

Increases in estrogen receptor- α concentration in breast cancer cells promote serine 118/104/106-independent AF-1 transactivation and growth in the absence of estrogen

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ABSTRACT A common phenotype in breast cancer is the expansion of the estrogen receptor- α (ER+) cell population and an inappropriate elevation of ER α protein, the latter predisposing patients for a poorer prognosis than those with lower levels of the receptor. A tetracycline-inducible ER α overexpression model was developed in the MCF-7 cell line to assess induction of endogenous gene activation and growth in response to elevations in ER α protein. Heightened levels of ER α resulted in aberrant promoter occupancy and gene activation in the absence of hormone, which was independent of ligand and AF-2 function. This increased receptor activity required the amino-terminal A/B domain and was not inhibited by tamoxifen, which supports an enhancement of AF-1 function, yet was independent of serine-104, 106, and 118 phosphorylation. Ligand-independent transcription was accompanied by an increase in growth in the absence of hormonal stimulation. The results suggest that elevated levels of ER α in breast cancer cells can result in activation of receptor transcriptional function in a manner distinct from classical mechanisms that involve ligand binding or growth factor-induced phosphorylation. Further, they describe a potential mechanism whereby increases in ER α concentration may provide a proliferative advantage by augmenting ER α function regardless of ligand status.—Fowler, A. M., Solodin, N., Preisler-Mashek, M. T., Zhang, P., Lee, A. V., Alarid, E. T. **Increases in estrogen receptor- α concentration in breast cancer cells promote serine 118/104/106-independent AF-1 transactivation and growth in the absence of estrogen.** *FASEB J.* 18, 81–93 (2004)

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ABNORMAL EXPRESSION of estrogen receptor α (ER α) is a striking pathological phenotype in many cases of breast cancer and is thought to contribute significantly to the natural history of the disease. Immunohistochemical analyses of malignant breast tumors frequently reveal an increase in both the number of cells

with detectable ER α protein and the staining intensity of ER α in individual cells compared with non-neoplastic breast tissue (1). Breast tumors with a high ER α content have been found to display features indicative of an increased proliferative rate, such as tumor cellularity (2, 3) and thymidine kinase activity (4, 5). The level of ER α expression appears to be an important factor in the natural history of breast cancer because patients with the highest ER α content who do not receive adjuvant therapy have as poor a chance for recurrence-free survival as patients with undetectable ER α (3, 6–8).

The prevalent molecular model constructed to explain these clinical observations is that high levels of ER α promote the growth of hormone-dependent tumors by potentiating receptor function as a ligand-dependent transcription factor. In vitro studies using heterologous (ER-negative) cell systems support the idea that increased steroid hormone receptor levels directly affect the cell's maximal capacity to mediate hormone-stimulated gene transcription. Specifically, investigators have determined that the magnitude of ER α - and glucocorticoid receptor-mediated transcriptional responses is directly proportional to the number of receptor molecules per cell (9, 10). However, other studies suggest that distinct ER α -dependent processes require vastly different amounts of receptor and that receptor concentration is not always the factor limiting the degree of estrogen responsiveness. Dose-response studies in estrogen-responsive human breast carcinoma (MCF-7) and pituitary lactotrope (PR1) cell lines showed that lower doses of estrogen are required for cell proliferation than for expression of certain estrogen target genes such as progesterone receptor and prolactin (11, 12). These observations suggest that receptor requirements may vary depending on the cell

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context and the response in question, making it difficult to extrapolate the functional consequences of elevated ER α in breast cancer cells from previous studies performed in heterologous systems.

Here, we directly investigated the consequences of an increase in ER α protein levels using a tetracycline (tet)-inducible gene expression system created in MCF-7 breast cancer cells. In the inducible expression system used in this study, ER α levels could be controlled by the amount of doxycycline (Dox) in the culture media. The magnitude of receptor activity before and after induction could then be determined such that the cellular context remains identical except for the levels of ER α . By this method, a causal relationship between receptor levels and function can be directly established and molecular mechanisms addressed.

MATERIALS AND METHODS

Cell culture

MCF-7 and human embryonic kidney (HEK) 293 cells were maintained at 37°C and 10% CO₂ in high glucose DMEM with phenol red and L-glutamine (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT, USA), 100 units/mL penicillin G (Life Technologies, Inc., Gaithersburg, MD, USA), and 100 μ g/mL streptomycin (Life Technologies). Tet-inducible cell lines and the parental cell line (see below) were maintained in the presence of 0.5 μ g/mL puromycin and/or 200 μ g/mL G418, respectively. Antibiotics were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Generation of a tet-regulated ER α HA cell line

Wild-type MCF-7 cells were stably transfected first with the pUHD172-1neo plasmid (13). This plasmid encodes a neomycin resistance gene used for clonal selection and a fusion product of the VP16 activation domain of herpes simplex virus and a mutated *Escherichia coli* tet repressor protein. Neomycin-resistant clones were tested for responsiveness to Dox (Sigma), a tetracycline derivative, by transient induction of a luciferase reporter gene. A clone that exhibited low background and high inducibility was chosen as the parental cell line (RTA-16). Overhang PCR was used to fuse an 11 amino acid fragment from the human influenza virus hemagglutinin (HA1) epitope (14) to the carboxyl terminus of human wild-type ER α to distinguish exogenous from endogenous receptor. Epitope-tagged full-length and Δ A/B ER α were subcloned into the pUHD10-3 plasmid downstream of the tet resistance operator and a minimal cytomegalovirus (CMV) promoter, then stably transfected into the parental clone along with a puromycin resistance plasmid (15). The Δ A/B ER α mutant was generated from the parental plasmid HE19 (16), kindly provided by Dr. P. Chambon.

Hormone treatments

Cells were washed with PBS and grown at 5% CO₂ in phenol red-free DMEM (Mediatech) supplemented with 4 mM L-glutamine (Life Technologies), 100 units/mL penicillin G, 100 μ g/mL streptomycin, and 10% steroid-depleted FBS for a minimum of 5 days prior to hormone treatment unless stated otherwise. Steroid-depleted serum was prepared by

undergoing the procedure outlined by Reddel et al. (17) six times. 17 β -Estradiol (E2) and 4-hydroxytamoxifen (4-OHT) were purchased from Sigma. The anti-estrogen ICI 182,780 was a gift from Dr. Jack Gorski. The final concentration of ethanol (EtOH) was 0.1% in all samples. Hormone incubation times for each experiment are indicated in the figure legends.

Northern blot analysis

Total RNA was isolated from cells using TRIZOL reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Ten micrograms of total RNA were electrophoresed in a 1.5% agarose gel containing formaldehyde and transferred to a nylon membrane (Gene-Screen; PerkinElmer Life Sciences, Boston, MA, USA) (18). The RNA was immobilized to the membrane by UV cross-linking (GS Gene Linker, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Prehybridization and hybridization of the membrane were performed in a hybridization oven (Robbins Scientific Corporation, Sunnyvale, CA, USA) at 55°C in a 25% formamide solution. The blot was probed with ³²P-radiolabeled cDNA of human *pS2*. Signal was quantified with a PhosphorImager (Storm 840 with ImageQuant software; Molecular Dynamics, Inc., Sunnyvale, CA, USA).

