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GRANT NUMBER 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 1996

TYPE OF REPORT: Annual

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

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 1996	Annual 1 Jul 95 - 30 Jun 96

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
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8. PERFORMING ORGANIZATION REPORT NUMBER

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

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

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 created a series of estrogen receptor (ER) mutants that were shown to function in a dominant negative manner. In year 2, we created more potent dominant negative ER mutants and developed a more selective estrogen-responsive reporter gene. We found that estrogen and growth factor signaling pathways are separable when studied with sensitive luciferase reporter gene assays. The effects of these dominant negative mutants are being evaluated in estrogen-dependent proliferation assays were in T47D breast cancer cells. Tetracycline-inducible ER expression vectors were created to allow induction of ER mutants in stably transfected cells. Preliminary results show potent inhibition of cell growth by mutant ERs. For transgenic studies, MMTV promoter-ER expression vectors have been created. Thus, we have completed each of the goals as outlined in the original proposal.

14. SUBJECT TERMS Breast Cancer

15. NUMBER OF PAGES
26

16. PRICE CODE

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

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J Larry Jameson 1/26/96

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

Estrogens and peptide growth factors are important regulators of breast cell proliferation. There is emerging evidence for complex interactions between these two types of mitogens. 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 estrogen-responsive DNA elements, they are expected to block 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 and other estrogen antagonists, 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 targeted to breast tissue in transgenic mice to assess their biological effects *in vivo*.

As described in our original application, the four major aims of this project are:

1. To create mutants of the estrogen receptor that function in an inhibitory, dominant negative manner.
2. To demonstrate the ability of dominant negative estrogen receptor mutants to block transcriptional activation of various estrogen responsive genes (by estrogen and growth factors) in transient expression assays.
3. To assess the ability of dominant negative estrogen receptor mutants to to inhibit cell proliferation in response to estrogen and growth factors in breast cancer cell lines.
4. To create a transgenic mouse in which dominant negative forms of the estrogen receptor are targeted 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.

Accomplishments during the previous (first) year of this project included: *a*) Creation of a series of estrogen receptor (ER) mutants with the potential for dominant negative activity. Although several dominant negative mutants were produced, we have continued to search for more potent ones during the second year. *b*) Creation of growth factor- and estrogen-responsive reporter genes for studies in transfected cells. *c*) Use of these reporter genes in transient expression experiments to examine interactions between growth factor signaling pathways and ER. *d*) Development of cell proliferation assays in cultured breast cancer cell lines. *e*) Development of inducible expression vectors to allow creation of stable cell lines that express mutant ERs. *f*) Initiation of construction of vectors using promoters

from mouse mammary tumor virus (MMTV) and whey acidic protein (WAP) for targeting expression of mutant ERs to mammary tissue in transgenic mice.

As detailed below, the accomplishments of the present (second) year of this project include: *a*) Creation of a more potent dominant negative ER mutant. *b*) Development of a more selective estrogen-responsive reporter that minimizes the confounding ER-independent effects of epidermal growth factor (EGF) on luciferase expression. *c*) Demonstration that estrogen and growth factor signaling pathways are separable when studied with sensitive luciferase reporter gene assays. *d*) Measurement of estrogen-dependent proliferation in T47D cells. *e*) Development of a Tet-Regulated inducible expression system in T47D cells. *f*) Creation of tetracycline-inducible ER expression vectors. *g*) Creation and screening of T47D stable cell lines carrying these vectors. *h*) Construction of vectors using the MMTV promoter for targeting expression of mutant ERs to mammary tissue in transgenic mice.

BODY

Experimental Methods

Cell culture.

MCF-7 (human breast adenocarcinoma, ER-positive) cells were obtained from Dr. Dean Edwards at the San Antonio Breast Cancer Group, Texas; the cells used in these experiments were subclone WS8, produced in the laboratory of V. C. Jordan, and grown in MEM supplemented with non-essential amino acids (NEAA), 10 mM Hepes and 5% calf serum (CS).

MDA-MB-231 (human breast adenocarcinoma, ER-negative) cells were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Subclone 10A, produced in the laboratory of V. C. Jordan, were used in these experiments. The cells were grown in MEM supplemented with NEAA, 10 mM Hepes, and 5% CS.

T47D (human breast cancer adenocarcinoma, ER-positive) cells were obtained from the ATCC. These cells were originally described by Keydar *et al.* (1979). The cells used in these experiments were subclone A18, produced in the laboratory of V.C. Jordan, and grown in RPMI 1640 supplemented with NEAA and 10% fetal bovine serum (FBS).

Ishikawa (human endometrial adenocarcinoma, ER-positive) cells were obtained from Dr. Erlio Gurpide of the Mt. Sinai School of Medicine, NY, and grown initially in MEM supplemented with NEAA, 10 mM Hepes and 10% FBS, later in DMEM/F-12 + 10% FBS for consistency with Ishikawa II cells.

Ishikawa II (an ER-negative strain of Ishikawa) and BG-1 (human ovarian adenocarcinoma, ER-negative) cells were obtained from Dr. Jeff Boyd of the University of Pennsylvania Medical Center and grown in DMEM/F-12 + 10% FBS.

Sera and media were purchased from Gibco/BRL. All FBS was heat-inactivated. Stripped sera were prepared by three-fold extraction with dextran-coated charcoal (DCC).

Creation of reporter plasmids.

Plasmids ERE-tk109-luc and ERE_2-tk109-luc (Catherino & Jordan, 1995) were constructed by William Catherino of the University of Wisconsin Comprehensive Cancer Center, by insertion of 1 or 2 copies of the *Xenopus* vitellogenin A_2 estrogen response element (ERE) into the *Hind* III site of the pt109 luciferase plasmid.

Plasmids tk109-A_3-luc and tk81-A_3-luc were constructed by Takashi Nagaya of J. L. Jameson's laboratory by inserting -109 to +52 or -81 to +52 fragments of the 5' sequence of thymidine kinase into pA3luc (Wood *et al.*, 1989). These plasmids contain three copies of the SV40 polyadenylation (poly A) sequence upstream of the promoter to inhibit nonspecific initiation. ERE_2-tk109-A_3-luc and ERE_2-tk81-A_3-luc were produced by cloning the doublet ERE from ERE_2-tk109-luc into these plasmids' *Hind* III sites.

ERE-SV40-luc was produced by cloning the vitellogenin ERE into the *Xho* I site of pGL3-promoter (Promega) 5' to the SV40 late promoter (for clarity, the latter plasmid is referred to as SV40-luc in the accompanying figures).

FOS-luc was constructed by Srividya Sundaresan of J. L. Jameson's laboratory by inserting -361 to +157 of the 5' sequence of the human *fos* gene into pA3luc (Sundaresan *et al.*, 1996). This region does not contain the ERE found in the full *fos* promoter.

Milligram-scale preps of all plasmids were prepared using Promega Wizard Maxi-prep kits. All restriction enzymes were purchased from Promega except as noted.

Site-directed mutagenesis of the ER.

The wild-type human ER expression vector pSG5-HEGO was the kind gift of Dr. Pierre Chambon of the Université Louis Pasteur, Strasbourg, France. Mutants ER_{1-536} and M543R were created using a variation of the polymerase chain reaction (PCR) overlap extension method (Ho *et al.*, 1989), in which nucleotide substitutions are introduced by synthetic oligonucleotide primers. pSG5-HEGO was used as a template. In mutant ER_{1-536}, codon 537 of the ER gene was changed from from TAT (tyrosine) to TAG (stop), while in ER-M543R, codon 543 was changed from ATG (methionine) to AGG (arginine). The use of a DNA polymerase with 3' to 5' proofreading exonuclease activity (*UlTma*™, Perkin-Elmer) instead of *Taq* polymerase was found helpful to avoid untemplated extension of the 3' ends, which can result in undesired mutations and/or poor amplification.

