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GRANT NO:

DAMD17-94-J-4082

TITLE:

Dominant Negative Mutants of the Estrogen Receptor as Probes of Estrogen Action and Inhibitors of Breast Cancer Growth

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REPORT DATE:

July 26, 1995

TYPE OF REPORT:

Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 26, 1995	Annual 1 Jul 94 - 30 Jun 95

4. TITLE AND SUBTITLE
Dominant Negative Mutants of the Estrogen Receptor as Probes of Estrogen Action and Inhibitors of Breast Cancer Growth

5. FUNDING NUMBERS
DAMD17-94-J-4082

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Northwestern University
Evanston, Illinois 60208-1110

8. PERFORMING ORGANIZATION REPORT NUMBER
0650-370-E400-01

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Estrogen provides one of the most important growth stimulators for breast epithelium. There is emerging evidence for complex interactions between estrogen and other growth factors that are important for the growth of breast cancer cells. Growth factor signalling pathways stimulate estrogen responsive genes, perhaps via the estrogen receptor. These findings open new opportunities to block breast cancer cell growth using mutant receptors that function as antagonists. In year 1 of this project, we proposed to develop the key components for initiating this project. This included: 1) Creation of a series of estrogen receptor (ER) mutants that might function in a dominant negative manner; 2) Create ER and growth factor responsive reporter genes for studies in transfected cells; 3) Begin transient expression studies for examining interactions between growth factor signaling pathways and the ER pathway; 4) Develop cell proliferation assays in response to estrogen; 5) Develop inducible expression vectors to allow creation of stable cell lines that express mutant ERs; 6) Obtain and begin construction of MMTV and whey acidic protein (WAP) expression vectors for targeting mutant ERs in transgenic mice. We have completed each of these goals as outlined in the original proposal.

14. SUBJECT TERMS
Breast Cancer, Estrogen Receptor, Transgenic mice

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRAC
Unclassified	Unclassified	Unclassified	Unlimited

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J Larry Jameson 7/28/95

PI - Signature Date

Dominant Negative Mutants of the Estrogen Receptor as Probes of Estrogen Action and Inhibitors of Breast Cancer Growth

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INTRODUCTION:

Estrogen provides one of the most important growth stimulators for breast epithelium. There is emerging evidence for complex interactions between estrogen and other growth factors that are important for the growth of breast cancer cells. Estrogen stimulates production of a variety of growth factors. In turn, growth factor signalling pathways can also stimulate estrogen responsive genes, perhaps via the estrogen receptor. These findings open new opportunities to block breast cancer cell growth using mutant receptors that function as antagonists. Based upon principles derived from dominant negative inhibitors of other members of the steroid/thyroid receptor superfamily, we propose to create mutant estrogen receptors capable of inhibiting wild type estrogen receptor action. Importantly, because these dominant negative mutants will act at the level of target estrogen responsive DNA elements, they will be capable of blocking receptor activity originating from estrogen activation, from constitutive receptor function, or from activation via growth factor pathways. This novel form of inhibition, at a step distal to the site of action of tamoxifen, should provide new insights into breast cancer cell biology as well as a new approach for inhibiting breast cancer growth. In parallel with in vitro characterization of the properties of these dominant negative mutants, they will be targetted to breast tissue in transgenic mice carrying oncogenes that predispose to breast cancer to assess their biological effects in vivo.

In year 1 of this project, we proposed to develop the key components for initiating this project. This included: 1) Creation of a series of estrogen receptor (ER) mutants that might function in a dominant negative manner; 2) Create ER and growth factor responsive reporter genes for studies in transfected cells; 3) Begin transient expression studies for examining interactions between growth factor signaling pathways and the ER pathway; 4) Develop cell proliferation assays in response to estrogen; 5) Develop inducible expression vectors to allow creation of stable cell lines that express mutant ERs; 6) Obtain and begin construction of MMTV and whey acidic protein (WAP) expression vectors for targeting mutant ERs in transgenic mice. We have completed each of these goals as outlined in the original proposal and as summarized below.

BODY:

Specific Aims

1. To create mutants of the estrogen receptor that function in an inhibitory, dominant negative manner.

2. Demonstrate the ability of dominant negative estrogen receptor mutants to block transcriptional activation of various estrogen responsive genes in transient expression assays.

3. Assess the ability of dominant negative estrogen receptor mutantsto to inhibit cell proliferation in response to estrogen and growth factors in breast cancer cell lines.

4. Create a transgenic mouse in which dominant negative forms of the estrogen receptor are targetted to the breast. Transgenic mice will be crossed with strains expressing breast cancer causing oncogenes to determine whether the inhibitory form of the estrogen receptor alters the incidence or progression of breast tumors.

Progress:

Selection of sites for ER mutagenesis.