Chromatin immunoprecipitation assay

ChIP assays were performed based on the protocol described by Weinmann and Farnham (19). Briefly, protein-DNA complexes were cross-linked by treating cells with formaldehyde. Nuclei were isolated and chromatin was sheared to an average length of 500 bp by performing three pulses of 20 s at output setting 7 using a Model 60 Sonic Dismembrator (Fisher Scientific, Hampton, NH, USA). Samples were centrifuged and the resulting supernatant was kept for input samples (10%) and subsequent immunoprecipitation. Protein A/G agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were blocked with bovine serum albumin (BSA) and herring sperm DNA (Promega Corporation, Madison, WI, USA) for 4 h at 4°C. Extracts were immunoprecipitated with 2 μ g of an anti-ER α antibody (HC-20) overnight at 4°C, followed by addition of blocked protein A/G agarose beads and another incubation for 1 h at 4°C. Immunoprecipitates were eluted from the washed beads and the cross-links were reversed. DNA fragments were then purified using the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA, USA) and amplified by PCR in the linear range using the following primers for *pS2*: (–533) 5'-GTTGGGATTA-CAGCGTGAG-3' (forward primer) and 5'-TGGAAGGATTT-GCTGATAGA-3' (–155) (reverse primer). PCR products were analyzed by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining.

Western blot analysis

To obtain whole-cell extracts, harvested cells underwent centrifugation, followed by a wash in PBS and lysis in a high-salt detergent buffer (Totex buffer; 20 mM HEPES, pH 7.9, 350 mM NaCl, 20% glycerol, 1% IGEPAL CA-630, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT). The concentration of protein in the supernatant was determined using the Bradford method (20). Proteins were separated by electrophoresis and Western blot analysis was performed as described previously (21). Membranes were immunoprobed with saturating concentrations of primary antibodies. These included an anti-HA antibody directed against the HA tag (1:2000, HA.11, Berkeley Antibody Co., Richmond, CA, USA) and anti-ER α antibodies that recognize the amino terminus

of human ER α (1:1000, NCL-ER-6F11/2, Novocastra Laboratories, Ltd., New Castle Upon Tyne, UK; 1:1000, H-184, Santa Cruz Biotechnology) or its carboxyl terminus (1:500, HC-20, Santa Cruz Biotechnology). Protein bands were visualized using the enhanced chemiluminescence detection method (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). To ensure equivalent protein sample loading, blots were reprobed with an anti-actin antibody (1:2000, C-11, Santa Cruz Biotechnology). Total ER α content in the parental, clone 3, and clone 9 cells was quantified using a secondary antibody conjugated to ^{125}I (NEN Life Science, Hounslow, Essex, UK), followed by PhosphorImager analysis (Storm 840 with ImageQuant software, Molecular Dynamics, Inc.).

ERK phosphorylation analysis

ER α HA cells were washed twice with PBS and incubated in serum-free media for 24 h before treatment with vehicle, 1 $\mu\text{g}/\text{mL}$ Dox for 4, 6, 8, and 24 h, 10^{-8} M E2 for 10 min, or 100 ng/mL epidermal growth factor (EGF; Sigma) for 10 min. Cell extracts were prepared as described by Watters et al. (22). Anti-phospho-Thr202/Tyr204-ERK1/2 (1:1000, E10; Cell Signaling Technology, Inc., Beverly, MA, USA) and anti-ERK1 antibodies (1:1000, C-16, Santa Cruz Biotechnology) were used for Western analysis.

Receptor localization studies

ER α HA cells were grown on confocal-ready tissue culture plates (MatTek Corporation, Ashland, MA, USA) and treated with vehicle or 1 $\mu\text{g}/\text{mL}$ Dox for 48 h. Cells were subsequently fixed overnight with 3.7% formaldehyde in PBS at 4°C. A solution of 3.7% formaldehyde in PBS with 0.2% Triton X (Sigma) was applied to the cells and incubated for 2 h at 4°C to increase membrane permeability. Cells were washed with 0.2% Tween[®] 20 (Fisher Biotech, Fair Lawn, NJ, USA) in PBS (PBST), then incubated with 5% goat serum (Sigma) in PBST at 37°C for 1 h to block nonspecific antibody binding. Primary antibodies were then added to all plates and incubated at 37°C for 1 h. These included an anti-HA (1:100, HA.11) and an anti-ER α antibody (1:100, HC-20). Cells were washed with PBST, then exposed to goat anti-mouse IgG Alexa Fluor[®] 488 and goat anti-rabbit IgG Rhodamine Red-X[™] conjugates (1:200, Molecular Probes, Inc., Eugene, OR, USA) for 20 min at 37°C. Cells were imaged using a laser confocal scanning microscope (Bio-Rad MRC 1024 and Laser Sharp software, Bio-Rad Laboratories).

Transient transfection and reporter gene assays

HEK293, MCF-7, and tet-inducible cell lines were transfected using the calcium phosphate method, Superfect[®] (QIAGEN), or Fugene[™] 6 (Roche Molecular Biochemicals, Indianapolis, IN, USA) transfection reagents, respectively. Reporter plasmids included an ERE-tk-Luc construct, which contains multimerized estrogen response elements (ERE) derived from the vitellogenin gene fused to a thymidine kinase (tk) promoter of herpes simplex virus, followed by the firefly luciferase gene (Luc), and an enhancerless tk-Luc plasmid (23). Transfection efficiency was normalized to β -galactosidase activity from a cotransfected CMV- β -galactosidase construct (CMV- β -gal). The wild-type ER α construct was generated as described previously (24). The mutant ER α construct pCMV5 ER α S104/106A was generously provided by Dr. B. Katzenellenbogen. The $\Delta\text{A/B}$, ΔLBD , S118A, and ΔNLS ER α mutants (HEG19, HE15, HEG457, and HEG5 respectively) were kindly provided by Dr. P. Chambon. Mutant ER α constructs were subcloned into the LHL-CA expression vector (25). This

vector contains a Moloney murine leukemia viral LTR driving the expression of a gene-encoding hygromycin resistance, a CMV enhancer, and an actin promoter controlling the expression of the receptor. It does not contain viral coding regions. The day after transfection, cells were treated with hormone and harvested after an additional 24 h. In the experiments involving mutant ER α constructs, MCF-7 cells were treated with 200 $\mu\text{g}/\text{mL}$ hygromycin B (Roche Diagnostics Corporation) for 24 h before harvesting to select against cells that did not become transfected. Luciferase (Luciferase Assay System, Promega Corporation, Madison, WI, USA) and β -galactosidase (Galacto-Light Plus[™] Chemiluminescent Reporter Gene Assay System, Tropix, Inc., Bedford, MA, USA) activity was measured as per manufacturer's instructions. EC₅₀ values were determined by nonlinear regression analysis for a sigmoidal dose-response curve (Prism 3.0; GraphPad Software, San Diego, CA, USA). Statistically significant differences were determined using a paired Student's *t* test.

Elk-1 transactivation assay

ER α HA cells were grown in steroid-depleted media for 24 h before transfection. DNA was transfected using Superfect reagent and included 0.5 μg CMV- β gal, 0.5 μg pFR-Luc (a luciferase reporter gene containing a 5X GAL4 binding site upstream of a minimal promoter), and 50 ng pFA2-Elk1 (an expression vector encoding a GAL4 DNA binding domain fused to the Elk-1 transactivation domain). Negative and positive control plasmids included pFC2-dbd and pFC-MEK1 expression vectors encoding the GAL4 DNA binding domain or a constitutively active MEK1 mutant, respectively. The above vectors were part of the PathDetect Elk-1 *trans*-Reporting System (Stratagene, La Jolla, CA, USA). Three hours after addition of the transfection mixture, the cells were washed twice with PBS and grown in serum-free media. Cells were harvested after 6 h incubation with EtOH, 10^{-8} M E2, or 10^{-7} M phorbol 12-myristate 13-acetate (PMA; CalBiochem, La Jolla, CA, USA) and after 6 and 24 h treatment with 1 $\mu\text{g}/\text{mL}$ Dox. Luciferase and β -galactosidase activity was measured as described above.

