Induction of Estrogen-Regulated Genes Differs in Immortal and Tumorigenic Human Mammary Epithelial Cells Expressing a Recombinant Estrogen Receptor

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Studies on estrogen receptor (ER)-positive human breast cancer cell lines have shown that estrogen treatment positively modulates the expression of the genes encoding transforming growth factor-α (TGFα), 52-kDa cathepsin-D, and pS2. To determine whether these genes would be similarly regulated by estrogens in normal human mammary epithelial cells, we stably transfected immortal nontumorigenic human mammary epithelial cells with an ER-encoding expression vector. ER-negative tumor cells were also transfected for comparison. Levels of TGFα and 52-kDa cathepsin-D mRNA were enhanced by estrogen treatment of both ER-transfected immortal and tumorigenic cells, demonstrating that the ER by itself is sufficient to elicit estrogenic regulation of the expression of these genes.

In contrast, expression of the pS2 gene was detected only in the ER-transfected tumor cells. The ER in both cell lines is capable of recognizing the pS2 promoter, however, since estrogen enhanced the activity of an introduced pS2-CAT reporter plasmid in transient expression analyses. These and other experiments with somatic cell hybrids between the immortal cells and ER+/pS2+ MCF-7 tumor cells, where pS2 gene expression is extinguished, support the conclusion that the immortal nontumorigenic cells encode gene products that block endogenous pS2 expression. These results also imply that such repressors are not active in the tumor cells. (Molecular Endocrinology 5: 1613–1623, 1991)

INTRODUCTION

Estrogen is essential for the growth of the normal human mammary gland (1, 2) as well as for most estrogen receptor (ER)-positive mammary carcinomas (3, 4). It is generally accepted that estrogen responsiveness in target cells is mediated by hormone binding to high affinity receptors. These nuclear receptors are estrogen-inducible transcription factors, which specifically recognize promoter sequences [estrogen-responsive elements (ERE)] that influence the transcriptional efficiency of estrogen-regulated genes. In the ER-containing breast tumor cell lines MCF-7 and ZR-75-1, estrogen has been shown to enhance expression of the genes for transforming growth factor-α (TGFα) (5, 6), 52-kDa cathepsin-D (7, 8), and pS2 (9, 10), a secreted polypeptide with unknown function. A role for these gene products in the estrogen-dependent growth and metastasis of tumor cells has been proposed (5, 7).

Few studies of estrogenic effects on normal human mammary epithelial cells (HMEC) have been reported (11, 12). When this work was initiated, it was not known whether estrogenic stimulation of these cells would induce the same set of genes as in ER-positive breast carcinomas. However, recent advances in techniques for culturing normal HMEC (13–15) make possible expression studies using normal, limited lifespan HMEC derived from reduction mammoplasty specimens. Immortal nontumorigenic HMEC have also been established (16, 17). We report here that the cultured normal and immortal HMEC do not express ER protein and do not show modulation of TGFα or 52-kDa cathepsin-D mRNA levels by estrogens. In addition, pS2 mRNA is not detectable in any of these cell lines (Ref. 18 and this study). To determine whether the synthesis of ER in the normal cells would elicit estrogen-regulated expression of genes that are induced in ER-positive tumor cells, immortal HMEC (184B5) (16) were stably transfected with the cDNA for the ER. Tumor cells (21MT-2) (19) derived from an ER-negative pleural effusion were also made ER positive by transfection with the ER cDNA to permit comparison of ER-negative immortal and tumor cells transfected with the same expression vector.

These studies show that estrogenic modulation of the genes for TGFα and the 52-kDa cathepsin-D can be reconstituted in both ER-negative immortal and tu-

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morigenic mammary epithelial cells by transfection of the ER cDNA. However, estrogenic induction of the pS2 gene occurs only in the tumor cells.

Furthermore, we have shown previously (20) that pS2 gene expression is extinguished in somatic cell hybrids formed by fusion of immortal HMEC with ER-positive MCF-7 cells. In this report we show that the hybrid cells produce barely detectable amounts of the ER. Yet, transfection of the ER expression vector into these hybrid cells fails to activate the endogenous pS2 gene. Thus, factors in addition to those that regulate ER expression appear to be involved in suppression of pS2 gene expression by the immortal HMEC genome.

RESULTS

Normal Mammary Epithelial Cells Do not Express ER

Cytoplasmic and whole cell extracts from normal HMEC (184, 76N) were analyzed for their ER content by ligand binding assays (Table 1) and immunoreactivity to antibodies raised against the human ER (Fig. 1). No estradiol binding or ER protein was detected in these samples under conditions in which both the MCF-7 and T47D extracts were positive. Immortalized derivatives of these cells (A1N4, B5, 18–2P-1, and 16–1–1) as well as a cell line originating from human milk (HBL-100) are also negative in these assays (Fig. 1 and Table 1). Northern blot analyses of total RNA confirmed the absence of detectable ER mRNA in these cells (data not shown).

Table 1. Estradiol Binding in HMEC, B5-ER, and 21T-ER Clones

Cell Line	[^{3}H]Estradiol Binding (fmol/mg protein)
184	UD
76N	UD
HBL-100	UD
A1N4	UD
B5	UD
B-C1	UD
-E5	599
-E17	798
-E38	918
21T	UD
21T-C9	UD
-E1	405
-E2	373
-E3	637
-E4	442
-E5	269
MCF-7	74

The 100,000 × *g* supernatants from the indicated cell lines were analyzed for their ability to bind [^{3}H]estradiol (5 nM) after incubation at 4 C for 16 h in the presence and absence of a 200-fold molar excess of diethylstilbestrol. The difference in binding under these two conditions after removal of free steroid by DCC treatment is tabulated. All values reflect the results of triplicate determinations of total and nonspecific binding. Ligand binding was assayed at least twice in all of these cell lines. UD, Undetectable. Values less than 20 fmol/mg protein are at the limit of detectability for this assay.

Transfection of ER-Negative Immortal and Tumorigenic Mammary Epithelial Cells with the ER Expression Vector

To assess the effects of estrogen administration on normal mammary epithelial cells, the human ER was constitutively expressed in immortal HMEC (B5) by transfecting these cells with an expression vector, pSV2neo/CMV-ER, bearing the human ER protein-coding sequences under the control of the murine cytomegalovirus promoter. We have also transfected ER-negative tumor cells (21T), derived from a pleural metastasis with the ER cDNA to permit comparisons of estrogen-regulated gene expression in immortal and tumor cells containing the same expression vector.