Selection of mutations to be made in the ER has been guided by three categories of pre-existing data: *(i)* There is significant structural homology between ER and thyroid hormone receptor, in which a large number (~50 distinct mutations) of naturally occurring dominant negative mutations that cause thyroid hormone resistance have been described (Refetoff *et al.*, 1993). Homologous mutations in ER should confer this inhibitory property. *(ii)* Mutagenesis studies of ER by Malcolm Parker and colleagues (Fawell *et al.*,1990; Danielian *et al.*, 1992) have delineated its functional domains (see Fig. 1). Mutations that eliminate the ligand-dependent transactivation function (TAF-2) without impairing dimerization or binding of estrogen and DNA should display a dominant negative phenotype. *(iii)* Dominant negative mutants have been identified among the products of random chemical mutagenesis of the ER by Benita Katzenellenbogen and coworkers (Ince *et al.*, 1993). By utilizing all three of these sources of information to direct our search we hope to find the most effective dominant negative mutation(s) for our purposes. The specific rationales for the creation of the various mutants is described below along with their functional characteristics.

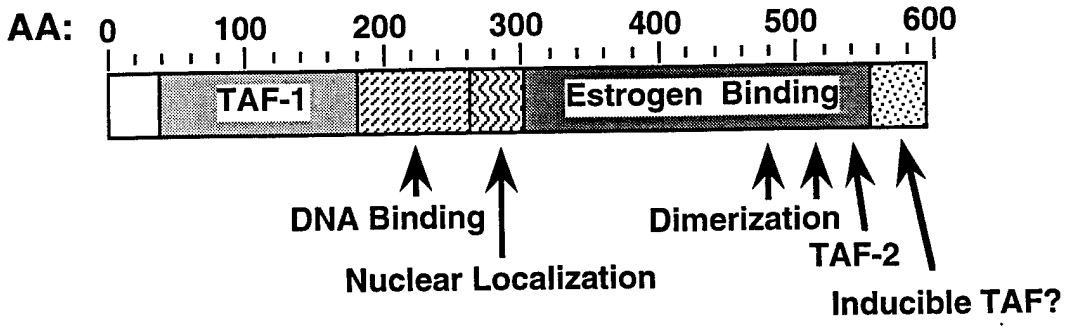


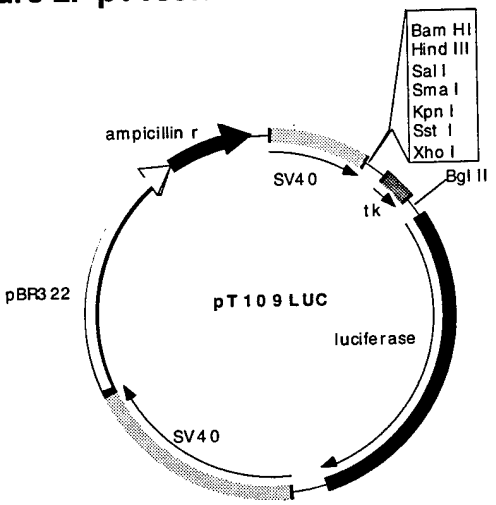
Figure 1. Functional Domains of the Estrogen Receptor

Creation and verification of ER mutations.

Mutations (except for 561fs, described below) were created by the unique-site elimination method (Deng and Nickoloff, 1992). Briefly stated, an oligonucleotide containing the desired base change but otherwise complementary to the appropriate region of the ER gene is annealed to a circular plasmid containing the gene. Simultaneously, another oligo is annealed that is complementary to a unique restriction site (*Sca* I) in a different region of same strand of the plasmid. The second oligo

contains a mismatch that destroys the restriction site. The annealed oligos and plasmid are incubated with T4 DNA polymerase and ligase to form an intact double-stranded plasmid in which one strand contains the original sequence and the other strand contains a mutation in the ER gene and in the *Sca* I site. When used to transform mismatch-repair-deficient (*mut S*) E. coli, these molecules give rise to daughter plasmids that are either wild-type or carry both mutations. Plasmid DNA prepared from the *mut S* cells is digested with *Sca* I, resulting in the linearization of wild-type plasmids. When the digested DNA is used to transform competent E. coli, the supercoiled mutant plasmids are taken up much more efficiently than the linear wild-type plasmids. Clones are selected and the presence of the mutation confirmed by DNA sequencing. To avoid the possible introduction of unintended mutations, a restriction fragment containing the mutation is then cloned into an unmutated ER expression vector (pSG5-HEGO, provided by Pierre Chambon).

Figure 2. pT109luc



Creation of estrogen responsive reporter genes

Estrogen responsive reporter vectors derived from the pT109LUC vector (Nordeen, 1988) (shown in Figure 2), were initially used to assess the effects of the dominant negative human estrogen receptor mutants on wild type estrogen receptor transactivation of estrogen responsive genes in transient transfection assays. These reporters were constructed by ligating Hind III linkers to either one or two copies of the *Xenopus* vitellogenin estrogen response element (ERE) (AGGTCAcagTGACCT) and inserting the fragments into the Hind III site of the vector. The plasmids created are referred to as ERE-tk109-luc and ERE2-tk109-luc. A disadvantage of these reporter constructs is that they have relatively high background luciferase activity.