Cell proliferation studies

ER α HA cells were washed with PBS and grown at 5% CO₂ in phenol red-free DMEM and 5% steroid-depleted FBS. After 3 days of estrogen withdrawal, 250,000 cells were plated per 10 cm dish. After a 2 day exposure to 1 $\mu\text{g}/\text{mL}$ Dox, cells were treated with EtOH or 10^{-8} M E2. Cells were harvested 2 and 6 days after hormone treatment. Culture media containing the appropriate treatments (vehicle alone, Dox alone, E2 alone, Dox and E2) was replaced 2 and 5 days after Dox treatment. Total genomic DNA was isolated from ER α HA cells using the Puregene[™] DNA purification kit (Gentra Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. DNA samples were quantified using UV spectrophotometry. Doubling times were determined by nonlinear regression analysis for an exponential growth curve (Prism 3.0; GraphPad Software, San Diego, CA, USA).

Cell cycle analysis

ER α HA cells were washed with PBS and grown at 5% CO₂ in phenol red-free DMEM and 10% steroid-depleted FBS. After 3 days of estrogen withdrawal, cells were plated at 10^6 cells per 10 cm culture plate. After a 2 day exposure to 1 $\mu\text{g}/\text{mL}$ Dox or vehicle, cells were treated with EtOH or 10^{-8} M E2 for 24 h. Experiments were also conducted in which cells were incubated in serum-free media for 24 h before treatment with

or without 1 $\mu\text{g}/\text{mL}$ Dox or 10^{-8} M E2 for 48 h. Cells were harvested by trypsinization, washed with PBS containing 1 mM EDTA and 0.1% BSA (PBS/EDTA/BSA), and fixed in 70% EtOH for 30 min at -20°C . Cells were incubated with propidium iodide stain (33 $\mu\text{g}/\text{mL}$ propidium iodide, 1 mg/mL RNase A, 0.2% IGEPAL CA-630 in PBS/EDTA/BSA) overnight in the dark at 4°C . Prior to flow cytometry, the cells were passed through a 41 μm nylon mesh screen (Sefar America Inc., Depew, NY, USA) to remove large aggregates. Cell cycle analysis was performed using a FACScan flow cytometer (Becton Dickinson and Co., San Jose, CA, USA) using ModFit LT data analysis software (Verity Software House, Inc., Topsham, ME, USA). The coefficient of variation of the G1 peak varied from 3 to 5% using this protocol.

RESULTS

Generation of a tet-inducible gene expression system to modulate ER α concentration in ER+ breast cancer cells

A tet-inducible system was developed in the well-characterized MCF-7 breast cancer cell line in which expression of an epitope-tagged ER α (ER α HA) could be controlled by the addition of Dox to the culture medium. To generate the double stable tet-on cell line, an expression vector encoding ER α HA under the control of a tet-responsive promoter was coinjected into a MCF-7 cell line containing the reverse tet transactivator construct along with a plasmid that confers resistance to puromycin. After drug selection, 12 stable colonies emerged and were screened for inducible expression of ER α HA by Western blot analysis. Three clones were found to express ER α HA after treatment with 1 $\mu\text{g}/\text{mL}$ Dox for 24 h.

The conditions that promote expression of the exogenous receptor were optimized by monitoring ER α HA protein levels in cells treated with varying doses and for varying times with antibiotic. As shown in Fig. 1A, ER α HA induction was detectable by stimulation with as little as 0.1 $\mu\text{g}/\text{mL}$ Dox, strong expression being attained with 1 and 5 $\mu\text{g}/\text{mL}$. We used a dose of 1 $\mu\text{g}/\text{mL}$ for time course experiments, as Gossen et al. (13) noted that prolonged exposure to doses in excess of 3 $\mu\text{g}/\text{mL}$ could result in cytotoxicity. Induction of ER α HA expression occurred within 4 h after application of Dox and increased progressively with longer treatment times (Fig. 1B). Similar time- and dose-dependent increases in ER α HA protein levels were observed in another positive clone (data not shown). Therefore, in all subsequent experiments treatment of cells consisted of 1 $\mu\text{g}/\text{mL}$ Dox for 48 h to induce high levels of ER α HA protein.

To quantify the changes in relative ER α levels in the absence and presence of Dox, ER α protein was measured by PhosphorImager analysis in two positive clones and in the tet-responsive parental cell line, which does not express ER α HA. Cells were grown with or without Dox and Western blot analysis was performed using a primary anti-ER α antibody, which can detect both receptor species, and an ^{125}I -radiolabeled secondary antibody. Induction of ER α HA expression by

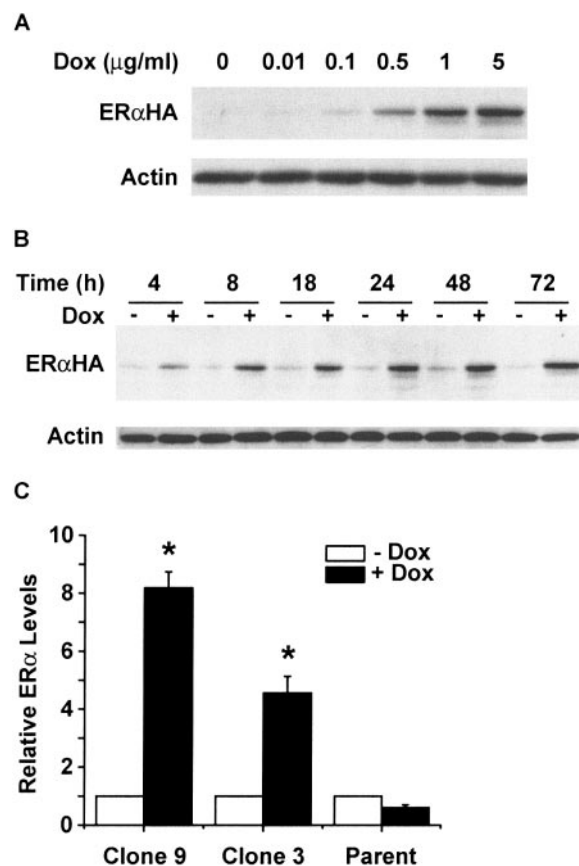


Figure 1. Dox-regulated expression of ER α HA. The induction properties of a tet-inducible HA-tagged ER α (ER α HA) were assessed by Western blot analysis of ER α HA protein in stable cell lines treated with varying doses of Dox for 48 h (A) and treated with 1 $\mu\text{g}/\text{mL}$ Dox for varying lengths of time (B) using an antibody directed against the HA epitope (HA.11). Shown is an analysis of a representative clonal line, clone 9. Blots were reprobbed with an anti-actin antibody (C-11) to verify equal loading between lanes. C) The fold increase in ER α concentration was determined by PhosphorImager analysis of Western blots, which used an ^{125}I -conjugated secondary antibody. The primary antibody was anti-ER α (NCL-ER-6F11/2), which detected both the HA-tagged and endogenous receptor. Quantitative analysis was performed on two clones and the parental cell line in the presence and absence of 1 $\mu\text{g}/\text{mL}$ Dox for 48 h. Increases in total ER α levels were determined relative to the amount of endogenous receptor present in cells grown in the absence of Dox, which was set at 1.0. Data represent the mean \pm SE for a minimum of 3 independent experiments. Statistical differences were determined by paired Student's *t* test. **P* < 0.01, relative to -Dox controls.

Dox dramatically increased the steady-state levels of ER α protein by 8- and 4.5-fold in clones 9 and 3, respectively (Fig. 1C). The presence of the antibiotic itself resulted in a modest decrease in ER α protein content in the parental cell line.