Mutant ER-L540Q was prepared by the PCR "megaprimer" method (Landt *et al.*, 1990; Kuipers *et al.*, 1991) using pSG5-HEGO as a template and a synthetic oligonucleotide primer to convert codon 540 from CTG (leucine) to CAG (glutamine).

pSG5-HEGO and the mutagenesis PCR products contain two *Bgl* II sites flanking the mutagenized region. The PCR products were digested with *Bgl* II and cloned into *Bgl* II-digested pSG5-HEGO to create mutant expression vectors. Orientation of the insert was checked by restriction digests. The mutated inserts and flanking regions in the resulting plasmids were sequenced in both directions using

an ABI 373 automated sequencer to verify the presence of the desired mutations and absence of any others.

pSG5 control vector was purchased from Stratagene.

Transient transfections.

Cells were seeded in 6-well plates ~24-48 hr before transfection to give a density of 30-40% confluence on the day of transfection. In experiments involving estrogen response, plating was done in estrogen-depleted medium (phenol-red-free medium with DCC-stripped serum).

In early experiments, cells were transfected using the calcium phosphate method (Sambrook *et al.*, 1989), but transfection efficiency was poor (as judged from light output with luciferase reporters) except in MCF-7 cells. In later experiments, cells were transfected using liposomes consisting of dioleyl phosphatidylethanolamine (DOPE) and dimethyldioctadecylammonium bromide (DDAB) (Rose *et al.*, 1991) in the ratio 300:500 (µg/ml of each component in stock solution) except as noted. Liposomes were prepared either by sonication or by injection of an ethanolic solution of the lipids into water (Campbell, 1995). For MDA-MB-231 cells, a DOPE:DDAB ratio of 500:200 gave superior transfections.

Luciferase assays.

Assays were performed ~24 hr after transfection except as noted. Cells were lysed by the addition of 500µl/well of 1% Triton X-100 in Buffer GG [25mM potassium glycylglycine, pH 7.8, 15mM $MgSO_4$, 4mM EGTA and 1mM dithiothreitol (DTT)]. 150µl aliquots of the lysate were mixed with 400µl of assay buffer (21mM potassium glycylglycine, 16mM potassium phosphate, 12mM $MgSO_4$, 3.3 mM EGTA, 2mM DTT, 2.1 mM ATP, pH 7.8) and assayed in an AutoLumat LB953 luminometer (EG&G Berthold). Reactions were initiated by the automated injection of 100µl of 200µM luciferin in Buffer GG.

Assay of estrogen-dependent T47D cell proliferation.

Cell proliferation was measured via the MTS assay (Cory *et al.*, 1991). T47D cells were maintained in phenol red free RPMI + 10% DCC-stripped FBS for 4 days and then trypsinized, counted using a hemocytometer, and plated at a density of 5000 cells/well into 96 well plates. Treatment media (200µl/well) were added on the following day, and replaced at 48 hr intervals until the end of the experiment. Treatment media consisted of culture medium plus 0.1% ethanol (vehicle control) or either 0.1 nM or 1 nM estradiol. Sixteen wells were treated with vehicle control media, and the two doses of estradiol were administered to 32 wells each. Control wells included wells that contained tissue culture media, but no cells. At 2, 4, 6, and 8 days of culture, a 20µl aliquot of a solution of tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS; Promega], 1.9 mg/ml, and an electron coupling reagent (phenazine methosulfate; PMS; Sigma), 0.044 mg/ml, in Dulbecco's PBS, was added to each well and the tissue culture plate was incubated for 4 hr at 37° C. These reagents are bioreduced by living cells into a formazan that is soluble in tissue culture medium. The absorbance of the formazan at 490 nm was measured directly from the 96 well

assay plate using an ELISA plate reader (Spectromax 250, Molecular Dynamics) without additional processing.

Evaluation of the Tet-Regulated Expression System in T47D cells.

The Tet-Regulated Expression System designed for the inducible expression of foreign proteins in mammalian cells (Shockett *et al.* 1995) was purchased from Gibco BRL (Catalog No. 10583-011, Grand Island, NY). T47D cells were grown to near confluency in a 150 mm tissue culture plate, trypsinized, centrifuged, resuspended in 1.2 ml complete medium, and placed in a 0.4 cm electrode gap electroporation cuvette (BioRad, Hercules, CA) containing 9 µg pTet-tTAk and 9 µg pUHC13-3 plasmid. Cells were pulsed once in a BioRad Gene Pulser at a setting of 300 V, 960 µF capacitance, and infinite resistance. After the pulse, cells were resuspended and plated into 6-well tissue culture plates and divided into 3 different treatment groups of 3 wells each: 72 hr tetracycline treatment, 48 hr tetracycline treatment followed by 24 hr without tetracycline, or complete absence of tetracycline for 72 hr. The tetracycline dose was 0.2 µg/ml. Cells were assayed for luciferase activity after 72 hr of treatment.

Creation of tetracycline inducible estrogen receptor expression vectors.

The wt hER cDNA, and the cDNAs of the dominant negative mutants ER_1-539 and ER_1-536 were subcloned into the pTetSplice plasmid using the following methods. 10 µg of pSG5-HEGO, pSG5-ER_1-536 and pSG5-ER_1-539 were digested separately with *Eco*R I to release the ER cDNAs from the pSG5 vector. The digestion products were run on a 1% agarose gel in 1x TAE buffer and the 1.8 kb bands containing the ER cDNAs were carefully excised from the gel, purified with a Geneclean II kit (Bio101, Inc., La Jolla, CA), and ligated into the *Eco*RI site of the pGEM9z plasmid (Promega). Resulting clones were screened by restriction digest analysis to determine orientation of ER cDNAs. Plasmid DNA from clones containing ER cDNAs in sense orientation was digested with *Spe* I and *Sal* I. Bands containing the ER cDNAs with flanking polylinker regions were purified by agarose gel electophoresis and recovered as described above, then ligated into the pTetSplice vector, which had been digested with *Spe* I and *Sal* I. Resulting clones were screened by restriction digest analysis to confirm orientation of ER cDNAs.

Stable transfection and screening of T47D cell lines.

T47D cells were electroporated with 4 µg pTet-tTAk, 1 µg pSV2neo, and 4 µg of either empty pTetSplice or the mutant or wtER pTetSplice constructs described above. Electroporation was performed as described above. Resuspended cells were plated into 150 mm tissue culture plates containing 0.2 µg/ml tetracycline, in order to suppress the activation of the pTetSplice constructs. Two days after electroporation, cells were split and diluted into 10 cm tissue culture plates containing 400 µg/ml G418 sulfate (Gibco BRL) and 0.2 µg/ml tetracycline. Media were changed 2-3 times per week and plates were observed daily for colony formation. Single colonies were isolated from the plates using 8 x 8 mm plastic cloning cylinders (Sigma, St. Louis, MO, Catalog No. C-1059), and placed into single wells of 48-well plates. Cells

were grown and subsequently transferred into single wells of 12-well plates, two wells of 6-well plates, 10 cm plates, and 150 mm plates.

In order to determine which stable clones contained pTet-tTAk, clones were transiently transfected by electroporation with 1 μg/well of the tetracycline-regulated luciferase reporter vector, pUHC13-3, and plated in 6-well plates. Three wells of each plate were treated for 72 hr with 0.2 μg/ml tetracycline, while the remaining three wells received media without tetracycline. Cells were assayed for luciferase activity after 72 hr in culture.