More sensitive estrogen responsive reporter gene constructs have been prepared by inserting either one or two copies of the *Xenopus* vitellogenin ERE into the Hind III site upstream of the thymidine kinase (tk) promoter (-81 to + 54 bp or -109 to + 54 bp, abbreviated as tk81 or tk109, respectively), which has been placed upstream of the firefly luciferase coding region in the plasmid pA3LUC. The promoterless pA3LUC vector has very low to no background luciferase activity. Differences between the tk81 and tk109 promoters include promoter length and the presence of a CCAAT box in the tk109 promoter fragment. The minimal tk promoters

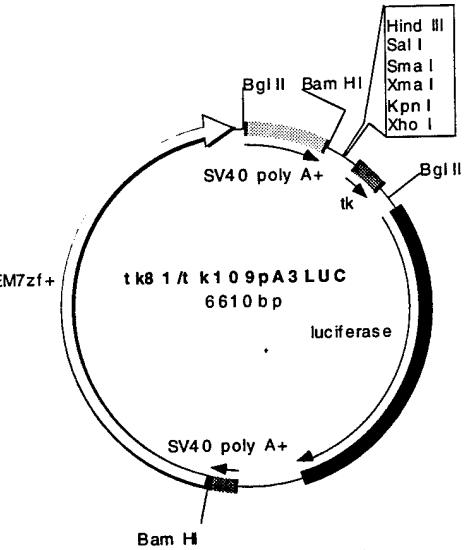


Figure 3. tk81/109pA3LUC

have no inherent estrogen responsiveness, nor does the pA3LUC plasmid. The tk81/tk109pA3LUC constructs are diagrammed in Figure 3.

The sensitivity of the estrogen responsive reporter gene constructs was assayed in human MCF-7 breast cancer cells. As Figure 4 illustrates, treatment with 1 nM estradiol resulted in a significant increase in light output by the ERE reporter gene constructs, as expected. When 1 µg of either pEREtk81pA3LUC, $pERE_2$tk81pA3LUC, or $pERE_2$tk109pA3LUC was transiently transfected into MCF-7 cells, the addition of estradiol resulted in a 10, 60, and 51 fold increase in luciferase activity, respectively. These very sensitive estrogen responsive reporter gene constructs are currently being used to evaluate dominant negative mutants of the human estrogen receptor.