Individual clones were analyzed for expression of the ER mRNA (data not shown) and protein. Western blot analysis demonstrated that the B5-ER transfectants (Fig. 2A, B-E clones) produce a 66-kDa protein recognized by anti-ER antibodies and indistinguishable in size from the ER protein found in ER-positive MCF-7 breast cancer cells. In contrast, the parental B5 population and a control transfectant (B-C3) receiving only the vector sequences show no detectable expression of ER protein (Fig. 2A). Similar results were obtained with the 21T-ER transfectants (Fig. 2B, 21T-E clones), which produce approximately the same amount of ER protein as B5-ER transfectants B-E17 and B-E38 and several-fold higher levels than the MCF-7 cells.

One measure of receptor activity is estradiol-binding capacity. No binding was detected in either the parental or control transfectants; the B5-ER and 21T-ER clones specifically bound amounts of [^{3}H]estradiol ranging

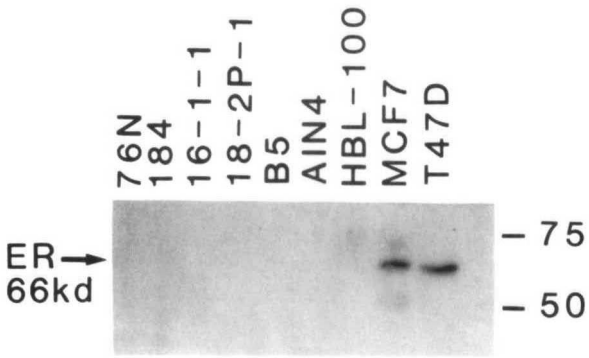


Fig. 1. Immunodetection of ER Protein in Normal, Immortal, and Tumor-Derived Mammary Epithelial Cells

Cell extracts (100 μg protein/lane) were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated with anti-ER antibodies, as described in *Materials and Methods*. The position of the 66-kDa band corresponding to the ER protein is indicated. Sizes (in kilodaltons) of the protein standards are indicated on the *right*.

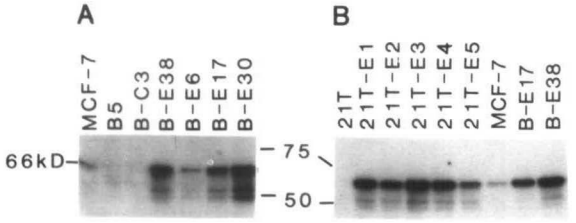


Fig. 2. Immunoblot Analysis of B5 and 21T ER Transfectants

A, Cell extracts (100 μg protein) from the parental (B5) and vector only (B-C3) transfectant are compared with MCF-7 and B5 ER-transfected (B-E6, 17, 30, 38) cells for production of ER protein by immunoreactivity with anti-ER antibodies (see Fig. 1 and *Materials and Methods* for details). The 66-kDa ER protein band and the sizes of the protein standards are indicated. B, Extracts from the parental 21T cells and ER-transfected clones (21T-E1, 2, 3, 4, 5) are compared with MCF-7 and B5-ER transfectants (B-E17 and 38) for levels of ER protein. The smaller mol wt bands (at approximately 50 kDa) are probably proteolytic degradation products.

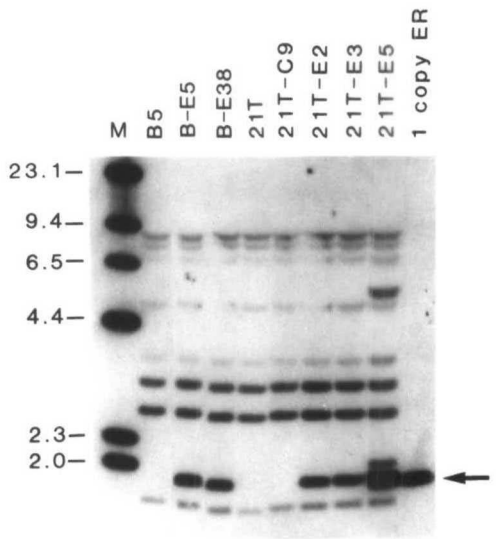


Fig. 3. Determination of pSV2neo/CMV-ER Plasmid Copy Number in ER Transfectants of B5 and 21T Cells

Ten micrograms of DNA from parental B5 and ER-transfected clones (B-E5, 38) as well as parental 21T, vector-transfected (21T-C9) and ER-transfected (21T-E2, 3, 5) clones were digested with *Eco*RI and analyzed by Southern blotting and hybridization with the probe for the ER cDNA. The *arrow* indicates the 1.8-kb band corresponding to the ER cDNA insert in the expression vector excised by *Eco*RI. *Eco*RI-digested pSV2neo/CMV-ER plasmid DNA (corresponding to one copy per genome) was analyzed in the *right lane*. M, size markers are ^{32}P-labeled DNA fragments from a λ-*Hin*dIII digest.

from 269–1000 fmol/mg protein (Table 1). These levels are approximately 5- to 10-fold higher than those in MCF-7 cells, in agreement with the relative protein amounts determined by Western blot analysis (see Fig. 2).

The high ER expression in these transfectants is not due to the integration of multiple copies of the ER expression vector sequences. Southern blot analysis of DNA from these clones showed that there is approximately one copy of the ER cDNA (encoded by the 1.8-kilobase (kb) *Eco*RI fragment) in all but the 21T-E5 transfectant, which has two additional *Eco*RI-generated fragments (Fig. 3).

The cellular localization of the ER was determined by immunoperoxidase staining of B5-ER cells. As shown in Fig. 4, A and B, the ER protein synthesized in these transfectants is sequestered in the cell nucleus, in agreement with reports for MCF-7 breast cancer cells (21). The B5 parental line and vector only transfectants showed undetectable staining (data not shown) similar to that observed for the control antibody reaction in Fig. 4C. The percentage of positive cells is estimated at 90% for B-E38 and 75–80% for B-E5, but is significantly less for other clones (*e.g.* 20–30% for B-E6; not shown). The receptor protein is similarly localized in the nucleus of the 21T-ER clones; the clones described here contain 80–95% cells positive for ER by immunoperoxidase staining (data not shown).

Estrogenic Modulation of Gene Expression in B5-ER Transfectants

Estrogen treatment of ER-positive breast cancer cell lines leads to increased expression of the genes for TGFα, 52-kDa cathepsin-D, and pS2 (6, 8, 9). To determine whether the presence of an ER in the B5 immortal mammary epithelial cells is sufficient to permit estrogen-mediated effects on gene expression, total RNA was isolated from the B5 and B5-ER transfectants treated with different concentrations of estradiol. As shown in the Northern blot analyses of Fig. 5A, both TGFα and 52-kDa cathepsin-D mRNA amounts increase in the B5-ER clones upon estrogen treatment, yet fail to be affected in the B5 parental population or in cells transfected with the vector alone (data not shown). In contrast, pS2 gene expression remains extinguished in the B5 cells and ER transfectants (Fig. 5B and data not shown).