High concentrations of receptor stimulate endogenous promoter occupancy and gene expression in the absence of hormone

By overexpression of ER α in MCF-7 cells, where estrogen regulates endogenous targets, we were afforded the

opportunity to ask how high concentrations of ER α protein affects gene activation in the context of higher order regulation by chromatin and cell- and promoter-specific factors. The effect of increased ER α levels on *pS2* expression was measured using Northern blot analysis. Estrogen-induced *pS2* expression in cells with higher levels of receptor was not different from controls (Fig. 2A, B). However, an increase in *pS2* mRNA was observed in the absence of hormone stimulation, reaching a level not significantly different from that induced by estrogen in control cells. The effects of increased ER α expression on insulin-receptor substrate-1 and progesterone receptor-B protein levels in the presence and absence of hormone were equivalent to that observed for *pS2* (data not shown).

Chromatin immunoprecipitation assays were performed to determine whether the increase in gene expression resulted from an alteration in ER α occu-

pancy of the *pS2* promoter, which contains an imperfect ERE (26). Stable receptor binding to estrogen target genes configured in chromatin is regulated by conformational changes that occur upon ligand binding. This dependence on hormone for receptor occupancy is demonstrated in control samples (Fig. 2C, -Dox). However, upon overexpression, ER α is strongly bound to the *pS2* promoter in the absence of hormone (Fig. 2C, +Dox), which indicates that high levels of ER α facilitate receptor occupancy of target gene promoters. This loss of dependence on hormone for recruitment to the DNA could explain, in part, the ligand-independent induction of the *pS2* gene resulting from ER α overexpression.

For comparison, transcriptional activation of an idealized ERE-driven reporter gene was also examined. ER α HA (clones 3 and 9) and parental cells were grown in the presence and absence of Dox, followed by stimulation with EtOH or 10^{-8} M E2 for 24 h. Similar to what was observed with endogenous target genes, elevation of ER α concentration in clone 9 increased reporter gene transcription by fivefold in the absence of estrogen (Fig. 3A). A rise in hormone-independent receptor activity (3.5-fold over uninduced cells) was also noted in clone 3. Dox treatment had no effect on ER α transactivation in the tet-responsive parental clone (data not shown). However, in contrast to endogenous gene regulation, increased ER α concentration in clone 9 potentiated estrogen-dependent transactivation of an ERE reporter by ~twofold (Fig. 3A). In clone 3, the effect of increased ER α content on estrogen-stimulated transcription was 1.5-fold. These findings reinforce the idea that transcriptional regulation of genes in the context of chromatin differs from that of naked DNA constructs, but they also demonstrate that the most pronounced effect of elevated levels of receptor, whether on endogenous or reporter genes, is the induction of gene expression in the absence of hormone.

Dose-response studies of clone 9 demonstrated that estrogen-induced transcriptional activity of an ERE was enhanced at all doses of ligand. Comparison of the dose-response curves in cells expressing endogenous and elevated levels of receptor indicates that, though the overall level of gene expression was greater in cells with higher levels of ER α , the dose required for half maximal response (EC_{50}) was not substantially different (6 pM) from uninduced cells (3 pM) (Fig. 3B). The direct overlap in dose-response curves under these varying receptor conditions indicates that the mechanism of hormone-induced gene activation in cells overexpressing ER α is not altered by either overexpression or epitope-tagging of the receptor.

To further confirm that the epitope tag does not impair ER α transactivation function, HEK293 cells were transiently transfected with wild-type ER α and/or ER α HA expression vectors along with an ERE reporter gene and were subsequently stimulated with either E2 or EtOH for 24 h. The epitope-tagged and wild-type receptor induced equivalent levels of gene transcrip-

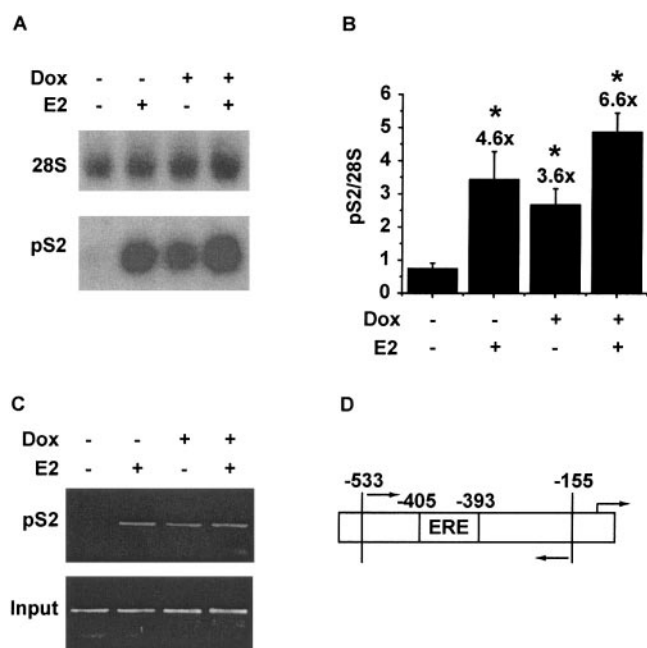


Figure 2. Expression and promoter occupancy of *pS2* gene in cells with increased concentrations of ER α . ER α HA (clone 9) cells were treated with (+) or without (-) Dox for 48 h, followed by 24 h incubation with (+) or without (-) 10^{-8} M E2. *A*) Expression of the *pS2* gene was determined by Northern blot analysis using a 32 P-radiolabeled cDNA probe. 28S rRNA served as a loading control. *B*) Data are presented as the mean \pm SE of the ratio of the signals of *pS2* to 28S RNA determined by PhosphorImager analysis of 3 independent experiments. Shown above each error bar is the fold increase in *pS2* expression relative to uninduced control cells treated with EtOH. Statistical differences were determined by paired Student's *t* test. **P* < 0.05, relative to -Dox, -E2 control. *C*) Chromatin immunoprecipitation assay was performed on cells treated in the presence (+) and absence (-) of Dox and E2 to examine recruitment of ER α to the *pS2* promoter in vivo, as described in Materials and Methods. Shown are the PCR amplification products from samples immunoprecipitated with an anti-ER α antibody (HC-20) (top panel) and from 10% of the input genomic DNA (lower panel). PCR was conducted using primers specific to the ERE-containing region of the *pS2* promoter shown in panel *D*.