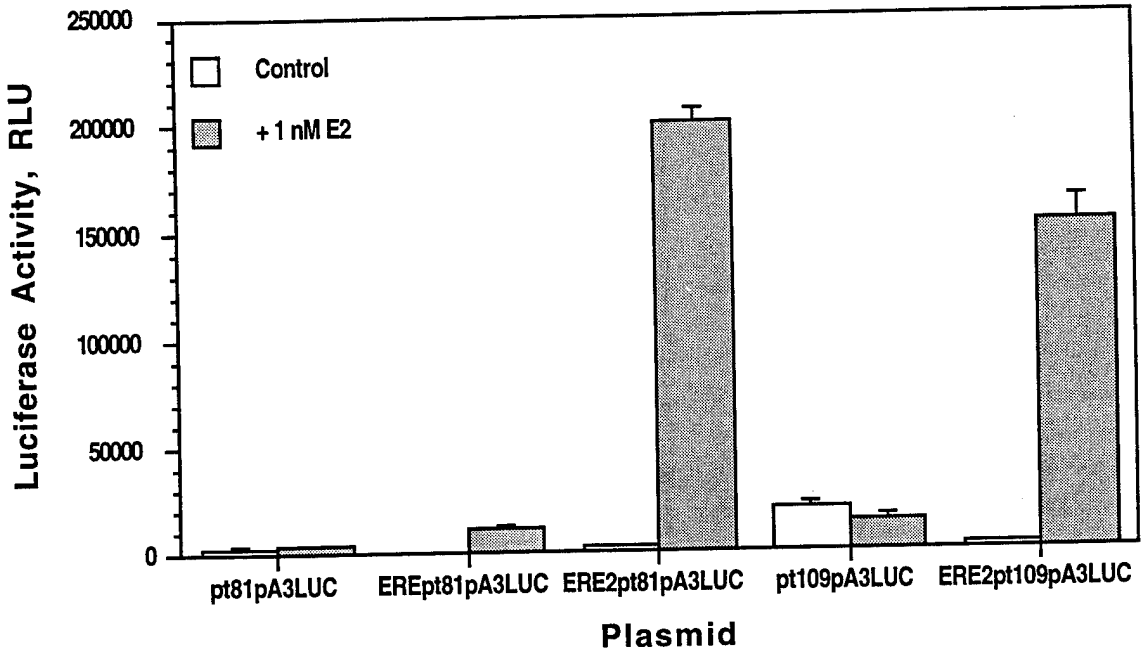


Figure 4. Comparison of Reporter Plasmids. MCF-7 cells were split into 6 well tissue culture plates and maintained in phenol red free Minimal Essential Medium (MEM) supplemented with 5% charcoal stripped calf serum for 2 days prior to transfection. Individual wells were transfected with 1 µg of reporter plasmid by the calcium phosphate-DNA coprecipitation method. Cells were incubated with calcium phosphate-DNA precipitates for 5 hours, rinsed twice with Hank's Balanced Salt Solution, and treatment media was added. Treatment media consisted of either ethanol vehicle control (0.1% ethanol v/v) or 1 nM estradiol in phenol red free MEM supplemented with 5% charcoal stripped calf serum. Cells were incubated with treatment media for 22 hours, and then lysed with 200 µl/well of a 1% Triton X-100 solution in 25 mM glyclglycine buffer, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM DTT, pH 7.8. An aliquot (150 µl) of the cell lysate from each well was pipetted into 12 x 75 mm plastic luminometer cuvettes containing 364 µl of luciferase assay buffer (25 mM glyclglycine buffer, 15 mM $MgSO_4$, 4 mM EGTA, 16 mM potassium phosphate, 1 mM DTT, and 2 mM ATP, pH 7.8). Negative controls included cell lysate aliquots of untransfected cells and cuvettes containing lysis and assay buffers alone. Light output was measured using an AutoLumat LB953 (EG&G Berthold) luminometer which injected 100 µl of 25 mM luciferin in 25 mM glyclglycine buffer, 15 mM $MgSO_4$, 4 mM EGTA and 10 mM DTT, pH 7.8 into each cuvette. The data shown are the means ± standard error of the mean of triplicate values in each treatment group, minus the average values obtained from the negative controls.

Creation of growth-factor-responsive reporters.

Several reporter plasmids have been created in our laboratory for monitoring activation of growth-factor signaling pathways. pA3Fos contains ~400 bp of the 5'-flanking region and ~30 bp of the 5'-untranslated region of the c-*fos* gene, cloned into the promoterless pA3LUC plasmid. In MCF-7 breast cancer cells, expression of this reporter is increased 20-fold or more by EGF. IGF-I and aFGF produce somewhat lower levels of expression.

Another reporter is based on the promoter of the *junB* gene, which is activated by serum and a number of growth factors. A 366-bp fragment, containing most of the major response elements in the promoter, was cloned into pA3LUC. pA3Jun is somewhat less responsive than pA3Fos to serum and growth factors when tested in JEG-3 and αT3 cells.

Reporters containing large fragments of natural promoters are likely to display complex regulatory behavior. A simpler growth-factor-responsive reporter has been produced by cloning a 28 bp fragment containing the serum response element from the human c-*fos* gene upstream of the minimal thymidine kinase promoter in pTK81-luc to generate pTK81-SRE. In preliminary experiments in MCF-7 cells, expression of this reporter is increased ~26 fold by EGF and ~15 fold by IGF-I.

Functional effects of the ER mutants.

Mutants are screened for dominant negative activity by examining their ability to inhibit expression of estrogen-dependent reporter genes (described above) in ER-negative cells cotransfected with wild-type ER. A list of available cells with known ER status is shown in Table 1. Screening of ER mutants has been done in JEG-3 and CHO cells , which are ER-negative and have high transfection efficiency using the calcium phosphate technique. Typically, cells are transfected with 1 μg of reporter plasmid, 2 ng of wtER (pSG5-HEGO) and an equal amount or up to 10-fold excess of mutant. Luciferase activity is measured as described in the legend of Fig. 4. Mutants which show dominant negative activity in this system are then tested for ability to inhibit the activity of endogenous ER in MCF-7 cells.

Table 1: Available cell lines with known ER status

Cell Line	Source	ER Status
MCF-7	Human breast	Positive
T47-D	Human breast	Positive
MDA-MB-231	Human breast	Negative
JEG-3	Human choriocarcinoma	Negative
CHO	Hamster ovary fibroblast	Negative
COS	Monkey kidney fibroblast	Negative
CV-1	Monkey kidney fibroblast	Negative
Ishikawa	Human endometrial adenocarcinoma	Positive
Ishikawa II	Human endometrial adenocarcinoma	Negative
BG-1	Human ovarian adenocarcinoma	Positive

We have also begun experimenting with liposome transfection methods, which should facilitate expressing ER mutants in some of the other cell lines shown in Table 1, many of which are resistant to transfection via calcium phosphate.