Stable clones which were found to contain pTet-tTAk were screened for the pTetSplice constructs using PCR. Genomic DNA was extracted from whole cells using the Puregene Kit (Gentra Systems, Minneapolis, MN). PCR was performed in a 50 μl reaction volume containing 100 ng of purified genomic DNA, 1.5 mM of each deoxynucleotide triphosphate, 67 mM Tris-HCl pH 8.8, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 10% dimethyl sulfoxide, 50 pmol of each PCR primer, and 5 U of Taq DNA polymerase. The reaction was carried out using an MJ Research PTC-200 thermalcycler with a temperature cycle of 3 min at 94° C, 1 min at 94° C, 1 min at 60° C, and 1.5 min at 72° C. After 40 cycles, the amplified materials were subjected to a 15 min extension at 72° C. Positive controls included plasmid DNA, and genomic DNA from untransfected T47D cells served as a negative control. Aliquots of the reactions were electrophoresed on a 1% agarose gel to visualize the products. The primers used to detect empty pTetSplice clones were: 5' TGA CCT CCA TAG AAG ACA CC 3' and 5' GGG CTG CAG GAA TTC GAT AT 3'. The primers used to detect the hER pTetSplice clones were 5' TGA CCT CCA TAG AAG ACA CC 3' and 5' TGG TAG CCT GAA GCA TAG TC 3'.

Construction of vectors using MMTV promoter for targeting expression of mutant ERs to mammary tissue in transgenic mice.

Transgenic targeting constructs containing the MMTV LTR, the ER cDNA (wtER, $ER_{1\text{-}536}$ or $ER_{1\text{-}539}$), and the SV40 polyadenylation signal were created in the following manner. The MMTV-driven mammalian expression vector pMAM was purchased from Clontech (Palo Alto, CA, Catalog No. 6100-1). Since the multiple cloning site (MCS) of pMAM did not contain any useful restriction sites for subcloning the ER cDNAs, a pair of oligonucleotides containing a unique *Eco*R I site were designed to be ligated into the *Xho*I site of the pMAM MCS. The oligonucleotides used had the following sequence: Oligo 1 5' TCG AGC GCT GAA TTC GCG ACT AGT AGT C 3'; Oligo 2 5' CGC GAC TTA AGC GCT GAT CAG AGC T 3'. The oligonucleotides were annealed in a 50 μl reaction volume containing 200 pmols of each oligonucleotide. The reaction was incubated in an MJ Research MiniCycler thermal cycler for 3 min at 72° C, then cooled to 20° C at a rate of 2°/min. Annealed oligonucleotides were phosphorylated by incubating with T4 polynucletide kinase and ATP at 37° C for 30 min. The phosphorylated, annealed product was ligated into the *Xho*I site of pMAM. The pMAM vector containing the new MCS was digested with *Hin*d III and the 2703 bp band containing the MMTV long terminal repeat (LTR), new MCS, SV40 poly A, SV40 origin and early promoter was isolated by agarose gel electrophoresis, purified from the agarose gel slice using Geneclean II, and ligated into the *Hin*d III site of pGEM7z (Promega), from which

the *Eco*RI site had been previously eliminated. Resulting clones were screened by restriction digest analysis to determine which clones contained the insert in sense orientation. This plasmid was designated MMTV-7z. 10 µg of pSG5-HEGO and the mutant expression vectors were digested separately with *Eco*R I to release the ER cDNAs, separated and purified as described above, and ligated into the *Eco*RI site of the MMTV-7z plasmid. Resulting clones were screened by restriction digest analysis to determine which clones contained the ER inserts in sense orientation. The transgenic targeting fragment, containing the MMTV LTR, the hER cDNA, and the SV40 polyadenylation signal, will be released from the vector by digestion with *Kpn* I and *Bam*H I.

Results

Creation of dominant negative ER mutants.

Recent studies indicate that hydrophobic residues in the conserved the C-terminal domain of the ER motif in this domain are critical for transactivation activity (Danielan *et al.*, 1992). Additional mutations in this region suggest that inactivation of this transactivation domain can create a dominant negative ER phenotype (Ince *et al.*, 1993).

In ER-M543R, a methionine in this domain is mutated to arginine. Surprisingly, this mutant not only failed to show dominant negative activity, it was able to produce estrogen-dependent transcriptional activation of an ERE-reporter plasmid in the absence of wild type (wt) ER (Fig. 1).

Another mutation in this area, L540Q, has been reported to have potent dominant negative activity (Ince *et al.*, 1993). We created this mutant by site-directed mutagenesis, but unexpectedly found that it lacked the ability to inhibit transcriptional activation by the wtER even when cotransfected in 10-fold excess

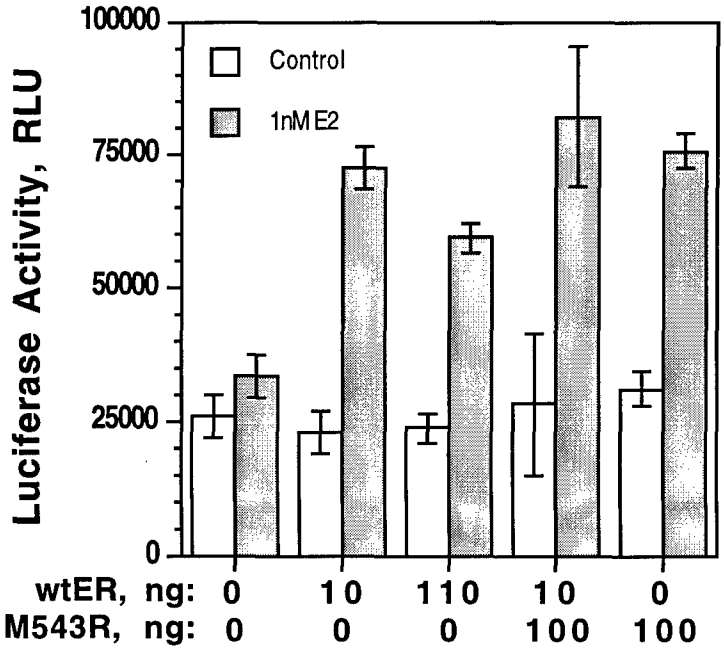

Fig. 1: ER-M543R has no dominant negative activity. Ishikawa II cells were grown in phenol-red free DME/F-12 + 10% DCC-FBS in six-well plates, liposome-transfected with 2µg/ well of ERE-SV40-luc and the indicated amounts of wtER (pSG5-HEGO) and mutant expression vector (plus sufficient pSG5 control vector to make the total DNA in each well equal), incubated 24 hr in growth medium + 1nM estradiol or 0.1% ethanol (control) and assayed for luciferase activity. Results are shown as mean ± s.e. of triplicate wells.

(Fig. 2). The presence of the L540Q mutation in our construct has been confirmed by the presence of an additional *Pvu* II site created by the nucleotide substitution (data not shown), as well as repeated sequencing of the cloning region, which shows the presence of the desired mutation and the absence of any others. The reasons for this disparity are unclear, although it is possible that expression of the L540Q mutant protein is variable in transfected cells. We plan to perform Western blotting experiments to verify expression of this and other ER mutants. Another possibility is that the previously reported mutant, which was created by random chemical mutagenesis, contains an additional, undetected mutation.

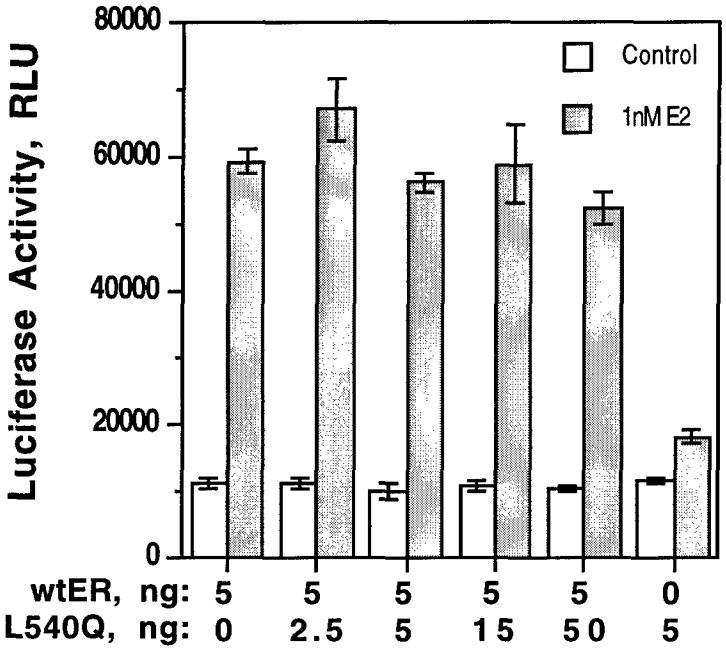

Fig. 2: ER-L540Q has no dominant negative activity. MDA-MB-231 cells were grown in phenol-red free MEM + 5% DCC-CS in six-well plates, liposome-transfected with 1µg/well of ERE_2-tk109-luc and the indicated amounts of wtER (pSG5-HEGO) and mutant expression vector (plus sufficient pSG5 control vector to make the total DNA in each well equal), incubated ~24 hr in growth medium + 1nM estradiol or 0.1% ethanol (control) and assayed for luciferase activity. Results are shown as mean ± s.e. of triplicate wells.