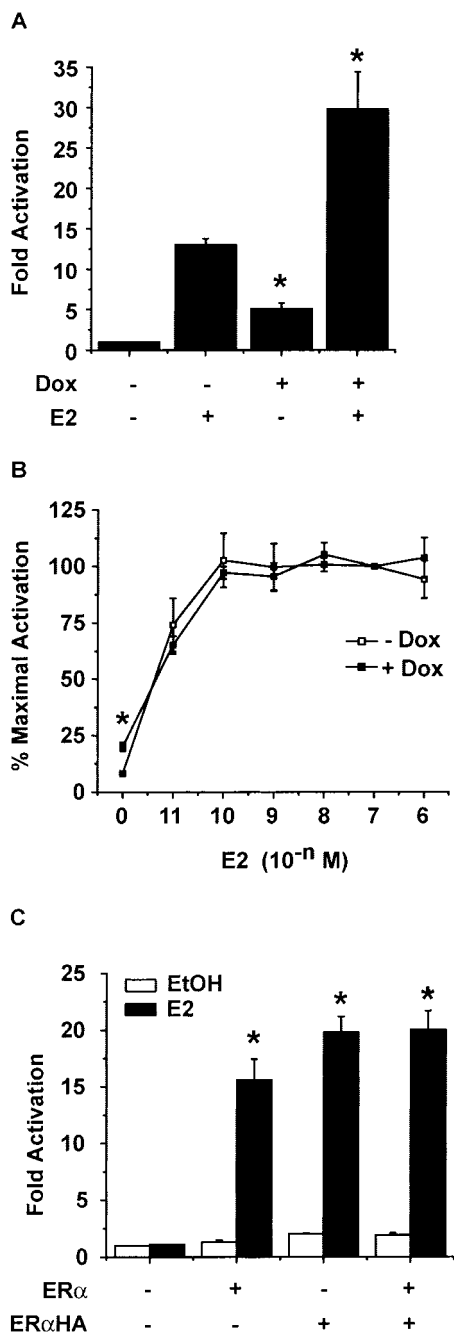


Figure 3. ER α transcriptional activity in cells with increased receptor concentration. *A*) Transcriptional activation of an ERE-tk-Luc reporter gene was assessed by transient transfection of ER α HA cells (clone 9) in the presence (+) or absence (-) of 1 μ g/mL Dox. To control for transfection efficiency, cells were cotransfected with a CMV- β -gal reporter gene. After transfection, cells were treated with 10⁻⁸ M E2 (+) or EtOH (-) for 24 h. Luciferase activity was normalized to β -galactosidase activity and an enhancerless reporter gene (tk-Luc). Fold activation was calculated relative to the EtOH-treated control in the absence of Dox, which was set at 1.0. Data represent the mean \pm SE for 6 independent experiments. Statistical differences were determined by paired Student's *t* test. **P* < 0.05, relative to the respective treatment in the absence of Dox. *B*) Transient transfection assays were performed as in panel *A* using varying doses of E2 and is presented as a percentage of maximal activation to illustrate the dose of estrogen required for half maximal response

(Fig. 3C). Coexpression of the tagged receptor with wild-type ER α did not result in any additional transcriptional activity than in cells expressing only the epitope-tagged receptor.

Increased transcriptional activity of unliganded ER α requires the A/B domain, but not the ligand binding domain

ER α protein is functionally organized into modular domains responsible for binding ligand, transactivation, and binding DNA (16). Receptor transactivation occurs through two separable activation functions, AF-1 and AF-2, located in the amino and carboxyl termini of the receptor, respectively. To determine whether AF-1, AF-2, or both are required for transactivation when unliganded ER α levels are elevated, mutant receptors lacking the amino-terminal A/B domain (Δ A/B) or the carboxyl-terminal ligand binding domain (Δ LBD) were used. The mutant receptors were transiently overexpressed in MCF-7 cells and transcriptional activity was compared with cells overexpressing wild-type receptor or vector alone. Transient overexpression of wild-type ER α and the Δ LBD receptor, which contains AF-1 but lacks the ligand binding domain, resulted in increased ligand-independent reporter gene activity that were not significantly different from each other (Fig. 4A). In contrast, high levels of the Δ A/B receptor, which retains the ligand binding domain and AF-2, were insufficient. The Δ A/B mutant receptor was however able to activate transcription in response to estrogen (data not shown) indicating that the lack of activity of this mutant receptor in the absence of estrogen was not due to a generalized defect in transactivation. Western blot analysis (Fig. 4B) confirmed that the expression levels of the mutant receptors were comparable to that of wild-type. These results provide three important findings: 1) the transcriptional induction by elevated ER α concentration is indeed independent of ligand, 2) the LBD is neither necessary nor sufficient for the activity arising from high concentrations of receptor, and 3) the acquired receptor function requires an intact A/B domain.

To independently examine the requirement for the A/B domain, we established a second stable tet-induc-

(EC₅₀) in cells overexpressing ER α compared with uninduced cells. 100% corresponds to 29- and 13-fold activation for cells treated with and without Dox, respectively, measured at the 10⁻⁷ M dose of E2. Statistical differences were determined as in panel *A*. **P* < 0.05, relative to -Dox controls. *C*) HEK293 cells were transiently transfected with wild-type ER α or ER α HA expression vectors and ERE-tk-Luc reporter plasmid, followed by treatment with 10⁻⁸ M E2 or EtOH for 24 h. Luciferase activity was normalized to β -galactosidase activity as in panel *A*. Fold activation in luciferase activity was calculated relative to the EtOH-treated cells transfected with vector. Data are shown as the mean \pm SE for 3 independent experiments. Statistical differences were determined by paired Student's *t* test. **P* < 0.01, relative to EtOH-treated controls.

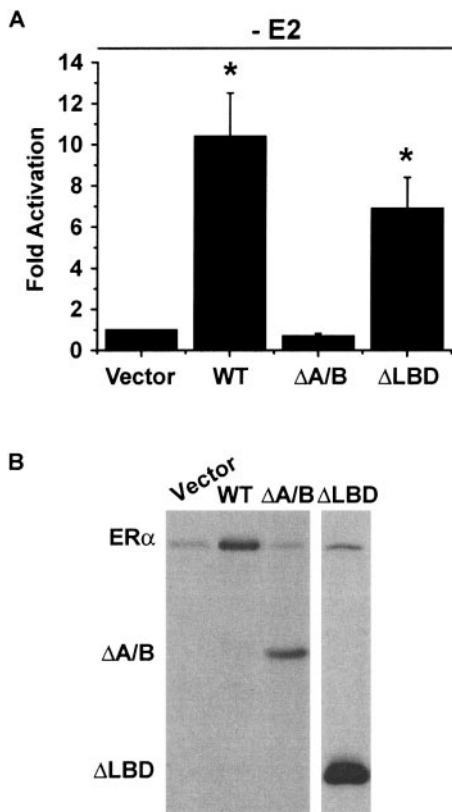


Figure 4. Ligand-independent ER α transactivation in cells with increased concentration of mutant forms of ER α . *A*) MCF-7 cells were transiently transfected with 3 μ g of expression vectors encoding wild-type (WT) or mutant ER α , as indicated. Transfection of the vector expressing only the hygromycin resistance gene was used as a negative control. Transcriptional activity was assessed by transfection of 0.5 μ g ERE-tk-Luc and 1.0 μ g CMV- β -gal. After transfection, cells were treated with EtOH and hygromycin B for 24 h to select against cells that did not become transfected. Luciferase activity was normalized to β -galactosidase activity and fold activation was calculated relative to activity measured in cells transfected with vector. Data are shown as the mean \pm SE for 5 independent experiments. Statistical differences were determined by paired Student's *t* test. **P* < 0.05, relative to vector control. *B*) Western blot analysis of the cell extracts obtained after transfection was performed using anti-ER α antibodies that recognize the carboxyl (HC-20) and amino termini (NCL-ER-6F11/2) of the receptor to visualize the mutant forms.