To date, five mutants have been prepared and screened: ER1-539, Y537H, G521R, R394C, and 561fs.

1-539: This mutation, in which the codon for aa 540 is replaced by a stop codon, resulting in the deletion of the 56 C-terminal amino acids, was chosen based on the observations that C-terminal deletion of thyroid hormone receptor (TR) produces a dominant negative phenotype. When cotransfected with wtER into JEG-3 cells, ER1-539 diminishes estrogen induction of the reporter plasmid (Fig. 5). It also appears to produce a small inhibition of endogenous ER signalling in MCF-7 cells (not shown).

Y537H: When the sequences of ER and TR are compared, this aromatic residue aligns with TR's phenylalanine 451, mutation of which causes a dominant negative phenotype. Additionally, this residue is a major site for tyrosine phosphorylation of the estrogen receptor (Castoria *et al.*, 1993). However, ER-Y537H displays transcriptional activation

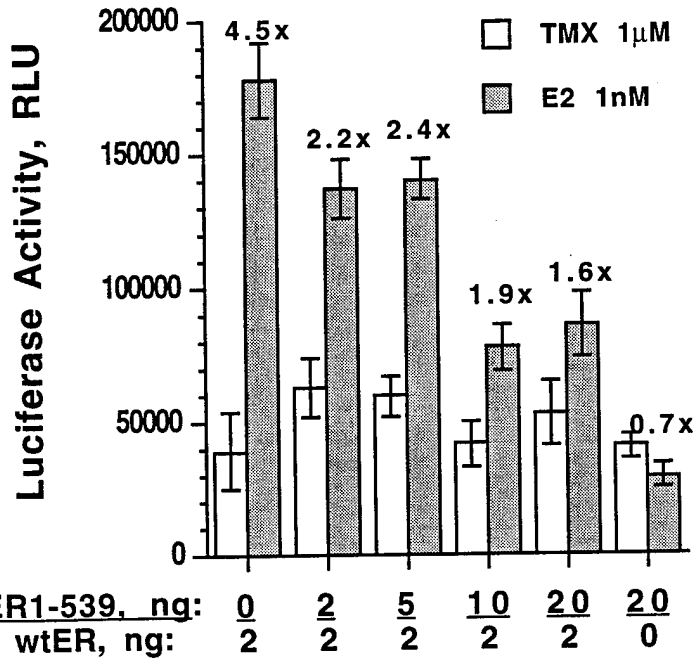


Figure 5. Dominant negative activity of ER1-539. JEG-3 cells were grown in 6-well plates in estrogen-depleted medium and transfected for 6.5 hrs with 1 μg/well of ERE-tk109-luc and the indicated quantities of wtER (pSG5-HEGO) and ER1-539. Empty pSG5 vector was added to maintain a constant total quantity of DNA in all wells. After 24 hr incubation in the presence of 1μM tamoxifen (to antagonize low levels of estradiol secreted by JEG-3 cells) or 1 nM estradiol, the cells were analyzed for luciferase activity. Results are shown as means ± s.e. of triplicate wells. Numbers at the top of the columns represent the ratio of activities in the estrogen-treated cells over the tamoxifen-treated.

and estrogen responsiveness equivalent to wtER. Apparently the Y537 residue is not essential for estrogen binding and transactivation.

G521R: This mutation was chosen based on the properties of the equivalent mutation (G525R) in mouse ER (Fawell *et al.*, 1990), which is reported to lack estrogen binding but retain DNA binding and the ability to dimerize. As expected, ER-G521R was unable

to mediate transcriptional activation of an estrogen-sensitive reporter gene, however, this mutant showed little or no ability to inhibit the activity of cotransfected wtER.

R394C: This residue aligns with R320 in thyroid hormone, which causes a dominant negative phenotype when mutated to a cysteine. This ER mutant lacks transcriptional activity and partially inhibits the activity of cotransfected wtER but appears to be less potent than ER1-539.

561fs: This mutation was created by cleaving pSG5-HEGO at the *BsmB* I site in the ER gene, filling in the resulting 5' overhangs, and religating, producing a frameshift mutation at amino acid residue 561. Based on the report by Ince *et al.* (1993) that a frame-shift ER mutant at residue 554, created by random chemical mutagenesis, had powerful dominant negative activity, we expected that this mutation would have a similar effect. However, although it lacks transcriptional activity of its own, ER-561fs has little or no dominant negative effect on the activity of cotransfected wtER.

Based on insights gathered from these mutants, a second group of mutants is currently being prepared, in hopes of obtaining a more powerful dominant negative than ER1-539. These include P535A, 537Stop (1-536), L540Q, M543R and L544Q.

Growth factor interactions with the ER pathway.