ER_{1-539} was the most potent dominant negative mutant identified in year 1 of this project. Although this is a C-terminal truncation mutant, it still contains one leucine from the hydrophobic C-terminal transactivation domain, as well as the tyrosine 537, a phosphorylation site proposed to play a role in ER function (Arnold & Notides, 1995; Castoria *et al.*, 1993). To test the effects of removing these residues, we created ER_{1-536}. This mutant also showed significant inhibition of the transcriptional activity of cotransfected wtER, both in Ishikawa II (Fig. 3) and MDA-MB-231 cells (Fig. 4). ER_{1-536} appears to be equally potent, as dominant negative activity is evident even when the mutant form was not present in great excess over wt. ER_{1-536} and ER_{1-539} were chosen for constructing inducible expression and transgenic animal vectors, as described below.

These dominant negative mutants have thus far displayed only weak inhibition of endogenous ER function in MCF-7 cells, as assessed by ERE-reporter expression. However, MCF-7 cells express a very high level of ER, and thus present an unusually difficult challenge for dominant negative mutants. We anticipate the mutants will be more effective in cells expressing more physiological levels of ER.

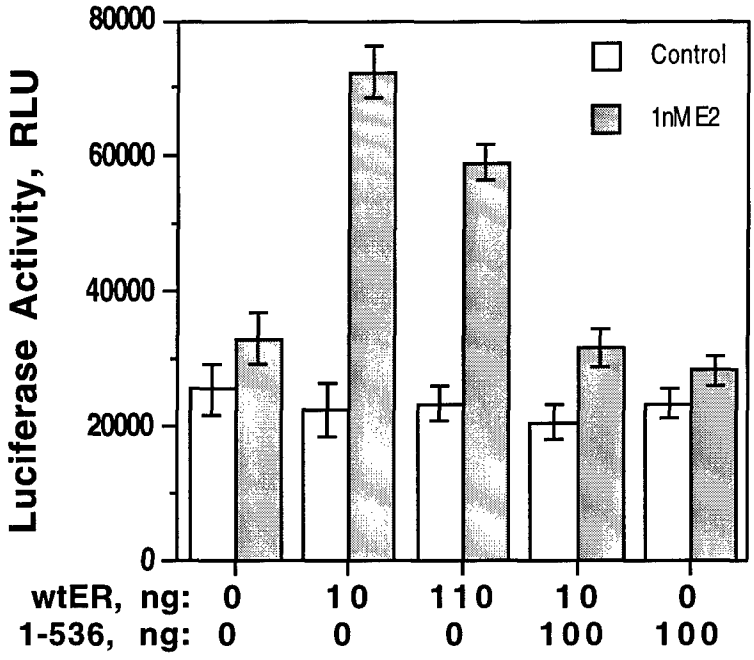

Fig. 3: Dominant negative effect of ER_{1-536}. Ishikawa II cells were grown in phenol-red free DME/F12 + 10% DCC-FBS in six-well plates, liposome-transfected with 2μg/ well of ERE-SV40-luc and the indicated amounts of wtER (pSG5-HEGO) and mutant expression vector (plus sufficient pSG5 control vector to make the total DNA in each well equal), incubated 24 hr in growth medium + 1nM estradiol or 0.1% ethanol (control) and assayed for luciferase activity. Results are shown as mean ± s.e. of triplicate wells.

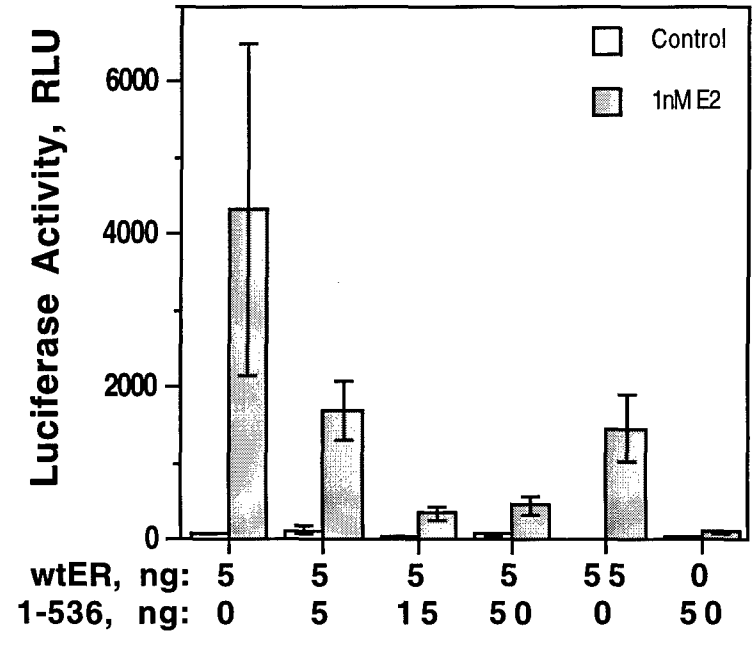

Fig. 4: Titration of wtER with ER_{1-536}. MDA-MB-231 cells were grown in phenol-red free MEM + 5% DCC-CS in six-well plates, liposome-transfected with 1μg/well of ERE_2-tk109-luc and the indicated amounts of wtER (pSG5-HEGO) and mutant expression vector (plus sufficient pSG5 control vector to make the total DNA in each well equal), incubated ~24 hr in growth medium + 1nM estradiol or 0.1% ethanol (control) and assayed for luciferase activity. Results are shown as mean ± s.e. of triplicate wells.

Development of a more selective estrogen-responsive reporter.

The estrogen-sensitive reporter plamids we used initially were created by insertion of one or more copies of the *Xenopus* vitellogenin ERE into pT109, which contains the firefly luciferase gene under the control of a portion of the thymidine kinase promoter (tk109). As noted in our first progress report, luciferase expression by the minimal pT109 promoter proved to be stimulated by EGF, making it difficult to attribute EGF stimulation of the ERE-reporters to ER-mediated signaling. However, the observation that EGF stimulation of the parental plasmid was consistently greater than or equal to that of the ERE-containing plasmids suggests that EGF stimulation was not mediated by ER. It has been reported that pUC-derived plasmids such as pT109 contain cryptic AP-1 sites (Lopez *et al.*, 1993), which could be the cause of the EGF stimulation. To address this possibility, we constructed new reporter genes containing one or two EREs and the tk109 promoter in the pA3luc backbone, as described in Experimental Methods. pA3luc contains SV40 polyadenylation signals to prevent adventitious transcription initiation. However, these reporters also showed EGF-stimulated transactivation by EGF in the absence of EREs (Fig. 5), suggesting that this stimulation was mediated by the tk109 promoter.