Estrogenic Induction of the pS2 Gene Occurs in 21T-ER Clones

The ability of estrogen to modulate gene expression in the tumorigenic 21T-ER cells was also analyzed. Results similar to those observed for the B5-ER transfectants were obtained for the expression of TGFα and 52-kDa cathepsin-D mRNA. Neither the parental 21T cells (Fig. 5C) nor the control transfected clones (data not shown) showed estrogenic modulation of the expression of these genes. Yet, levels of TGFα and 52-kDa cathepsin-D mRNAs were significantly enhanced by estrogen addition to the ER-transfected 21T clones (21T-E in Fig. 5C). Similar results were obtained with the other 21T-ER clones (data not shown). The magnitude of the estrogen-dependent increase in steady state mRNA levels is comparable to that observed in MCF-7 and ZR-75-1 cells (6, 8, 22).

In striking contrast to the B5-ER results, where the

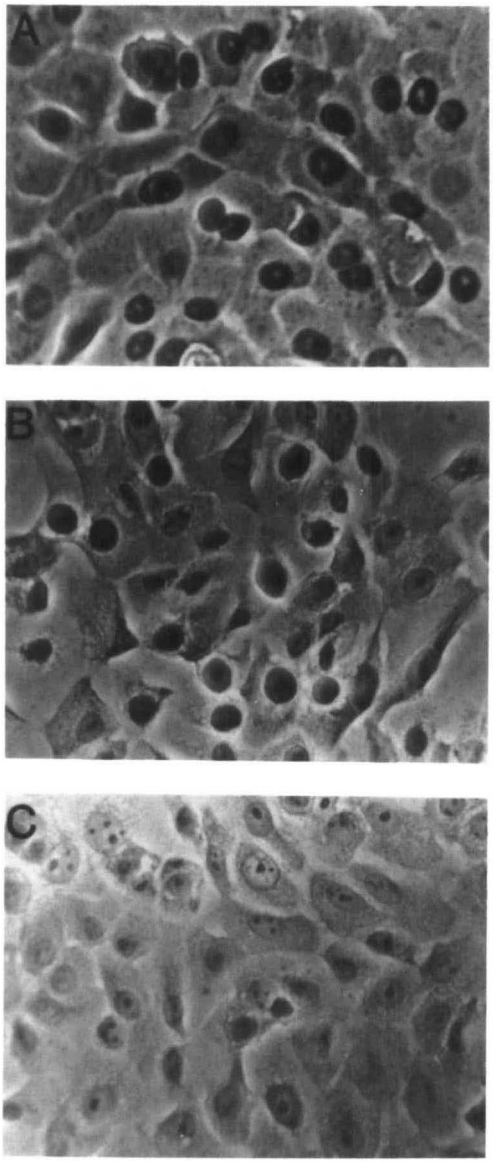


Fig. 4. Immunocytochemical Localization of the ER

B5-ER transfectants B-E38 (A and C) and B-E5 (B) cells were plated in chambered slides and treated with 5 μg/ml anti-ER antibody H222 (A and B) or normal rat IgG (C), as described in *Materials and Methods*. Phase contrast micrographs (×230) of the immunoperoxidase-stained cells are shown. Note the dark nuclei of the positively stained cells in A and B.

pS2 gene was extinguished regardless of ER expression, estrogen treatment of 21T-ER cells (Fig. 5C), but not of the parental 21T or control transfectants (not shown), resulted in the generation of detectable levels of pS2 mRNA. The amount of pS2 is at least 50-fold lower than that in either MCF-7 (data not shown) or ZR-75–1 cells (Fig. 5C).

Immortal and Tumor Cells Show Different Sensitivities to Estrogen Regardless of Growth Medium

Since the B5 and 21T cells require different medium conditions for optimal growth, it was possible that the ability of the 21T-ER cells to synthesize pS2 mRNA in response to estrogen treatment depended upon their growth in α+HE medium (see *Materials and Methods*). Cultivation of B5-ER clones B-E17 and B-E38 in α+HE medium (Fig. 5B) and 21T-ER clones 21T-E2 (data not shown) and E3 (Fig. 5C) in DFCI-1 medium generated results identical to those described above for each cell line.

Titration of the estradiol concentration required for induction of pS2 or augmentation of 52-kDa cathepsin-D and TGFα mRNA levels demonstrated a significant difference between the B5-ER and 21T-ER clones (Fig. 5). No modulation of 52-kDa cathepsin-D or TGFα mRNA levels was observed at estrogen concentrations less than 10^{-7} M for the B5-ER cells (Fig. 5B), whereas increased expression of these gene products as well as pS2 induction are detected at estradiol levels at least 100-fold lower (10^{-9} M) in the 21T-ER cells (Fig. 5C). Increased expression of the 52-kDa cathepsin-D gene is observed at 10^{-11} M estradiol in MCF-7 and ZR75–1 cells (8, 22). Augmentation of the TGFα gene mRNA levels has been reported to occur with a dose of 10^{-10} M estradiol (6).

The pS2-Driven CAT Activity Is Increased by Estrogen in B5-ER Cells

To determine whether the lack of pS2 expression in the B5-ER transfectants was due to an inability of the introduced ER to recognize the ERE sequences of the pS2 promoter, a pS2-CAT construct (23), bearing 1100 basepairs (bp) of pS2 promoter sequence was transfected into B5 and B5-ER cells. Chloramphenicol acetyltransferase (CAT) activity was analyzed in the presence and absence of estrogen. The parental B5 as well as the B5-ER cells produce low, but detectable, CAT enzyme in the absence of estrogen (Fig. 6A). Only the B5-ER transfectants (B-E17 and B-E38), however, show increased CAT activity when grown in the presence of estrogen. Treatment with 10^{-8} M estradiol resulted in increased pS2 promoter activity, albeit lower than that obtained at 10^{-7} M estradiol. Similar results were obtained using the *Xenopus* vitellogenin gene promoter (24), which also has an ERE (data not shown). In contrast, CAT activity in cells transfected with a control plasmid (pCMV-CAT), where the CAT gene is under the control of the cytomegalovirus promoter, is not modulated by estrogen in either the parental B5 cells or the ER-transfected clone (B-E38; Fig. 6B). Thus, the introduced ER is indeed functional, since it is capable of activating the pS2 promoter present on transfected plasmid DNA. Yet, it fails to induce the endogenous gene.