Based on the report by Ignar-Trowbridge *et al.* (1993) that EGF induced expression of an ERE-containing reporter plasmid via ER in ovarian and endometrial adenocarcinoma cells, we expected to obtain similar results in breast cancer cells. To date, however, we have not observed growth factor signaling through the ER. In some experiments the reporter ERE-tk109-luc shows several-fold induction by EGF; however, this induction is not mediated via the ERE since the parental plasmid shows equal or larger induction (Fig. 6). Moreover, activation of ERE-tk109-luc by EGF is not inhibited by anti-estrogens (Fig. 7), and occurs in JEG-3 cells, which lack ER. ERE-containing reporter plasmids based on the EGF-unresponsive tk81 promoter do not respond to EGF (data not shown). We are testing other reporter plasmids and expanding our experiments to include the cell lines used by Ignar-Trowbridge *et al.* in order to resolve the discrepancy between our findings and theirs.

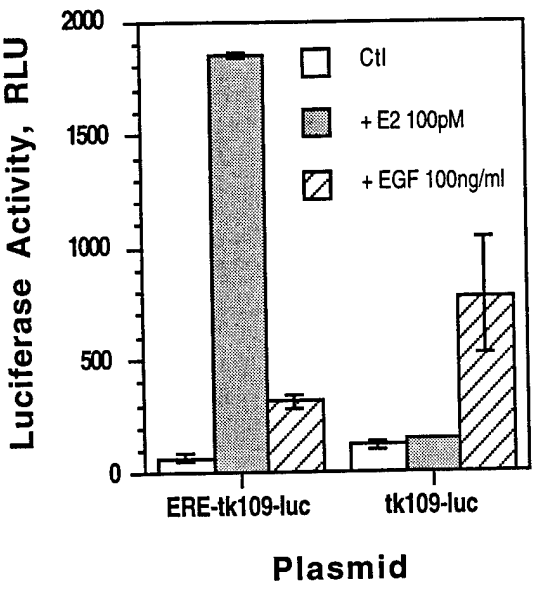


Figure 6. Response of Reporter Plasmids to EGF and Estradiol.
MCF-7 cells were grown in 6-well plates in estrogen-free medium and transfected with 1μg/well of the indicated plasmid. 18hr after transfection, cells were treated for 6 hr with the indicated agonists and assayed for luciferase activity. Results are shown as mean and range of duplicate wells.

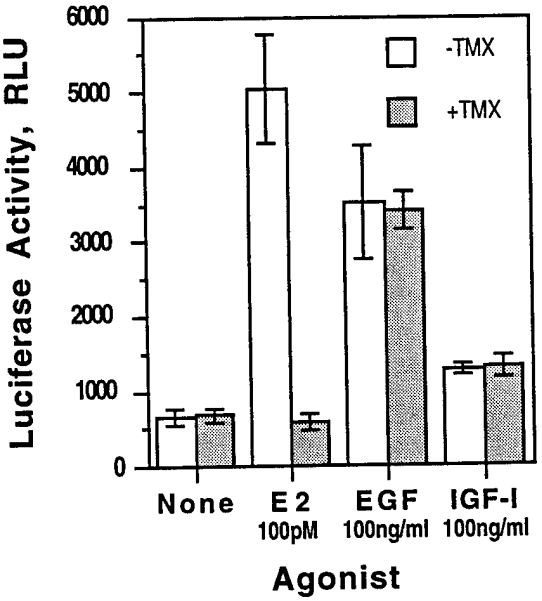


Figure 7. Effect of Tamoxifen on Response of ERE Reporter to EGF.
MCF-7 cells were grown in 6-well plates in estrogen-free medium and transfected with 1μg/well of ERE-tk109-luc. 18hr after transfection, cells were treated for 6 hr with the indicated agonists in the presence or absence of 1 μM tamoxifen, then assayed for luciferase activity. Results are shown as mean ± s.e. of triplicate wells.

Development of breast cancer cell growth assays.

In addition to their ability to inhibit wild type activation of estrogen responsive genes, the dominant negative mutants will also be assessed by their effects on estrogen-dependent breast cancer cell growth. It is expected that dominant negative mutants of the human estrogen receptor will inhibit estrogen-stimulated cell proliferation. Two types of cell proliferation assays have been validated for this purpose. These include the cellular conversion of a tetrazolium salt into a formazan product that is detected using an ELISA plate reader (MTT Assay) and [^{3}H]thymidine incorporation into DNA. The MTT Assay is conducted in 96-well culture plates. Cells are maintained in phenol red free MEM supplemented with 5% charcoal stripped calf serum for 3 days and then trypsinized, counted using a hemocytometer, and plated at a density of 5000 cells/well into 96 well plates. Treatment media is added on the following day, and at 48 hour intervals until the end of the experiment. At 4, 6, and 8 days of culture, the tetrazolium salt, ([3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide]) (MTT), which is a yellow dye dissolved in phosphate buffered saline, is added to each well (15 μl/well) and the tissue culture plate is incubated for 4 hours at 37 C. A solubilization /stop solution containing SDS, acetic acid, and *N,N*- dimethylformamide is then added to all of the wells (100 μl/well) and the plate is incubated for 1 hour at room temperature. The wells are mixed to dissolve the blue formazan product, and absorbance at 570 nm is

measured using an ELISA plate reader. In a preliminary experiment, treatment of MCF-7 cells with 1 nm estradiol for 6 days resulted in a significant ($P < 0.0001$) increase in absorbance as compared to control wells (data not shown).