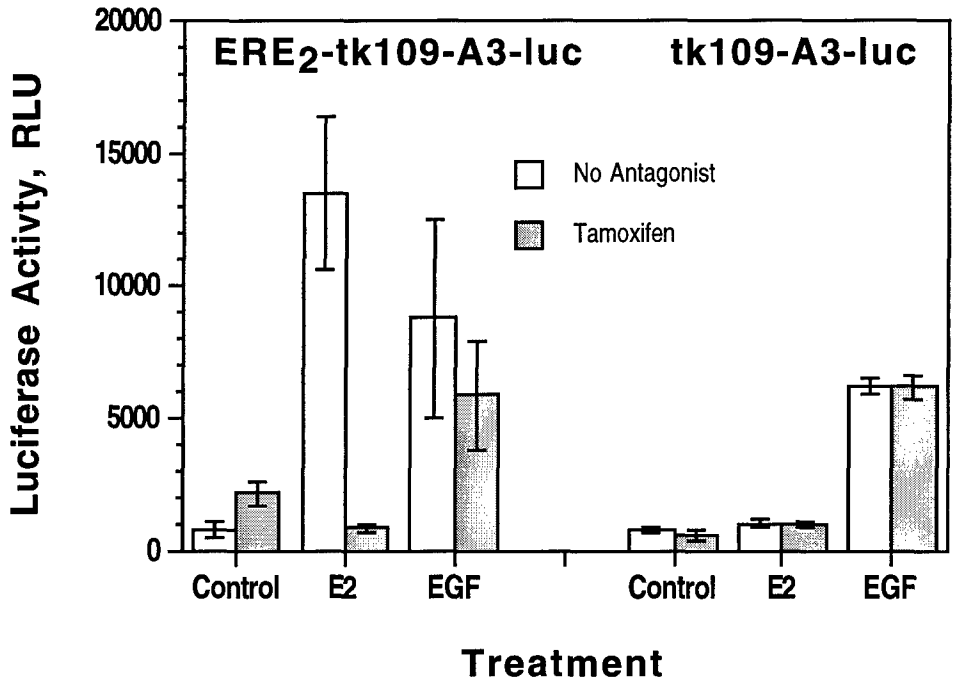

Fig. 5: EGF stimulation of ERE$_2$-tk109-A$_3$-luc is not dependent on EREs. Ishikawa cells were grown in 6-well plates in phenol-red-free MEM + 5% charcoal-stripped calf serum, liposome transfected with 5 μg/well of the indicated plasmids, and incubated 24 hr in growth medium with the indicated additions (tamoxifen, 1 μM; E2, 100 pM; EGF, 100 ng/ml). Luciferase assay results are expressed as fold induction over no-addition control for each reporter plasmid, shown as mean ± s.e. of triplicate wells.

An analogous family of reporter genes were created using a shorter fragment of the thymidine kinase promoter (tk81). The double-ERE member of this family (ERE_2-tk81-A3-luc) showed good induction by estrogens, but the overall level of light output was unsatisfactorily low in most cell lines, including MCF-7. It was usually difficult or impossible to accurately measure the light output of the single-ERE or no-ERE members of this series, or that of the double-ERE in unstimulated cells, making it impossible to accurately assess the fold activation produced by agonists acting on the basal promoter. This family of reporters was used successfully for transfecting BG-1 cells, however (described below).

Ideally, a reporter plasmid should have a promoter powerful enough to produce accurately measureable amounts of light even without stimulation by agonists, be unresponsive to the agonists in the absence of specific response element sequences, and give a good fold response to agonists when the specific response element sequences are present. The pGL3-promoter plasmid (Promega) contains a luciferase gene under the control of an SV40 early promoter, and it is unresponsive to estradiol or EGF in MCF-7 cells. Insertion of an ERE upstream of the promoter (described in Experimental Methods) produced a selective estrogen-responsive reporter with good light output (Fig. 6).

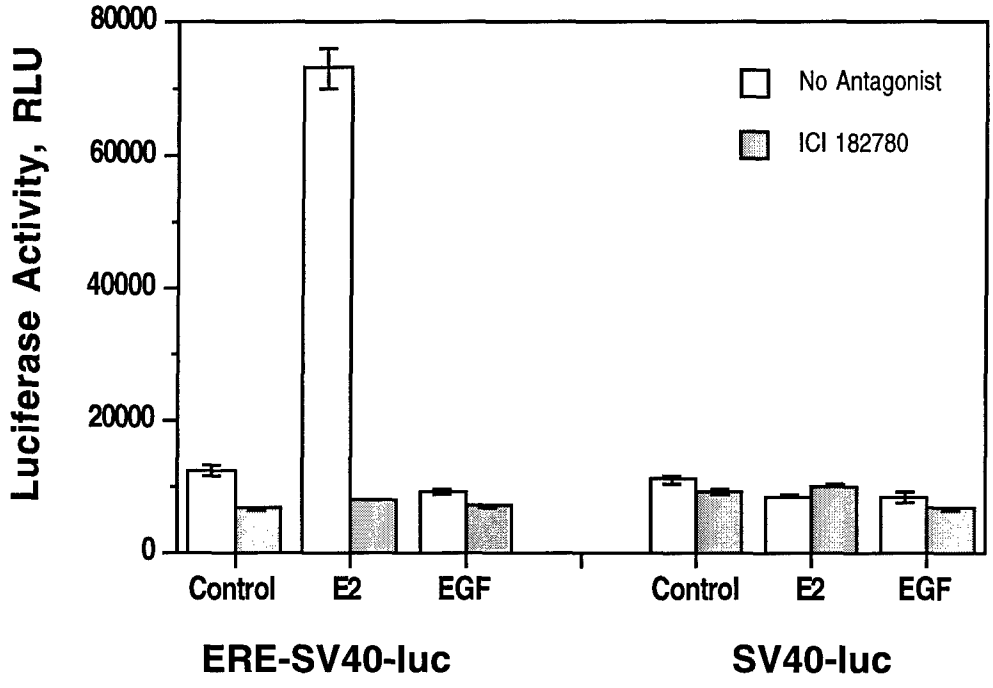

Fig. 6: Effect of estradiol and EGF on a selective estrogen-sensitive reporter plasmid. MCF-7 cells were grown in 6-well plates containing phenol-red-free MEM + 5% DCC-CS, calcium phosphate transfected with 1µg/well of ERE-SV40-luc or SV40-luc (pGL3-promoter), and incubated ~24 hr in growth medium with the addition of no agonist, 100 pM estradiol or 100 ng/ml EGF and with or without 100 nM ICI 182780. (As a vehicle control, 0.1% ethanol was added to the control and EGF wells). Cell extracts were assayed for luciferase activity; results are shown as mean ± s.e. of triplicate wells.

Demonstration that estrogen and growth factor signaling pathways are independent.

As described in our original application, one of our hypotheses was that growth factors, particularly EGF, are able to transcriptionally activate ER in the absence of ligand. For example, it was shown previously that EGF induced expression of an ERE-containing chloramphenicol acetyltransferase (CAT) reporter plasmid in Ishikawa and BG-1 cells (Ignar-Trowbridge *et al.*, 1993). Based upon this result, we tested whether dominant negative ER mutants could inhibit growth-factor induced transactivation in breast cancer cells. However, we did not demonstrate ER-mediated transcriptional activation of ERE-containing reporter constructs. As noted in our first progress report, EGF activation of tk109-based ERE-reporters in MCF-7 cells was less than or equal to the EGF activation of the respective ERE-less plasmids. In view of these results, we developed a new ERE luciferase reporter (described above) based on the SV40 early promoter, which is the basal promoter of one of the ERE reporter plasmids used by Ignar-Trowbridge *et al.* We also expanded our experiments to include the same cell lines used in previous studies.

As shown above in Fig. 6, the basal SV40-luc plasmid was unresponsive to both estradiol and EGF in MCF-7 cells. Addition of an estrogen response element upstream of the promoter resulted in activation by estradiol, but not by EGF. In the same experiment, cells transfected with the EGF-responsive reporter FOS-luc (not shown) produced a >10-fold increase in luciferase activtity when treated with EGF, indicating that the EGF signaling pathway is functional in these cells.