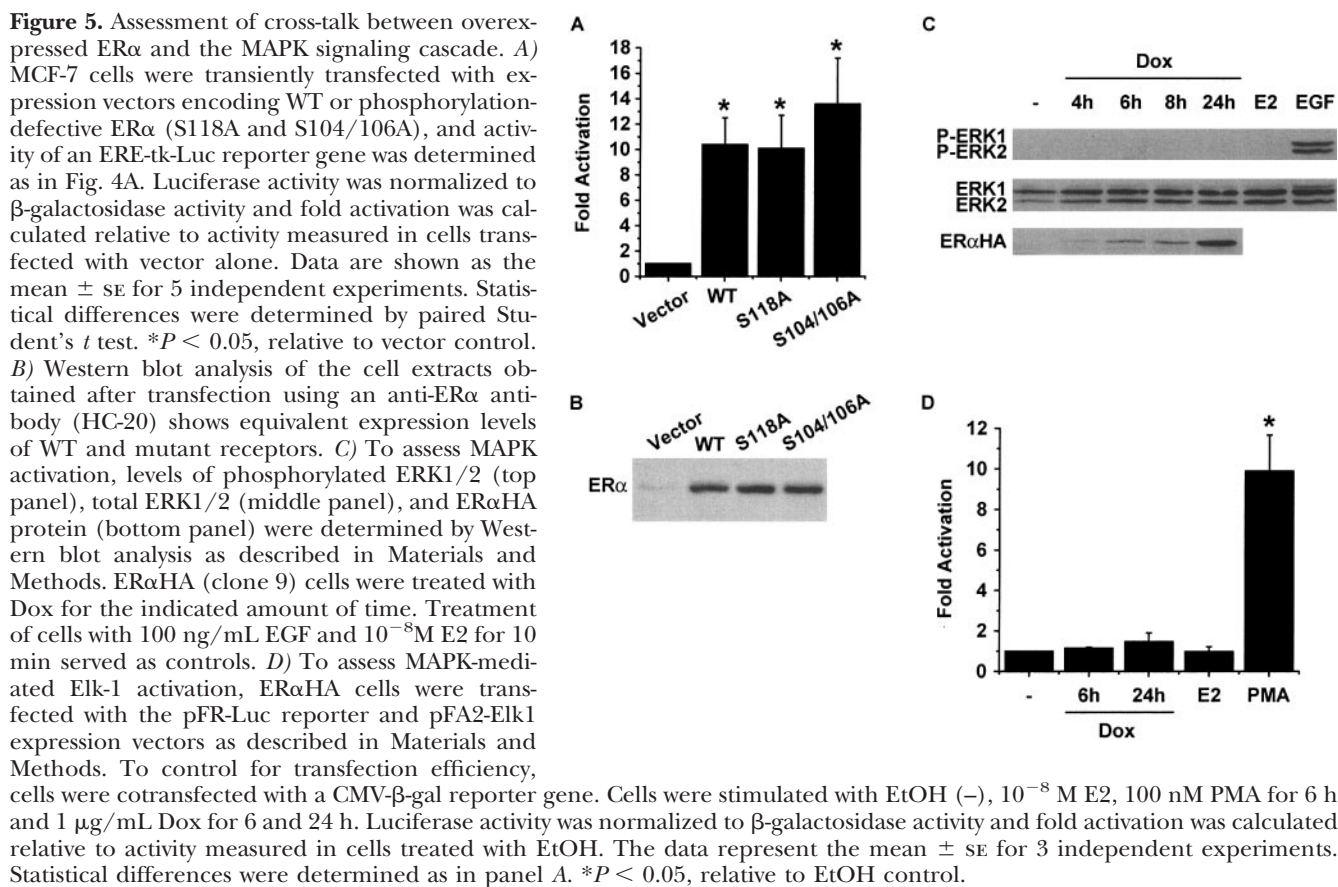
ible MCF-7 cell line in which expression of an epitope-tagged receptor lacking the A/B domain (Δ A/B ER α HA) is inducible by Dox. The effect of high levels of Δ A/B ER α HA protein on transcriptional activation of an idealized ERE-driven reporter gene was examined using the same experimental protocol as that for the wild-type ER α HA cells in Fig. 3. Identical results were obtained as in the transient overexpression experiments; a receptor that lacks the A/B domain cannot induce reporter gene transcription in the absence of ligand when overexpressed (data not shown). These findings support the conclusion that the A/B domain is essential in mediating the effects of overexpression of unliganded ER α in the cell systems used in this study.

Increased transcriptional activity of unliganded ER α does not involve cross-talk with the MAPK pathway

Numerous intracellular signaling pathways can stimulate ER α transcriptional activity through serine phosphorylation in the receptor amino terminus (reviewed in refs 27–29). For example, activation of the mitogen-activated protein kinase (MAPK) cascade increases ER α phosphorylation at serine 118, which enhances AF-1 activity (30, 31). To address the possibility that the heightened transcriptional activity of unliganded ER α could result from cross-talk with signaling cascades that depend on receptor phosphorylation, ER α mutants with serine-to-alanine replacements at residues 118 and 104/106 were transiently overexpressed in MCF-7 cells as described above. Hormone-independent reporter gene activation measured in cells overexpressing the phosphorylation-defective mutants was not significantly different from that of the wild-type receptor (Fig. 5A). Western blot analysis (Fig. 5B) confirmed that the expression levels of the mutant receptors are similar to that of wild-type. These data indicate that phosphorylation at serines 104, 106, or 118 is not critical and thus argues against possible activation of ER α by growth factors. This conclusion is further supported by experiments performed in the ER α HA cell line using serum-free culture media, in which heightened transcriptional activity was observed upon overexpression of the receptor despite the absence of exogenous growth factors (data not shown).

Various growth factor signaling cascades have also been shown to be induced downstream of ER α activation, depending on the cell context and conditions (32–34). To assess whether high levels of ER α could lead to hormone-independent activation of the MAPK pathway in the ER α HA cell line, extracellular regulated protein kinase (ERK) phosphorylation was measured in the presence and absence of ER α overexpression. While treatment with EGF induced robust ERK phosphorylation, ERK activation in the Dox-treated cells could not be detected by Western blot analysis (Fig. 5C) or by immunofluorescent staining for nuclear translocation of phospho-ERK (data not shown). E2 treatment was also ineffective at inducing ERK phosphorylation in the ER α HA cell line.

To further investigate the possibility that high levels of unliganded ER α could cross-talk with the MAPK signaling pathway, a more sensitive technique was used. Transcriptional activity of Elk-1, a transcription factor activated by MAPK via phosphorylation (35), was assessed in transient transfection assays. PMA induced significant Elk-1 activation (Fig. 5D). However, neither treatment with E2 nor overexpression of unliganded ER α resulted in Elk-1 activation. Taken together, these results suggest that ER α is uncoupled from MAPK signaling in this particular subclone of MCF-7 cells, and therefore the hormone-independent transcriptional activity of ER α acquired by overexpression is unlikely a result of cross-talk with growth factors that signal through this pathway.



Increased transcriptional activity of unliganded ER α requires nuclear localization

Unliganded ER α resides mainly in the nuclear compartment in both the presence and absence of hormone (36, 37). However, the receptor has also been detected in association with the plasma membrane (38–40) and can be sequestered in the cytoplasm under certain conditions (41). To examine the subcellular localization of ER α under overexpression conditions, immunocytochemistry was performed on ER α HA cells in the presence and absence of Dox (Fig. 6A). In uninduced cells, ER α HA was undetectable and endogenous ER α was seen predominantly in the nucleus with pronounced nucleolar exclusion (i and iii). Upon treatment with Dox, ER α HA protein could be visualized in the majority of cells and remained predominantly nuclear (ii and iv). These results illustrate that ER α HA protein, though overexpressed, is retained in the nuclear compartment and indicate that increased expression of an epitope-tagged receptor does not cause a major shift in the subcellular localization of the receptor.

To determine whether nuclear localization is required for transactivation when unliganded ER α levels are elevated, the activity of a mutant lacking two nuclear localization signals in the hinge region of the receptor (Δ NLS ER α) was examined. The loss of these signals results in a receptor that is expressed in the cytoplasm and is no longer predominantly nuclear (42,

43). The Δ NLS mutant receptor was transiently overexpressed in MCF-7 cells and transcriptional activity was compared with cells overexpressing wild-type receptor or vector alone. Overexpression of Δ NLS ER α did not support hormone-independent transactivation (Fig. 6B). Thus, nuclear localization of ER α is essential for the heightened transcriptional activity of the receptor when present at high levels.