The [^{3}H]thymidine uptake assay is performed in the following manner. Cells are maintained in estrogen-free conditions as described for the MTT Assay for 3 days and then trypsinized, counted using a hemocytometer, and plated at a density of 15,000 cells/well into 12-well tissue culture plates. Treatment media is added on the following day, and at 48 hour intervals until the end of the experiment. At 4, 6, and 8 days of culture, 2 µCi of ^{3}[H]thymidine diluted in culture media is added to each well. The tissue culture plate is then incubated for 4 hours at 37 C. All media is removed, and cells are washed once with PBS. Cells are solubilized with a 0.1% SDS solution, and the DNA is precipitated by the addition of a 1% BSA solution and a 10% trichloroacetic acid solution. The resulting pellet is isolated and solubilized in 1 N NaOH, and an aliquot is diluted into 5 ml scintillation fluid. Samples are counted for 1 minute using a scintillation counter and disintegrations per minute are recorded.

Creation of inducible estrogen receptor expression vectors.

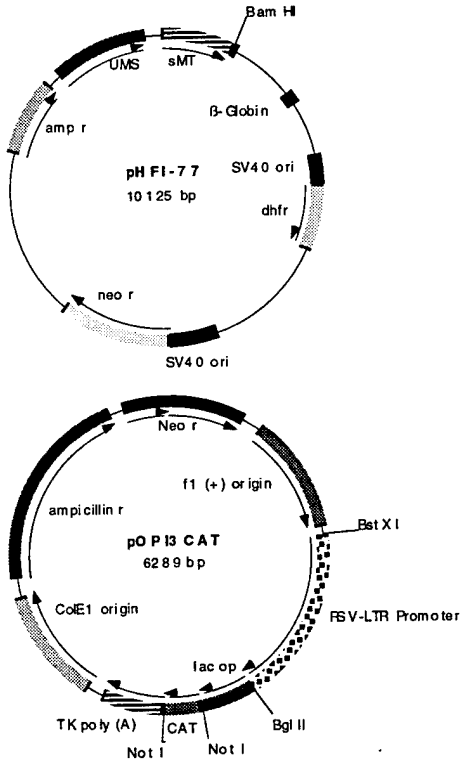


Figure 8. Inducible vectors.

In addition to their ability to inhibit wild type activation of estrogen-responsive genes, the dominant negative mutants will also be assessed by their effects on estrogen-dependent breast cancer cell growth. For these experiments, the dominant negative human estrogen receptor cDNAs will be cloned into inducible promoter expression vectors, and cell proliferation assays will be conducted as described above. Two of these vectors are shown in Figure 8. The pHFI-77 vector contains the ovine metallothionein promoter which is induced by heavy metals such as zinc and cadmium. Dominant negative human estrogen receptor cDNAs will be cloned into the *BamH* I site, which will put their expression under the control of the inducible metallothionein promoter. The other vector, pOP13CAT, contains an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible promoter from the *lac* operon. The CAT reporter gene sequences will be removed by *Not* I digestion, and the dominant negative human estrogen receptor cDNAs will be cloned into the *Not* I site, which will put expression of the dominant negatives under the control of the *lac* operon (indicated as lac op on the vector map), which is activated by IPTG treatment. *Not* I polylinkers will be utilized if necessary. The human estrogen receptor positive breast cancer cell lines MCF-7 and T47D will be stably transfected with dominant negative estrogen receptors. Transient transfection assays could be used, however, variable transfection efficiencies from well to well and

between experiments could make interpretation difficult. Therefore, stable cell lines containing the dominant negative estrogen receptor constructs under the control of inducible promoters will be made. The inducible expression vectors will be used to prevent the dominant negative mutant receptors from initially interfering with normal cell growth. Both the pHFI-77 and pOP13CAT vectors contain the neomycin resistance gene, which will be used to select stable clones. Stably transfected cells will be selected by treatment with the neomycin analog, G418 sulfate. Several clones of each dominant negative mutant receptor will be examined for dominant negative effects on breast cancer cell growth. Effects on cell growth in response to treatment with estrogen will be examined 24-96 hours after induction of dominant negative ERs. Cell growth will be measured by the cellular conversion of a tetrazolium salt into a formazan product (MTT Assay) and [^{3}H]thymidine incorporation into DNA as described above.