It is possible that our results reflect cell-specific differences in the ability of EGF to activate transcription by the ER. For example, tamoxifen has been found to act as an estrogen antagonist in breast epithelia, but it has agonistic effects on in uterine endometrium (reviewed by Friedl & Jordan, 1994). In order to address this possibility, we examined the ability of EGF to activate transcription of luciferase reporters in Ishikawa and BG-1 cells, which are derived from uterine and ovarian adenocarcinomas, respectively. As shown above in Fig. 5, tk109-A3-luc in Ishikawa cells was substantially activated by EGF, but the addition of a double estrogen response element did not significantly increase this response. Even more clear-cut results were obtained with the SV40-luc reporters (Fig. 7). The ERE-less basal reporter is only modestly activated by EGF; the activation of the ERE-containing reporter is no greater and is not antagonized by the estrogen antagonist ICI 182780. In BG-1 cells, ERE_2-tk81-A3-luc was activated by estradiol but not by EGF (Fig. 8).

In an effort to clarify the discrepancy between our results and those of Korach's laboratory, we are exchanging reporter plasmids, and each laboratory has agreed to the effect of EGF using the other's reporter genes.

We have also examined the potential of estradiol to activate, and tamoxifen to inhibit, the EGF signaling pathway in MCF-7 cells, as assessed by expression of the FOS-luc reporter. No effects were observed (Fig. 9).

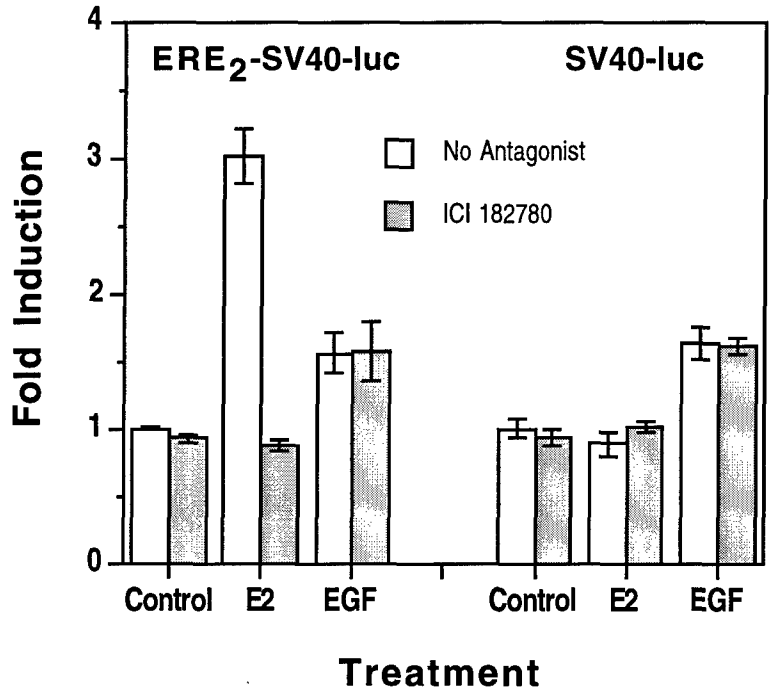

Fig. 7: Effect of EGF and estradiol on SV40-based reporters in Ishikawa cells. Ishikawa cells were grown in phenol-red-free DME/F-12 + 10% charcoal-stripped FBS, liposome transfected with 1 µg/well of the indicated plasmids, and incubated 24 hr in serum-free, phenol-red-free medium with the indicated additions (ICI 182780, 100 nM; E2, 100 pM; EGF, 100 ng/ml). Luciferase assay results are expressed as fold induction over no-addition control for each reporter plasmid, shown as mean ± s.e. (n=3).

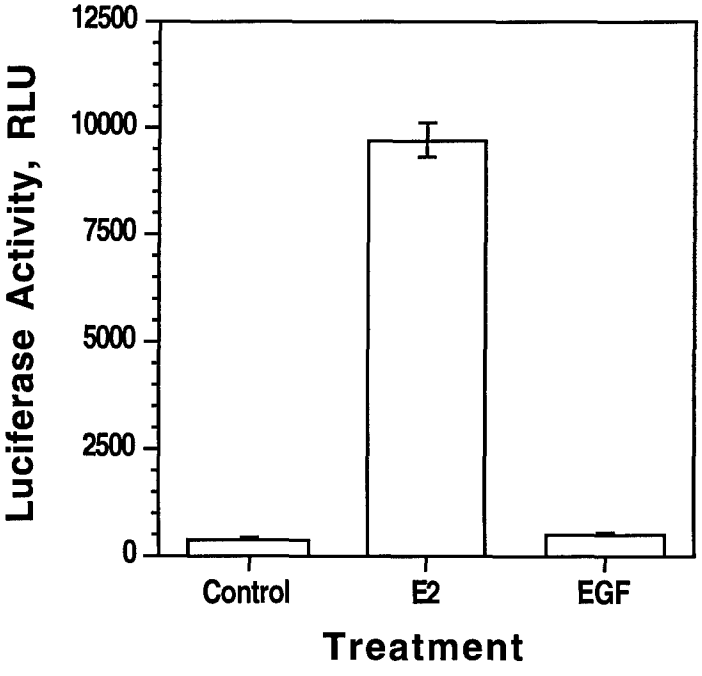

Fig. 8 Effect of EGF and estradiol on ERE_2-tk81-A_3-luc in BG-1 cells. BG-1 cells were grown in 6-well plates in phenol-red-free DME/F-12 + 10% charcoal-stripped FBS, liposome transfected with 5 µg/well of ERE_2-tk81-A3-luc, incubated 24 hr in phenol-red-free medium + 1% DCC-FBS with the indicated additions, and assayed for luciferase activity. Results are shown as mean ± s.e. for triplicate wells.

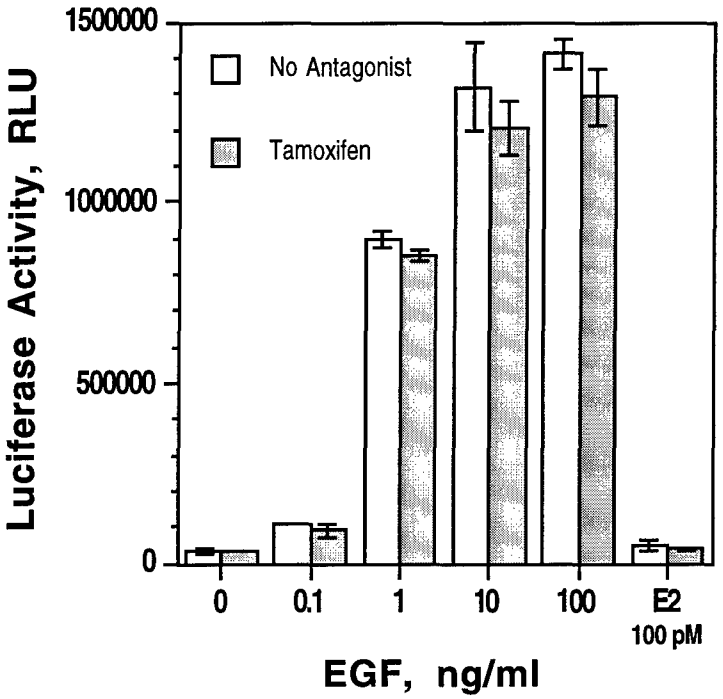

Fig. 9: Effect of estradiol and tamoxifen on EFG signaling. MCF-7 cells were grown in estrogen-depleted medium in 6-well plates, calcium-phosphate transfected with 2 µg/well FOS-luc, serum-starved overnight and treated for 6 hr with the indicated concentrations of EGF or estradiol with or without 1 µM tamoxifen. Luciferase assay results are shown as the mean and range of duplicate wells.