Increased hormone-independent receptor function is antagonized by ICI, but not tamoxifen

Results so far indicate that sequences within the A/B domain are responsible for the ligand-independent activity of the receptor that arises upon overexpression. The specific role of AF-1 function within this domain was examined using two ER α antagonists. ICI 182,780 is considered a full antagonist because it blocks both AF-1 and AF-2 activity by rapidly depleting cells of ER α protein (24, 44, 45) as well as affecting receptor dimerization and localization (46, 47). In contrast, tamoxifen specifically inhibits the receptor's ligand-dependent transactivation function, AF-2, by preventing coactivator recruitment to the ligand binding domain (48, 49). ER α HA cells were grown in the presence and absence of Dox and were stimulated with EtOH, ICI, or 4-hydroxytamoxifen. Western blot (Fig. 7A) analysis demonstrates that ICI treatment induces degradation of ER α HA and endogenous ER α , resulting in a marked

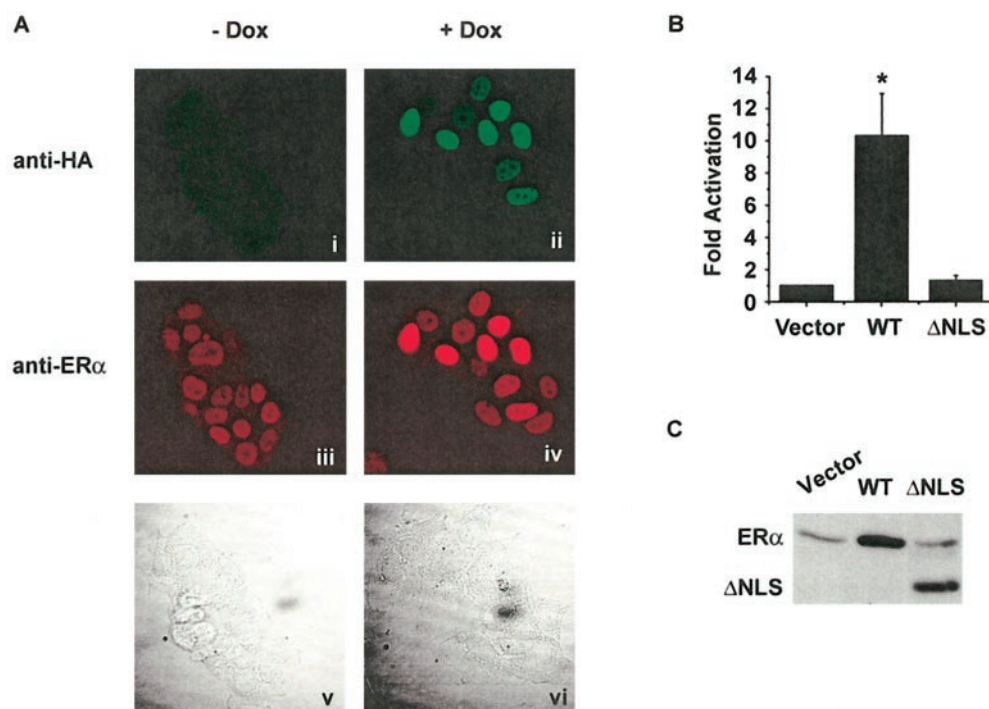


Figure 6. Functional localization of overexpressed ER α . *A*) ER α HA (clone 9) cells were treated with vehicle (i, iii, and v) or 1 μ g/mL Dox (ii, iv, and vi) for 48 h and fixed in 3.7% formaldehyde at 4°C. Immunocytochemistry for ER α HA protein was performed using an anti-HA (HA.11) antibody (panels i, ii) or anti-ER α (HC-20) antibody (panels iii, iv). Signals were visualized on a laser scanning confocal microscope. Corresponding bright-field light micrographs of the vehicle- and Dox-treated cells are presented in v and vi, respectively. *B*) MCF-7 cells were transiently transfected with 3 μ g of expression vectors encoding WT or NLS-defective ER α (Δ NLS), and activation of an ERE-tk-Luc reporter gene was assessed as in Fig. 4A. Luciferase activity was normalized to β -galactosidase activity

and fold activation was calculated relative to activity measured in cells transfected with vector alone. Data are shown as the mean \pm SE for at least 3 independent experiments. Statistical differences were determined by paired Student's *t* test. **P* < 0.05, relative to vector control. *C*) Western blot analysis of cell extracts obtained after transfection was performed using an anti-ER α antibody (HC-20) shows that wild-type and mutant ER α were expressed at equivalent levels.

reduction in total receptor protein. Tamoxifen, on the other hand, maintains the receptor at levels similar to EtOH-treated controls. Evaluation of the effects on receptor transcriptional activity in the absence of added estradiol showed that only ICI was effective at inhibiting the enhanced hormone-independent receptor function gained by overexpression (Fig. 7B). This inhibition was dose-dependent. Tamoxifen could not block the activity even at high doses (Fig. 7B). These results indicate that overexpression of ER α causes constitutive receptor activity that is resistant to treatment with tamoxifen. In addition, they strengthen the conclusion that the enhanced activity of the receptor is mediated through increased AF-1 function encoded in the A/B domain.

Heightened expression of ER α stimulates growth in the absence of hormonal stimulation but does not enhance the proliferative response to estrogen

Last, we investigated how an increase in the steady-state level of ER α protein affects cell proliferation. After maintenance in 5% steroid-depleted serum for 3 days, vehicle or Dox was added to ER α HA cells. After 2 days, cells were treated with EtOH or 10^{-8} M E2 (designated day 0 in Fig. 8). Cells were harvested after 2 and 6 days of hormone treatment, and total genomic DNA was isolated. Despite higher steady-state levels of ER α , estrogen treatment resulted in similar increases in DNA content in Dox-treated cells compared with uninduced cells (Fig. 8A). Doubling times were comparable in

control cells (2.5 days) and those overexpressing ER α (3.0 days). When the cells were treated with a lower dose of estrogen (10^{-10} M), estrogen again stimulated similar increases in DNA content (data not shown). Cell cycle analysis using flow cytometry on day 2 indicated that estrogen treatment increased the fraction of cells in S and G2/M (from 22% to 58% for -Dox; 27% to 42% for +Dox) with no greater increase in the percentage of cells entering the cell cycle when receptor levels were elevated by Dox treatment.

Although a proliferative advantage was not gained by overexpression of ER α in the presence of estrogen, growth in the absence of hormone was stimulated by an increase in ER α protein concentration. As early as 2 days after maximal induction of receptor levels, an increase in DNA content was observed in cells with high ER α levels compared with uninduced cells (Fig. 8A, B, day 0). Cell cycle analysis of serum-starved ER α HA cells treated with and without Dox for 2 days showed that a greater percentage of cells had entered S and G2/M in the group treated with Dox than those maintained in the absence of Dox (+Dox=27% compared with -Dox=21%). By day 6, proliferation of cells with elevated ER α levels maintained in the absence of hormone achieved 45% the response induced by estrogen (Fig. 8A, B at day 6). Increased ER α expression caused a decrease in hormone-independent doubling times from 4.4 days in control cells to 3.3 days in Dox-treated cells. These findings illustrate that if receptor levels are inappropriately elevated, hormone-independent growth becomes significant.