Extinction of pS2 Expression in B5xMCF-7 Cell Hybrids Is not Reversed by Introduction of the ER

The existence of tissue- and cell type-specific repressors of gene expression has frequently been inferred from studies of somatic cell hybrids between express-

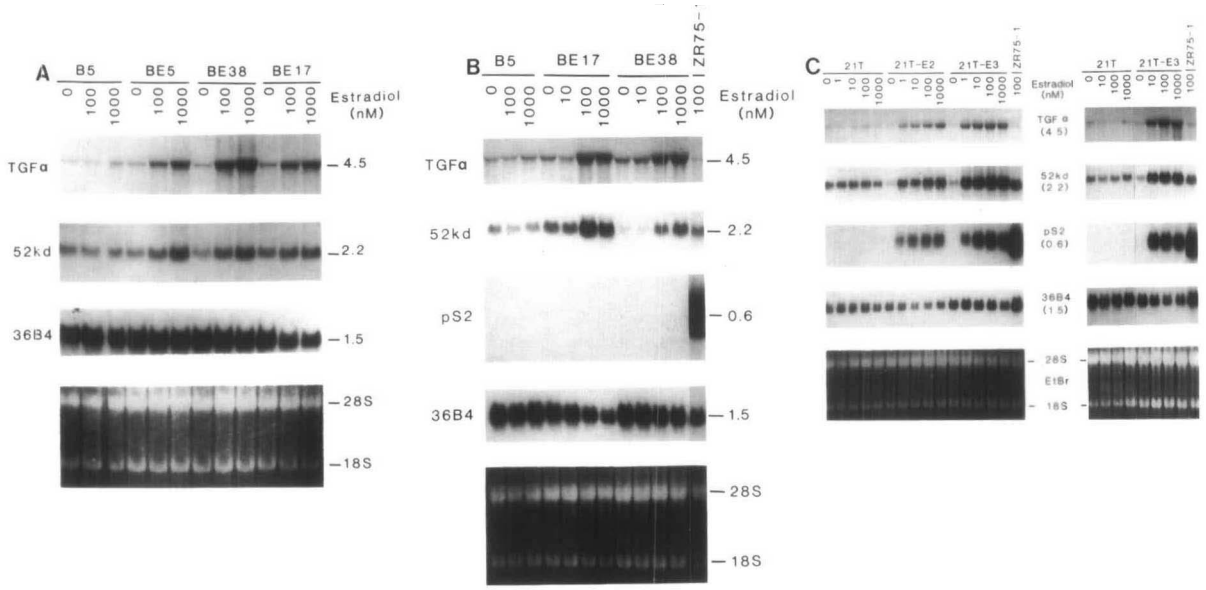


Fig. 5. Effects of Estrogen on TGFα, 52-kDa Cathepsin-D, and pS2 mRNA Levels in B5-ER and 21T-ER Cells

A, Total RNA (20 μg/lane) isolated from B5 and B5-ER transfectants B-E5, 38, and 17, which were cultured for 72 h in modified DFCI-1 medium containing the indicated concentrations of estradiol, was fractionated on 1.3% agarose-formaldehyde denaturing gels. Autoradiograms of the same filter hybridized with probes for TGFα, 52-kDa cathepsin-D, and 36B4 (control RNA, whose levels are not enhanced by estrogen) are shown *above* the photograph of the ethidium bromide-stained gel (indicating the ribosomal 28S and 18S RNA bands). The size (in kilobases) of the specific mRNA species recognized by each probe is given on the *right*. B, Total RNA from the indicated cell lines grown in modified α+HE medium with various concentrations of estradiol was analyzed as described in A. C (*Left side*), Total RNA from 21T parental and ER transfectants (21T-E2 and 3) grown in modified α+HE medium with the indicated concentrations of estradiol was analyzed as described in A. C (*Right side*), Same as in C, *left side*, except that the cells were cultured in modified DFCI-1 medium.

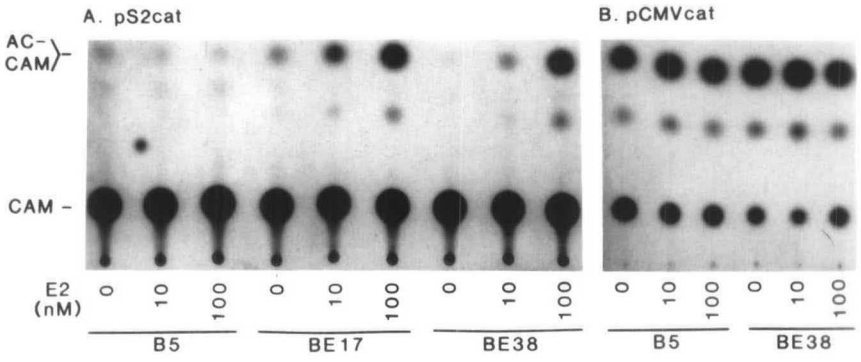
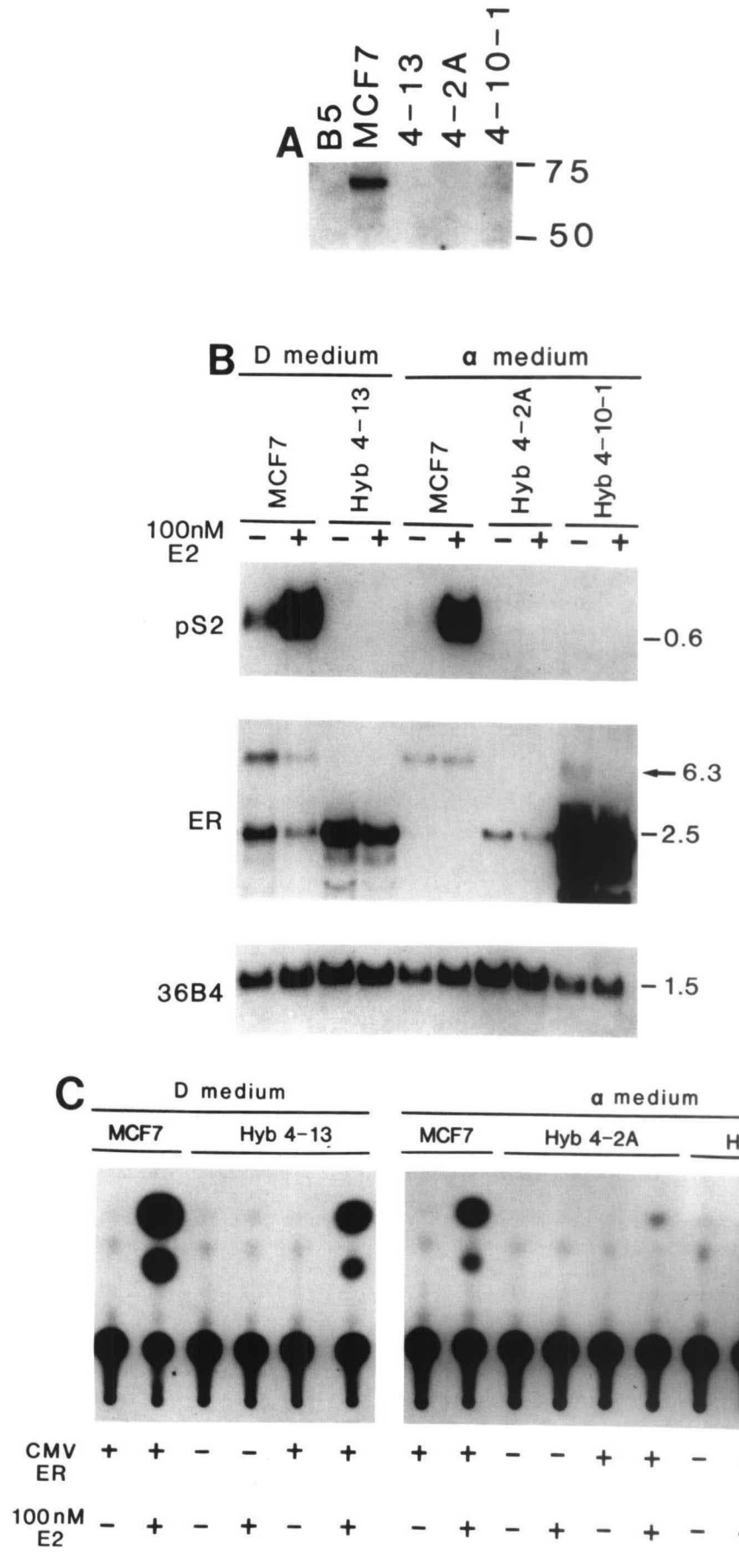