Measurement of estrogen-dependent T47D cell proliferation using the MTS assay.

T47D cells were evaluated for estrogen-dependent cell proliferation. As shown in Fig. 10, treatment of T47D cells with either 0.1 nM or 1 nM estradiol resulted in a significant increase in cell number, as measured by the MTS Assay, compared to the ethanol-treated control cells.

In order to study the effects of the dominant negative estrogen receptor mutants on estrogen- and growth factor-stimulated cell proliferation in human breast cancer cell lines, an inducible expression vector was used so that mutants could be stably transfected into T47D cells without effects on cell growth during the process of selection. After selection of transfected clones, the dominant negative ER mutants can be induced to evaluate acute effects on cell growth. We elected to use the recently described autoregulatory tetracycline inducible system (Shockett *et al.*, 1995) whic has been reported to have minimal leakiness in the absence of induction.

Evaluation of Tet-Regulated Expression System in T47D cells

The Tet-Regulated Expression System consists of two plasmids. The pTetSplice plasmid contains the Tetp promoter (consisting of the regulatory sequence (tetO) from the tetracycline-resistance operon of Tn10 upstream of a minimal hCMV promoter), and intron and polyadenylation signal from SV40, and a multiple cloning site (MCS). The pTet-tTAk plasmid contains the gene for the tetracycline transactivator (tTA) protein under the control of Tetp. When the tTA binds to tetO, transcription of the target gene is activated. In the presence of tetracycline, tTA cannot bind to the DNA, and the target gene is silent; removal of tetracycline reverses the process and activates the target gene. To use the system, the gene of interest is cloned into the MCS of the pTetSplice plasmid. The pTetSplice plasmid containing the recombinant gene and pTet-tTAk are then co-transfected

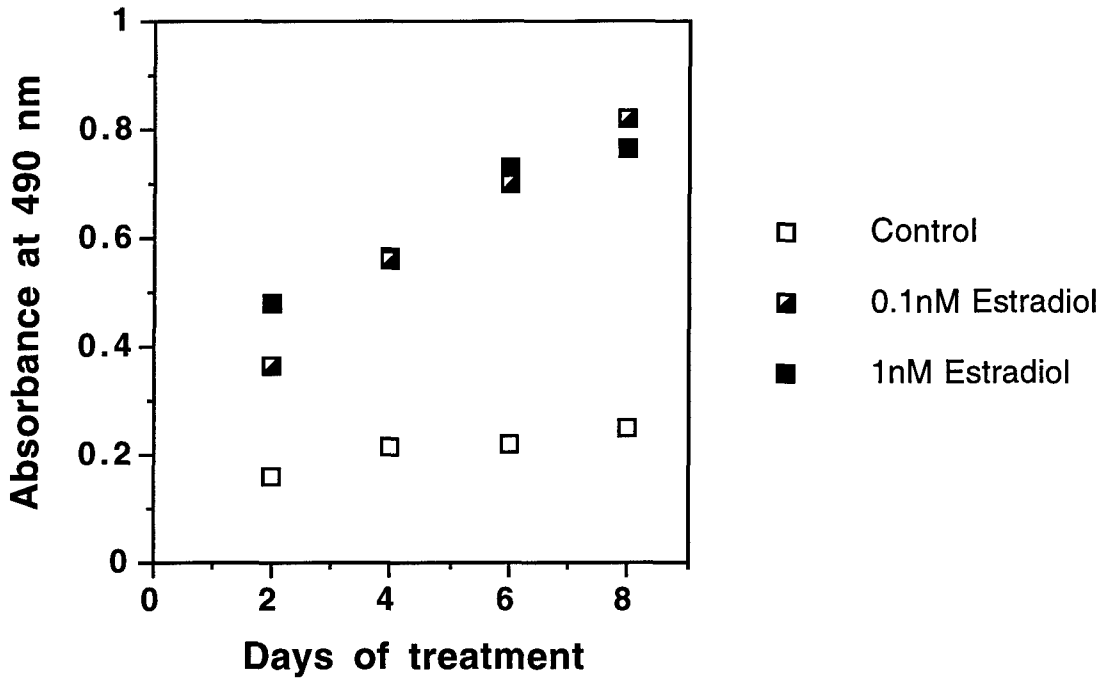

Fig. 10: Effect of estradiol on T47D cell proliferation.
T47D cells were grown in estrogen-depleted medium for 4 days, then split into 96-well plates at a density of 5000 cells/well. Cells received either control medium (n=16) or medium supplemented with 0.1 or 1 nM estradiol (n=32 for each dose). MTS assays were performed after 2, 4, 6 or 8 days of treatment to determine cell growth. Results are shown as mean values; error bars (s.e.) are smaller than the plot symbols.

into mammalian cells in the presence of tetracycline. Expression is induced by withdrawal of tetracycline from the medium.

The Tet-regulated Expression System was tested in T47D cells to determine if the system is capable of responding in a regulated manner in these cells. T47D cells were transiently transfected with equal amounts of the pTet-tTAk plasmid and the pUHC13-3 plasmid, which contains the luciferase gene under the control of Tetp. Transfected cells were treated either with or without tetracycline for 72 hr, and then assayed for luciferase activity (Fig. 11). As expected, cells treated with tetracycline for 72 hr had a relatively low level of luciferase activity (mean: 15292 RLU), while cells which were not treated with tetracycline had a luciferase activity 213 fold higher than the 72 hr tetracycline treatment group. An intermediate level of luciferase activity was seen in cells treated with tetracycline for the first 48h, but not for the last 24 hr of the experiment. Based upon these results, it was concluded that the Tet-regulated expression system is potently inducible in T47D cells.

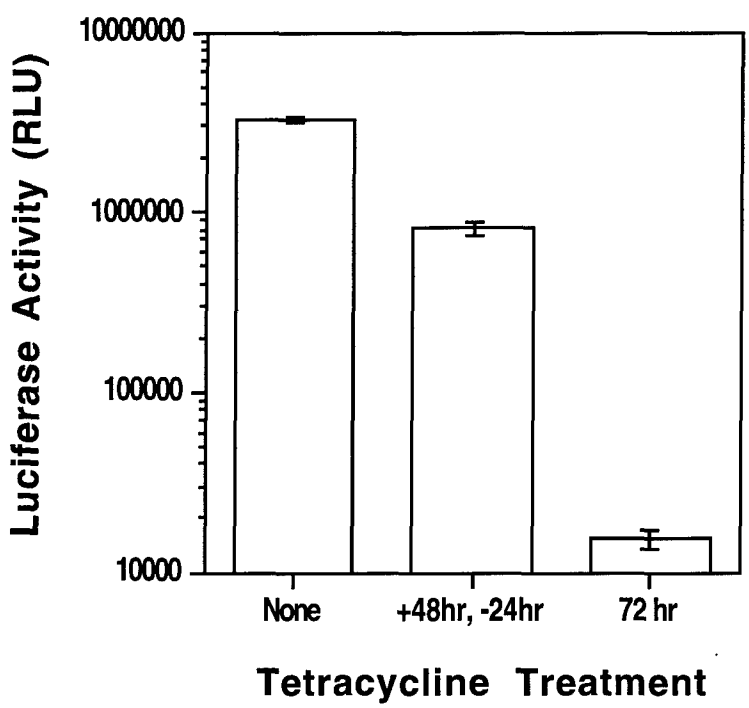

Fig. 11: Evaluation of the Tet-Regulated Expression System in T47D cells. T47D cells were transiently transfected by electroporation with 1 µg/well of pTet-tTAk and pUHC13-3 plasmid DNA, plated into 6-well plates, divided into 3 treatment groups (72hr with no tetracycline, 48hr with tetracycline [0.2 µg/ml] followed by 24 hr without, or 72 hr with tetracycline) and assayed for luciferase activity. Results are shown as mean ± s.e. of triplicate wells. Note logarithmic scale of vertical axis.

Creation and screening of T47D stable cell lines.

