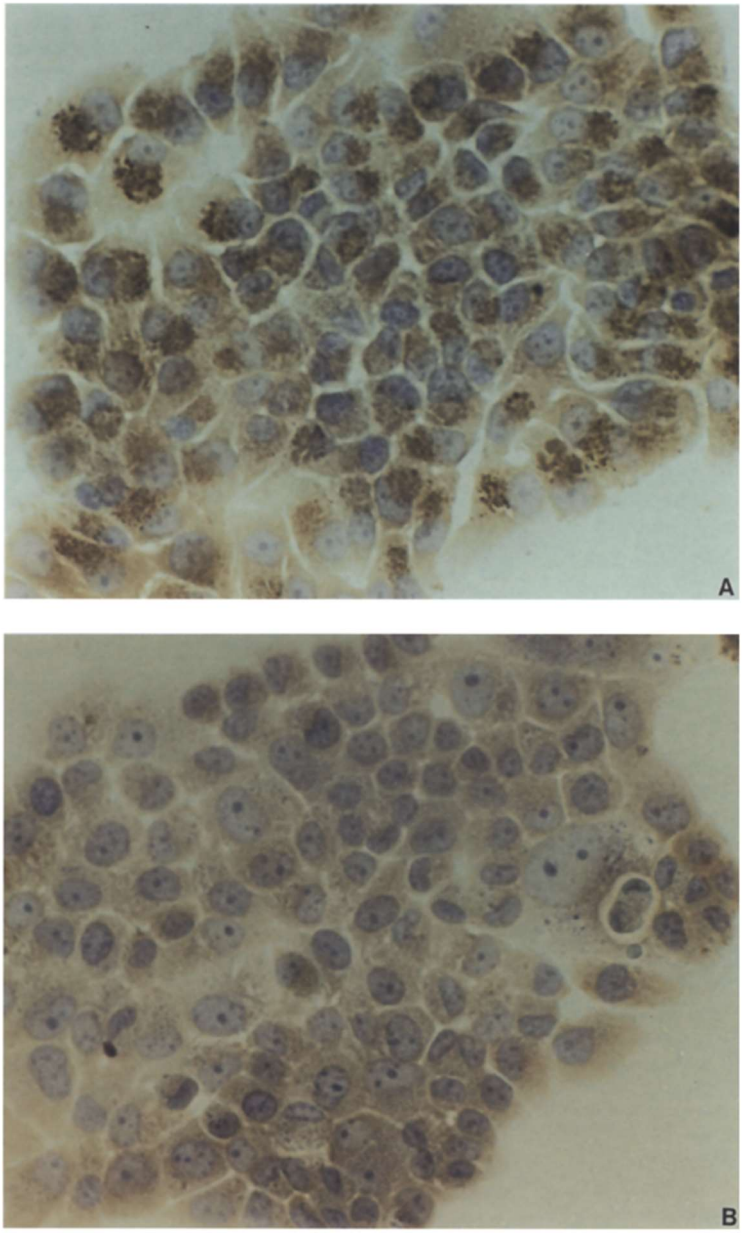

Fig. 6. immunocytochemical localization of Zn-α_2-glycoprotein in T-47D cells cultured in the presence of dihydrotestosterone. T-47D cells were grown on coverslips and treated for seven days with 10^{-7} M DHT. Zn-α_2-gp was stained using specific antibodies as described in Materials and methods. A brown-colored reaction product was clearly visible within the cytoplasm of cells treated with DHT (A) compared to control cells (B).