Fig. 6. Estrogen Dependency of pS2-CAT Expression in B5-ER Cells

A, B5 and ER clones B-E17 and 38 were transfected with 10 μg pS2-CAT, 2 μg pCMVβgal, and 8 μg pSP65 carrier DNA. After growth (36 h) in modified DFCI-1 medium (see *Materials and Methods*) containing the indicated concentrations of estradiol, cytoplasmic extracts (equivalent units of β-galactosidase activity) were analyzed for CAT activity. The autoradiogram of the TLC-fractionated extracts from a representative experiment is shown. The positions of the chloramphenicol substrate (CAM) and acetylated product (Ac-CAM) are indicated. B, B5 and B-E38 cells were transfected with 5 μg pCMV-CAT, 2 μg pCMVβgal, and 13 μg pSP65 carrier DNA and processed as described in A.

ing and nonexpressing cells. We have previously shown that somatic cell hybrids formed by fusion of B5 (ER-/pS2−) cells with MCF-7 (ER+/pS2+) cells do not express the pS2 gene (20). These hybrids express TGFα and 52-kDa cathepsin-D mRNA at levels comparable to those observed in the parental 184B5 cells, but there is no increase upon estrogen treatment (data not shown). Western blot analysis of whole cell extracts from these hybrid cell populations (Fig. 7A) as well as estradiol-binding assays (data not shown) revealed that they produce very low (hybrid 4–10–1) to undetectable (hybrids 4–2A and 4–13) levels of ER.

Since the chromosome bearing the active pS2 gene of the MCF-7 cell parent is present in the hybrid cells (determined by restriction fragment length polymorphism analysis; data not shown), lack of pS2 expression



could be due to inadequate levels of the ER. To test this possibility, the ER was transiently introduced into the hybrid clones by transfection of the ER expression vector. Total RNA was isolated 48 h later, but no expression of pS2 mRNA was detectable in the ER-transfected hybrid cells (Fig. 7B).

Evidence that functional ER were indeed expressed in this transient transfection include the detection of recombinant 2.5-kb ER mRNA (Fig. 7B). The levels of this mRNA varied dramatically in the different cell lines, with hybrid 4–10–1 showing the highest amounts. This may be due to different transfection efficiencies, since both the β-galactosidase activity (from the cotransfected plasmid control) of 4–10–1 extracts and the number of ER-expressing cells determined by immunocytochemistry were at least 10-fold greater than in the other transfected cells (data not shown). The activity of the transfected ER was ascertained by cotransfection of the pS2-CAT plasmid in parallel plates. Estrogenic enhancement of CAT activity occurred in the parental MCF-7 cells as well as in each of the ER-transfected hybrid cell lines (Fig. 7C). These results demonstrate that pS2 extinction in the hybrid cells is not due to the very low ER levels, since elevating ER expression by transfection failed to induce the endogenous pS2 gene.

DISCUSSION

Much evidence indicates that estrogens are important for the proliferation of the normal mammary gland (1, 2). We have found, however, that cultured normal HMEC do not express receptors for this steroid hormone. This finding is not surprising in view of reports which show that the levels of ER protein in the human breast fluctuate during the menstrual cycle (25). Furthermore, immunohistochemical analyses of sections from normal mammary gland indicate that only 7–10% of the epithelial cells are ER positive (26, 27). Taken together, these data imply that regulated expression of the ER occurs in the normal breast. The failure of the cultured HMEC to express ER protein may be due to nonpermissive culture conditions or to their identity as a stem cell population (28) which has not yet differentiated to an ER-positive cell type. They may also represent an ER-negative normal cell population.

Two distinct but related questions have been addressed by the studies described here. 1) Does stable expression of an ER in these cells permit estrogen-regulated gene expression to occur? 2) Does estrogen have different effects on gene expression in immortal and tumorigenic mammary epithelial cells? The answer to both questions is affirmative. We have constitutively expressed the ER protein in immortal nontumorigenic B5 human mammary epithelial cells and in ER-negative 21T tumor cells. A 66-kDa ER protein identical in size to that extracted from MCF-7 cells is found in the ER-transfected clones from both of these cell types. This receptor is appropriately localized in the cell nucleus and is expressed at levels ranging from 5- to 10-fold higher than those found in MCF-7 cells.

Estrogen treatment of both immortal and tumorigenic ER transfectants increased the steady state levels of the mRNA for TGFα and 52-kDa cathepsin-D. Thus, estrogenic regulation of these gene products is dependent upon the presence of an ER and does not require additional factors. The same conclusion was reached for expression of the 52-kDa cathepsin-D gene in the cervical cancer cell line, HeLa, which was transfected with a similar ER expression vector (29). To date, the regulation by estrogen of expression of the TGFα and the 52-kDa cathepsin-D genes in HMEC has only been shown in the ER-positive breast cancer cell lines ZR-75–1, T47D, and MCF-7 (6, 8, 22).