Design of mammary specific expression vectors for targeting ER mutants in transgenic mice.

The mouse whey acidic protein gene will be used to target and drive the expression of the dominant negative human estrogen receptors in the mammary glands of transgenic mice. The WAP gene is very tissue specific and is only expressed during lactation. Therefore, normal mammary gland development can take place before the activation of the dominant negative mutants. If necessary, the dominant negative mutants could be expressed under the control of the MTTV promoter, however, the MMTV promoter is not as tissue specific as the WAP gene; there is also expression in several secretory glands of the head including lacrimal and salivary. The human dominant negative mutant estrogen receptor cDNAs will be cloned into the mouse WAP expression vector pBSWAP (Burdon *et al.,* 1991), shown in Figure 9. This vector contains the entire mouse WAP gene. The dominant negative cDNAs will be cloned into the unique *Kpn I/Asp*718 site located in the first exon. The WAP gene containing the dominant negative insert will then be excised from the vector by restriction endonuclease digestion with *Nsi* I and *BamH* I and isolated by agarose gel electrophoresis. The band containing the *Nsi* I-*BamH* I fragment will be excised and purified using the Geneclean II kit (Bio 101, Inc., La Jolla, CA), and the DNA purified using a Qiagen tip #5 (Qiagen, Inc., Chatsworth, CA). The DNA will then be phenol/chloroform extracted, ethanol precipitated, resuspended in 100 µl of 1 x PBS injection buffer, passed though a Millipore syringe filter for particle removal, and subsequently microinjected into fertilized mouse eggs. Generation of the founder mice will be accomplished by the Northwestern University transgenic animal core facility, under the direction of Dr. Philip Iannaccone. Southern blots or PCR of tail DNA will be used to identify founder mice that contain the transgene of interest. Mice will then be bred to confirm germ line transmission of the transgene. Expression of the human ER in mammary tissue will be confirmed by RT-PCR using mammary tissue mRNA from normal and lactating animals

Rodent models of breast cancer include administration of carcinogenic agents such as dimethylbenz[a]anthracene (DMBA) and *N*-methyl-*N*-nitrosourea (NMU), which cause mammary tumors, and several transgenic mouse strains in which protooncogenes are driven by the mouse mammary tumor virus (MMTV) or the mouse whey acidic protein (WAP) are overexpressed in the mammary gland and result in the development of mammary tumors. The protooncogenes that have been used in the past to cause mammary tumor formation include v-Ha-ras, c-myc, and activated c-erbB_2 (c-neu). Animals transgenic for both MMTV-v-Ha-ras and MMTV-c-myc have a tumor incidence greater than the sum of the two individual rates. The mutant ER transgenic mice will be bred with mice transgenic for both MMTV-v-Ha-ras and MMTV-c-myc. Female mice will be kept lactating for as long as possible by a steady supply of newborn pups. The onset of tumors, incidence, total number of tumors, size of tumors, and tumor morphology will be analyzed. It is expected that the expression of the dominant negative mutant estrogen receptor will inhibit the development of mammary tumors.

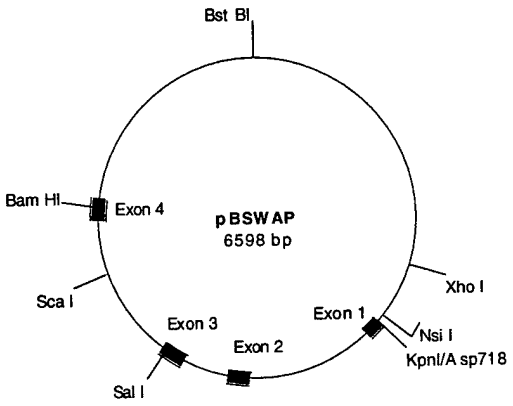


Figure 9. pBSWAP vector

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CONCLUSIONS:

As outlined in the original grant, we have developed most of the tools that are necessary for carrying out the proposed in vitro and in vivo experiments to examine the functional effects of dominant negative mutants of the ER.

1. We have created several dominant negative mutants of the ER and have demonstrated that they block wild-type ER function in co-transfection assays. It is desirable to increase the potency of the current group of dominant negative mutants and we are currently creating a second generation of mutants.

2. We find little evidence for cross-talk between growth factor pathways and the ER. Because this result is in contrast to the reports of some laboratories, we are continuing to explore the possibility that such effects may be cell-specific or limited to a certain group of reporter genes. However, we are also considering strategies for creating dominant negative mutants of growth factor receptors, such as the EGF-R and the IGF-R to provide alternative means to block the effects of these growth factors.

3. With a first generation of dominant negative ER mutants in hand, we are now ready to initiate studies to examine their effects on cell proliferation in stably transfected cell lines (using inducible promoters). We have also completed the first transgenic construct and will begin these experiments within the next several months.

PUBLICATIONS:

No publications have been generated to date.