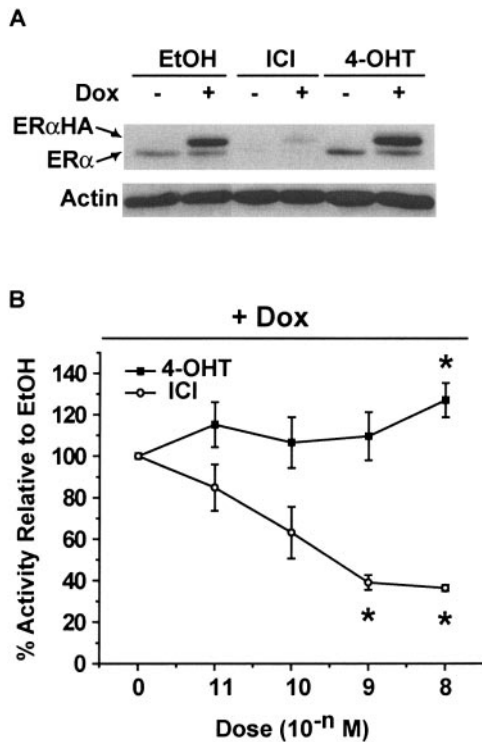


Figure 7. Effect of ER α antagonists on hormone-independent transcriptional activity resulting from increased receptor concentration. *A*) ER α HA (clone 9) cells were treated with (+) or without (-) 1 μ g/mL Dox for 48 h, followed by treatment with EtOH, 10⁻⁸ M ICI 182,780 (ICI), and 10⁻⁷ M 4-hydroxytamoxifen (4-OHT) for 3 h. Receptor levels were determined by Western blot analysis using an anti-ER α antibody (NCL-ER-6F11/2). Equivalent protein loading was verified by reprobing the blot with an anti-actin antibody (C-11). *B*) ER α HA (clone 9) cells were treated with vehicle or 1 μ g/mL Dox for 48 h. Transcriptional activity was assessed by transient transfection of an ERE-tk-Luc and CMV- β -gal, as an internal control for transfection efficiency. After transfection, cells were treated with various concentrations of 4-OHT (■) and ICI (○), as indicated, for 24 h. Luciferase activity was normalized to β -galactosidase activity and is shown as a percentage of the activity measured in the EtOH-treated cells (100% corresponds to fourfold activation). Data represent the mean \pm SE for a minimum of 3 independent experiments. Statistical differences were determined by paired Student's *t* test. **P* < 0.01, relative to EtOH-treated control.

DISCUSSION

Results presented here demonstrate that elevated concentrations of ER α in breast cancer cells can lead to acquisition of hormone-independent induction of estrogen target gene expression through an increase in AF-1 transactivation. While AF-1 activity is commonly associated with ligand-independent receptor function, these studies indicate that AF-1 activity arising from high expression levels of ER α is independent of receptor phosphorylation of serines 118, 104, and 106 and does not involve cross-talk with the MAPK cascade. Moreover, we demonstrate that elevated ER α concentration promotes stable binding of the receptor to endogenous estrogen target gene promoters without

the requirement for ligand-induced recruitment to DNA. With the tet-inducible model system, a direct causal relationship could be drawn between these heightened responses and an increase in ER α concentration, expanding on previous studies where heightened ER α levels were also associated with greater induction of endogenous estrogen target gene expression (12, 50–54). Furthermore, mutagenesis studies demonstrated that the increased receptor activity is truly ligand independent since the ligand binding domain is neither necessary nor sufficient for this response, providing additional mechanistic insight.

AF-1 activity of ER α has been shown to vary depending on the cell type and promoter context (16, 55–58). In general, this notion is based on the relative transactivation capacity of wild-type vs. receptors lacking the A/B domain in ER-negative cell types. Our studies reveal that ER⁺ MCF-7 cells are permissive of AF-1 activity under conditions of ER α overexpression. This is supported by mutagenesis analysis of the ER α domains harboring the two transactivation functions and by pharmacological studies with receptor antagonists ICI

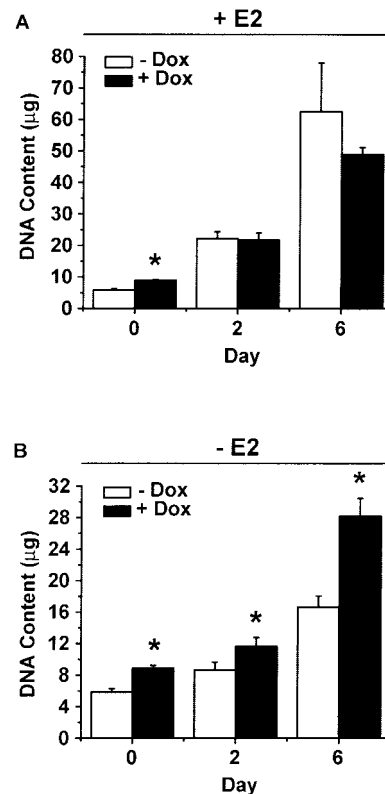


Figure 8. Consequence of elevated levels of ER α on cell proliferation. 250,000 ER α HA (clone 9) cells were plated in 10 cm cell culture plates containing 5% steroid hormone-depleted serum and pretreated with vehicle or 1 μ g/mL Dox for 48 h prior to stimulation with hormone. On day 0, 10⁻⁸ M E2 (*A*) or EtOH (*B*) was added to the culture medium. Cells were harvested 2 and 6 days after hormone treatment. Total genomic DNA was isolated and quantified using UV spectrophotometry. Data represent the mean \pm SE for a minimum of 3 independent experiments. Statistical differences were determined by paired Student's *t* test. **P* \leq 0.05, relative to -Dox control.

182,780 and tamoxifen. Previous studies examining ligand-independent AF-1 function have focused on phosphorylation-induced activation of the receptor by growth factor signaling cascades (30, 31). Elevations in receptor content, however, can promote AF-1 activity in the absence of such phosphorylation events, suggesting that a distinct mechanism is responsible for receptor activation under these conditions. How increased ER α might promote AF-1 function is not certain, but it is conceivable that, by mass action effects, overexpression could stabilize receptors in an active conformation or increase association with coactivators such as p72/p68 RNA helicases (59, 60), p160 coactivators (61, 62), and hMMS19 (63) with the A/B domain. An alternative hypothesis is that increased expression of ER α might compete away or effectively squelch a repressor that maintains the endogenous receptor in an inactive state. Either scenario could account for the stable occupancy of target gene promoters by ER α in the absence of hormone.

An eightfold increase in receptor concentration did not grossly disrupt cellular localization of ER α , which remained predominantly nuclear. Nonetheless, high levels of expression could conceivably increase cytoplasmic or membrane association. To address this possibility, the ability of a nuclear localization signal mutant (42, 43) to induce ligand-independent transactivation was examined. Our results indicate that receptor localization outside the nucleus does not support the heightened ER α function. Together with data that showed a lack of significant MAPK activation, these findings suggest that the ligand-independent transactivation results from a genomic action of ER α .

While the precise mechanism to explain how estrogen acts as a mitogen remains controversial, transcriptional control by ER α plays an important role. For instance, estrogen-activated ER α regulates the expression of several key cell cycle regulatory genes, such as *c-myc* (64), *c-fos* (65), and cyclin D1 (66). Furthermore, antagonists of ER α transactivation function such as tamoxifen and Faslodex inhibit breast cancer cell growth. We found that the consequence of ER α overexpression on growth was similar to its effects on endogenous gene activation. Therefore, heightened induction of gene expression acquired from increased unliganded ER α could be responsible in part for the observed increase in hormone-independent cell growth. However, it cannot be excluded that hormone-independent growth may represent a phenomenon that is independent of ER α transcription and might be mediated through nongenomic actions of the receptor (39, 67).

The steady-state level of ER α protein within cells is tightly controlled by a battery of mechanisms including transcriptional, post-transcriptional, and post-translational regulatory events. A well-recognized example of the potential value of restricting cellular ER α concentration is desensitization, which provides a means of preventing overstimulation by hormone. This study provides an additional explanation for why it is critical for cells to

precisely regulate ER α levels in which the control of ER α concentration is essential in restricting AF-1 receptor activity and preserving hormone-dependence. FJ

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