Our results with the immortal B5-ER cells show that estrogenic regulation of TGFα and 52-kDa cathepsin-D is not limited to tumor cells. Yet, it may be important that the doses of estradiol required to elicit these increases in mRNA level are much higher in the immortal cells than in the tumor cells. This is unlikely to be due to receptor number, since the levels are similar in all of the transfectants studied. It is possible that the estradiol is metabolized differently by these two cell types or that the apparent affinity for estradiol of the receptor differs depending on the cell type. It is noteworthy that the ER cDNA employed in these studies has a single nucleotide mutation in the ligand-binding domain (30). This altered ER protein has been shown to have a lower affinity for estradiol than the wild-type receptor (31). However, the different estradiol sensitivities of the B5-ER and 21T-ER cells cannot be due to this mutation, since the same cDNA was introduced into both cell types.

High levels of TGFα, 52-kDa cathepsin-D, and pS2 gene products have been found in subsets of breast

Fig. 7. Expression of ER in Hybrid B5 × MCF-7 Cells Enhances pS2-CAT but not the Endogenous pS2 Gene Activity

A, Immunoblot analysis of ER protein in total cell extracts (100 μg protein/lane) from parental (B5 and MCF-7) and hybrid (4–13, 4–2A, and 4–10–1) cells; see Fig. 1 for details). B, Northern blot analysis of total RNA (20 μg/lane) isolated from MCF-7 or hybrid cells transfected with pSV2neo/CMV-ER and cultured in either modified DFCI-1 medium (D medium) or modified α-Medium (see *Materials and Methods*) with or without 100 nM β-estradiol. The filter was sequentially hybridized with probes for the pS2, ER, and 36B4 mRNAs. The size of each transcript (in kilobases) is indicated at the *right*. The *arrow* points to the endogenous ER mRNA found in the MCF-7 cells. C, CAT activity was assayed in extracts from parallel plates of the same cells transfected with 10 μg pS2-CAT with or without 1 μg pSV2neo/CMV-ER (plus carrier pSP65 to a final concentration of 20 μg DNA). Medium conditions were identical to those in B. The amounts of extracts that yielded equivalent β-galactosidase activity were analyzed for each medium condition for all but 4–10–1, which contained 10-fold higher levels of β-galactosidase.

carcinomas (32–35). Yet, of these and other potential prognostic indicators in breast cancer, only the pS2 protein has been shown to be absent in the normal breast and benign fibroadenomas (32). Thus, it is remarkable that the ability of the introduced ER to mediate estrogenic induction of pS2 is strikingly different in the immortal and the tumor cells. The B5-ER cells do not produce pS2 mRNA, whereas the tumor-derived 21T-ER cells express pS2 mRNA only in the presence of estrogen. The B5 block to pS2 gene expression is not due to a failure to recognize the ERE of the pS2 promoter, since CAT expression after transient transfection of pS2-CAT constructs into the B5-ER cells is enhanced by estrogen. One explanation for these apparently contradictory findings is that the chromatin configuration of the endogenous gene in the B5 cells may be inaccessible to the transcriptional machinery. In addition, posttranscriptional modulation of the pS2 gene (*e.g.* instability of the pS2 mRNA in B5-ER cells) cannot be ruled out by the experiments presented here. In contrast, the pS2 gene in the 21T-ER cells must be poised for transcriptional activation, since transfection of the ER into those cells is sufficient to induce pS2 expression.

Studies of the mechanism of tissue-specific and estrogen-dependent expression of the vitellogenin gene have similarly shown that induction of the endogenous gene in chicken hepatocytes (36) or *Xenopus* kidney cells (37) or oocytes (38) does not occur upon introduction of ER. Yet, the transfected ER in the kidney cells and hepatocytes were shown to be functional, since estrogen treatment enhanced the activity of transfected vitellogenin promoter-CAT reporter plasmids (36, 37) or vitellogenin minigene constructs (37). Other analyses have pointed to a role for additional positive (39, 40) as well as negative transcription factors (40) in the control of tissue- and hormone-dependent vitellogenin gene expression.

Our experiments with the B5 × MCF-7 somatic cell hybrids suggest a role for inhibitory factors in preventing estrogenic activation of endogenous pS2 gene expression in B5-ER cells. Although the hybrid cells contain the pS2-bearing chromosomes from both B5 and MCF-7 parents, introduction of the ER failed to permit estrogenic induction of the pS2 gene. These results strongly support the possibility that extinction of pS2 expression by the B5 cells may involve a repression mechanism in addition to the down-regulation of ER expression. A similar mechanism has been proposed for the tissue-specific expression of the rat GH gene (41). Here, loss of a specific *trans*-activator and activation of a negative regulatory element may be responsible for extinction of GH gene expression in fibroblast-pituitary somatic cell hybrids. Our ability to measure estrogenic enhancement of pS2 promoter-driven CAT activity, but not endogenous pS2, in the ER-transfected hybrid cells may be explained by titration of the putative repressor molecules by the high copy number of the promoter introduced by transient transfection. Alternatively, and perhaps more likely, estrogenic induction of CAT activity would also occur if the negative regulatory element is absent from the pS2 promoter sequences in the construct or if sequences in the pS2-coding region are important for its regulation.

In summary, this study has shown that the ER is itself capable of mediating estrogenic enhancement of TGFα and 52-kDa cathepsin-D mRNA expression in two human mammary epithelial cell lines, one immortal and the other tumorigenic. In contrast, pS2 gene expression is blocked in the immortal ER-transfected HMEC, but induced by estrogen in the tumorigenic ER-transfected cells. These data and those obtained from analysis of the somatic cell hybrids suggest that the immortal B5 cells encode gene products capable of suppressing pS2 gene activity even in the presence of functional ER. This putative repressor of the pS2 gene is, however, absent or inactive in the tumorigenic 21T cells. The product of the pS2 gene is not detected in the normal mammary gland or in benign lesions (32), but is found in 35% of all ER-positive breast cancer biopsies and appears to predict an overall excellent prognosis (33). The expression of another estrogen-regulated gene, the progesterone receptor, is also indicative of a favorable prognosis (for review, see Ref. 50). It will be interesting to test whether progesterone receptor regulation is similar to that of TGFα and 52-kDa cathepsin-D or to pS2 in these ER-transfected HMEC. Understanding the mechanisms that regulate estrogen-dependent gene activity may provide insights into the means by which tumor progression occurs.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Normal HMEC (76N, 184) were derived from patient reduction mammoplasty specimens, as previously described (14, 15). 184B5 (B5) and 184A1N4 (A1N4) are immortal cell lines established by benzopyrene treatment of 184 normal cells (16). Immortalization of 76N normal cells by human papilloma virus 16 (HPV16) DNA transfection generated the 16AP cell line (17), from which 16–1–1 was subcloned. 18–2P-1 is a clonal population of HPV18-immortalized 76N cells (17). 21MT-2 (referred to as 21T) is a tumor cell line established from a pleural effusion specimen obtained from a breast cancer patient (19, 42). B5 × MCF-7 hybrid cells were produced by polyethylene glycol-mediated cell fusion (20). HBL-100, T47D, and ZR-75–1 cells were obtained from the American Type Culture Collection (Rockville, MD), and MCF-7 cells from the Michigan Cancer Foundation. All normal and immortal mammary epithelial cells were cultured in DFCI-1 medium, as previously described (14). MCF-7, T47D, and ZR-75–1 were grown in α-Minimal Essential Medium (GIBCO) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES (pH 7.0), 1 μg/ml insulin, and 50 μg/ml gentamycin sulfate (α medium). 21T and HBL-100 cells were routinely cultured in α medium supplemented with 2.8 μM hydrocortisone and 12.5 ng/ml EGF (α+HE medium).

Construction and Transfection of the ER Expression Vector

The 1.8-kb *Eco*RI fragment encoding the translated sequences of the MCF-7 ER gene (in Bluescribe M13+) (43) was cloned

into the unique *Eco*RI site of the eucaryotic expression vector pSV2neo/CMV (Yaswen, P, unpublished; details of construction available upon request). In this construct, expression of the ER sequences is controlled by the mouse cytomegalovirus immediate early promoter (Genetics Institute, Boston, MA; pmCMV X/H), and selection in G418 of successfully transfected clones is permitted by the simian virus-40 (SV40) promoter-driven neomycin resistance gene, which is included in the same plasmid.

Introduction of pSV2neo/CMV-ER or the vector DNA into B5 cells was performed by electroporation of 1×10^7 cells with 10 μg linearized plasmid DNA. Treated cells were plated at 1×10^6/100-mm tissue culture dish (Falcon, Oxnard, CA), and selection was initiated 2 days later in medium containing 100 μg/ml G418 (Gibco, Grand Island, NY). Individual clones were trypsinized and carried in DFCI-1 plus 50 μg/ml G418. These clones are called B5-ER.

Calcium phosphate-mediated transfection (44) of 21T cells (10^6 cells/100-mm plate) was carried out with 10 μg linearized pSV2neo/CMV-ER or the vector DNA. Selection of G418-resistant clones was begun 48 h later by growth in α+HE medium plus 200 μg/ml G418. Individual colonies were picked and maintained in the same medium with 100 μg/ml G418. These clones are designated 21T-ER.

ER Quantitation (Dextan-Coated Charcoal Assay)

Subconfluent cells were treated with estrogen-free medium for 12–24 h. The appropriate culture medium free of phenol red (Gibco) was supplemented with 1% (for DFCI-1) or 5% (for α medium) dextran-coated charcoal-treated FCS (DCC-FCS), which was prepared by heating the FCS (Hyclone, Logan, UT) at 56 C for 30 min, followed by one extraction with 0.1 vol 10% Norit-A (100–400 mesh; Sigma, St. Louis, MO)/0.1% dextran-10 mM Tris-HCl, pH 7.4 (50 C; 90 min). Cells were detached by trypsinization, treated with 0.0375% trypsin inhibitor-0.01 mM EDTA, and centrifuged 5 min at 800 × *g*, and cell pellets were resuspended in 400 μl 10 mM Tris-HCl (pH 7.4)-1.0 mM EDTA-1.5 mM dithiothreitol at 4 C and lysed using a Dounce homogenizer (Kontes Co., Vineland, NJ). Extracts (1.0–2.0 mg protein/ml) assayed for receptor levels were the supernatants obtained after centrifugation at 100,000 × *g* (30 min; 4 C). Extracts (100 μl) were incubated with β-[2,4,6,7-^{3}H] estradiol (5 nM final concentration; 105 Ci/mmol Amersham, Arlington Heights, IL) with or without a 200-fold molar excess of diethylstilbestrol (to measure nonspecific binding) overnight at 4 C. Unbound estradiol was removed by treatment with 200 μl 0.25% Norit-A-0.0025% dextran-10 mM Tris-HCl (pH 8.0) for 10 min at 4 C, followed by centrifugation for 5 min. Supernatants were counted in Biofluor at 42% efficiency in a Packard scintillation counter (Downers Grove, IL). The indicated levels of estradiol binding reflect correction for the amount of protein remaining in the supernatant after DCC extraction.

ER Characterization by Western Blot Analysis

Subconfluent monolayer cultures were treated as described for ligand binding assays (see above) and lysed in 20 mM Tris (pH 7.5) containing 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium desoxycholate, 0.5 U/ml aprotinin (Sigma), and 3 mM phenylmethylsulfonylfluoride at a final cell density of 2×10^7/ml. After centrifugation in an Eppendorf microfuge (5 min; 4 C), supernatants were recovered and frozen at −70 C.

Aliquots of the cell lysates corresponding to 100 μg protein [determined by a Coomasie blue reagent assay (Bio-Rad, Richmond, CA), using BSA as standard] were denatured by boiling for 5 min in sample buffer [10% glycerol, 2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, 80 mM Tris (pH 6.8), and 0.004% bromophenol blue] and analyzed by electrophoresis in 8% SDS-polyacrylamide gels using Bio-Rad low mol wt prestained markers as standards. Transfer of gel-fractionated proteins onto 0.2-μm nitrocellulose membranes (Schleicher and Schuell, Keene, NH) was performed in a Bio-Rad minielectroblotting apparatus at 200–300 mamp for 2 h in 20% methanol-25 mM Tris-192 mM glycine (pH 8.3). Immunoblotting was carried out essentially as described by Greene *et al.* (45). Briefly, nitrocellulose filters were blocked by incubation (2 h; 25 C) in 3% Blotto (Carnation Instant Milk) in 50 mM Tris (pH 7.5) containing 150 mM NaCl and 0.2% Tween-20 (TBS). The overnight labeling (4 C) with anti-ER antibody H222 Spγ (1 μg/ml in 1% Blotto-TBS; Abbott Laboratories, North Chicago, IL) was followed by bridging antibody [2 μg/ml rabbit antirat immunoglobulin G (IgG); 1 h; 25 C], [^{125}I]protein-A (1 μCi/ml; 30 mCi/mg for 1 h at 25 C; Amersham), and autoradiography.

ER Immunocytochemistry

B5-ER cells were trypsinized and plated in chamber slides (Lab-Tek, Naperville, IL). After 1–2 days, cells were treated using a modification of the immunoperoxidase staining technique of King *et al.* (46). After fixation in freshly prepared 3.7% formaldehyde-PBS, cells were permeabilized in methanol (4 min; −20 C) and methanol-acetone (1:1) (4 min; −20 C). Endogenous peroxidases were inhibited by incubation in methanol-3% hydrogen peroxide (4:1) at 25 C for 20 min. Nonspecific binding was blocked by incubating the cells in 3% normal goat serum (NGS) in PBS (30 min; 25 C). They were then treated with either anti-ER antibody H222 Spγ (5 μg/ml; Abbott Laboratories) or normal rat IgG in 1% NGS overnight at 4 C. Sequential treatment with goat antirat IgG (10 μg/ml in 1% NGS for 30 min at 25 C) and peroxidase-antiperoxidase complex [Sternberger-Meyer, Jarretsville, MD; 1:100 in 50 mM Tris-HCl (pH 7.4)-0.85% NaCl (TNS) plus 1% NGS for 30 min at 25 C] preceded the visualization of peroxidase activity by reaction with 0.6 mg/ml diaminobenzidine-0.06% hydrogen peroxide in TNS for 6 min at 25 C.

Studies of Gene Expression in B5-ER and 21T-ER Clones

B5, 21T, and transfected clones were plated (5×10^5/100-mm dish for B5 and derivatives; 1×10^6/150-mm dish for 21T and derivatives) in either modified DFCI-1 medium (lacking estradiol, phenol red, and bovine pituitary extract) containing 5% DCC-treated FCS) or in modified α+HE medium (without phenol red and containing 5% DCC-treated FCS). The following day, cells were fed with fresh medium containing the appropriate estradiol concentration (see figure legends). Medium was changed 48 h later, and cells were harvested approximately 72 h after estrogen addition.

Northern and Southern Blot Analyses

Total cellular RNA from subconfluent cell monolayers was isolated, fractionated on agarose gels, electroblotted onto nylon filters (Zeta-Probe), and hybridized with the specified probes, as previously described (18).

DNA was isolated from confluent cell monolayers, and 10 μg were digested by *Eco*RI, fractionated on a 0.8% agarose gel, and processed for Southern blot analysis, essentially as previously described (18) except that the DNA was transferred to Immobilon-N filters (Millipore, Bedford, MA). Hybridization of ^{32}P-labeled probes was performed in 6 × SSC (1 × SSC = 150 mM NaCl-15 mM sodium citrate, pH 7.0), 0.5% SDS, 5 × Denhardts solution (0.1% each of Ficoll, polyvinylpyrrolidone, and BSA), 10 mM EDTA, and 100 μg/ml denatured salmon sperm DNA at 65 C for 20 h.

The DNA restriction fragments used to probe Northern blots for expression of the TGFα and pS2 genes have been previously described (18). The 1.45-kb *Eco*RI fragment from p52k9 (22) hybridizes to the 52-kDa cathepsin-D mRNA, and the *Pst*I 800-bp fragment from the 36B4 cDNA clone, which corresponds to a relatively uncharacterized mRNA whose levels are unaltered by estrogen treatment (32), was used to quan-

titate 36B4 mRNA levels. The ER mRNA and genomic sequences were probed by the 1.8-kb *Eco*RI fragment (43).

Transient Transfections with CAT Reporter Plasmids

Plasmid pCMVβgal was constructed by ligating the 4.0-kb *Bam*HI-*Hin*dIII β-galactosidase-encoding fragment from pCH110 (Pharmacia, Piscataway, NJ) and the 1.4-kb *Hin*dIII-*Eco*RI fragment containing the mouse cytomegalovirus promoter from pmCMVX/H (Genetics Institute) into pSP64 (Promega, Madison, WI) cut with *Bam*HI and *Hin*dIII. In a similar transfer of the CMV promoter fragment, pCMV-CAT was constructed by replacing the SV40 promoter/enhancer of pSV2cat (47). The pS2-CAT reporter plasmid (23) was kindly provided by P. Chambon.

B5 cells and ER transfectants were seeded in DFCI-1 medium at 1×10^6 cells/100-mm dish and incubated at 37 C for 16–20 h. The culture medium was replaced with a modified DFCI-1 medium (without estradiol, phenol red, and bovine pituitary extract; containing 5% DCC-treated FCS, and 0.02 μM hydrocortisone). Cells were exposed to a calcium phosphate-DNA coprecipitate containing either pS2-CAT (10 μg) or pCMVCAT (5 μg), pCMVβgal (2 μg; used as a control for transfection efficiency), and pSP65 (to bring the total DNA to 20 μg/plate) for 6 h. At this time, the cells were osmotically shocked in 15% glycerol (in solution A, a balanced salts solution) (13) for 4 min, and fresh medium was added with and without the indicated concentrations of estradiol (diluted from a 5×10^{-4} M stock dissolved in absolute ethanol). Approximately 36 h later, the plates were rinsed with solution A, harvested in 250 mM Tris, pH 8.0, and frozen in dry ice-ethanol. After two cycles of freeze-thaw to lyse the cells, the cytoplasmic extracts were obtained by centrifugation in an Eppendorf centrifuge (10 min), and aliquots were assayed for protein concentration, β-galactosidase activity, and CAT activity.

The MCF-7 parent and hybrid cells were plated (5×10^5/100-mm dish) in α or DFCI-1 medium, as indicated. Transfections with 1 μg pSV2neo/CMV-ER and 19 μg pSP65 (carrier DNA) for RNA analysis or 10 μg pS2-CAT and 2 μg pCMV-βgal, with and without 1 μg pSV2neo/CMV-ER (and pSP65 to increase the total DNA to 20 μg) were performed as described above. The culture medium was either modified α-medium (without phenol red or insulin; containing 5% DCC-stripped FCS) or the DFCI-1 counterpart described above. Cells were grown in the presence or absence of 100 nM estradiol for 36 h before harvesting for RNA or β-galactosidase/CAT analysis.

β-Galactosidase and CAT Activity Measurements

Cytoplasmic extracts (5–20 μg total protein) from transfected cells were assayed for β-galactosidase levels essentially as previously described (48), but using chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim, Indianapolis, IN) as substrate and the modifications of Fridovich-Keil *et al.* (49).

CAT activity was analyzed according to previously described procedures (47), using 0.1 μCi [^{14}C]dichloroacetyl-1,2-chloramphenicol (New England Nuclear; 60 Ci/mmol)/assay volume of 120 μl. Cytoplasmic extracts were incubated for 60 min at 37 C to generate the acetylated product, which was extracted with ethyl acetate and chromatographed in chloroform-methanol (95:5) on silica gel TLC plates (Baker flex 1B).

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