Human breast cancer T47D cells were stably cotransfected with empty pTetSplice, wt and dominant negative hER pTetSplice constructs, pTet-tTAk, and, since the tet regulated plasmids do not contain a selective marker, pSV2neo, which contains the neomycin resistance gene and allows for selection by treatment with the neomycin analog, G418 sulfate.

In order to determine which stable clones contained pTet-tTAk, clones were transiently transfected by electroporation with 1 µg/well of a 6 well plate of the tetracycline regulated luciferase reporter vector, pUHC13-3. Clones which contained pTet-tTAk were expected to produce an increase in luciferase activity in the absence of tetracycline in the media. Three wells of a 6 well plate were treated for 72 hr with 0.2 µg/ml tetracycline, while the remaining three wells received media without tetracycline. Cells were assayed for luciferase activity. A typical result of the screening assay is shown in Fig. 12 . Cells which were shown to contain pTet-tTAk were screened for pTetSplice constructs by PCR. Stable clones containing the pTet-tTAk and pTetSplice constructs are currently being used to study the effects of the dominant negative human estrogen receptor mutants on estrogen and growth factor dependent cell proliferation.

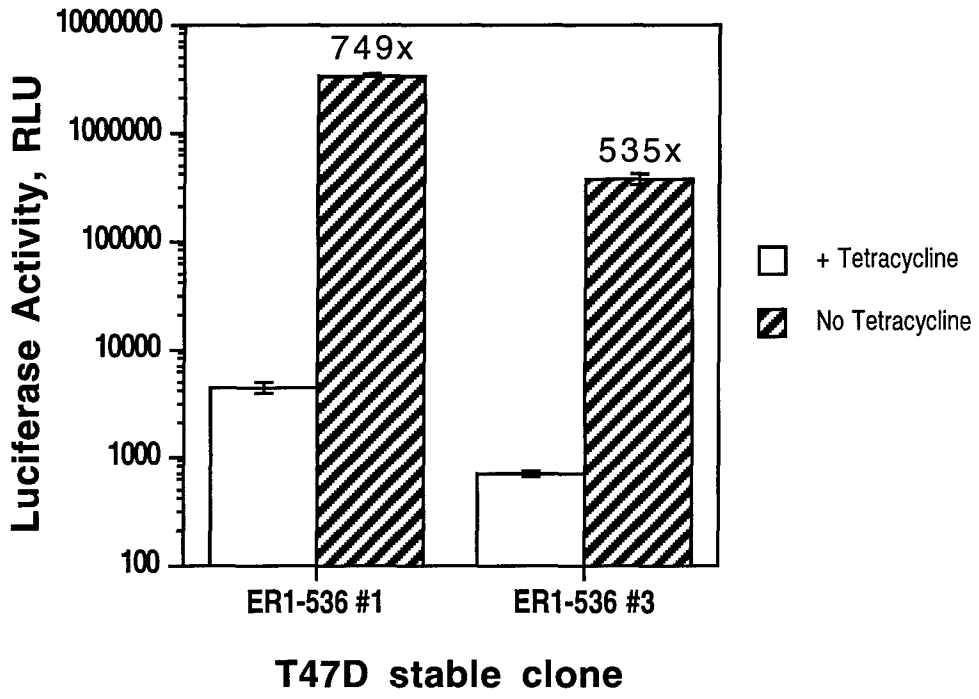

Fig. 12: Screening of T47D stable clones for pTet-TAk.
T47D stable clones carrying mutant ER were transiently transfected by electroporation with the tetracycline-regulated luciferase reporter pUHC13-3 (1 μg/well of a 6-well plate), cultured for 72 hr in the presence or absence of 0.2 μg/ml tetracycline, and assayed for luciferase activity. Results are shown as mean ± s.e. of triplicate wells. Note logarithmic scale of vertical axis.

Construction of vectors using MMTV promoter for targeting expression of mutant ERs to mammary tissue in transgenic mice.

Vectors using the MMTV promoter to target expression of both wt and mutant hERs to the mammary tissue of transgenic mice have been prepared as described in Materials and Methods. The construct format is shown in Figure 13. Transgenic mice will be made using these transgenic constructs in the Northwestern University Transgenic Facility, under the direction of Dr. Phillip Iannoccone.

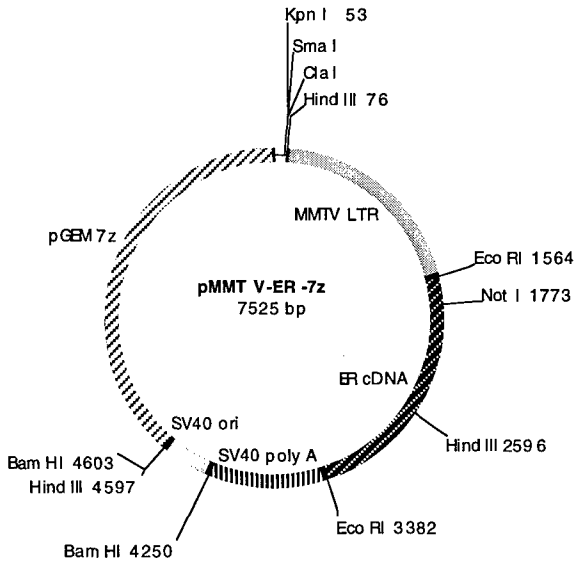

Fig. 13: Transgenic targeting plasmid

CONCLUSIONS

After testing several reporter systems, we have produced a selective estrogen-responsive reporter based on the SV40 early promoter. The basal promoter shows little or no activation by EGF or estradiol in the cell lines tested. Introduction of an ERE conveys responsiveness to estradiol but not to EGF. Based on results with this and other reporters, we find no evidence that EGF can activate transcription by ER. Our results indicate that the estrogen and EGF signaling systems for transcriptional activation are independent using our assay conditions, and suggest that the previously reported activation of ER-mediated transcription by EGF may be less robust and direct than previously suggested.

These results have focused our efforts on the direct effects of estrogen rather than EGF-induced transcriptional activation of the ER. However, the effect of such mutants on less direct effects of growth factors, such as endogenous gene expression and cell proliferation, are still of great interest, as well as the effects on estrogen-induced transcription and cell growth.

To this end, C-terminal truncation mutants of the ER have been prepared that inhibit the transcriptional activity of wtER in several cell lines using a number of different reporters. These mutants have been cloned into an inducible expression system and stably transfected into T47D cells, which have been identified as a good model for studying the effects of ER mutants on estrogen- and growth factor-induced cell proliferation. Preliminary results suggest that these mutants are potent inhibitors of breast cell proliferation.

In addition, constructs of targeted expression of the dominant negative mutant ERs in mammary tissue of transgenic mice have been created. Strains of mice carrying these transgenes will be used to characterize the effect of dominant negative ER mutants on mammary development and tumorigenesis.

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APPENDIX: Abbreviations

ATCC, American Type Culture Collection
ATP, adenosine triphosphate
CAT, chloramphenicol acetyltransferase
cDNA, complementary DNA
CS, calf serum
DCC, dextran coated charcoal
DCC-CS, DCC-stripped calf serum
DCC-FBS, DCC-stripped fetal bovine serum
DDAB, dimethyldioctadecylammonium bromide
DMEM, Dulbecco's modified essential medium
DNA, deoxyribonucleic acid
DOPE, dioleyl phosphatidylethanolamine
DTT, dithiothreitol
E2, estradiol
EGF, epidermal growth factor
EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
ELISA, enzyme-linked immunosorbent assay
ER, estrogen receptor
ERE, estrogen response element
FBS, fetal bovine serum
Hepes, *N*-2-hydroxyethylpiperazine]-*N'*-2-ethanesulfonic acid
IPTG, isopropyl β-D-thiogalactopyranoside
LTR, long terminal repeat
MCS, multicloning site
MEM, minimal essential medium
MMTV, mouse mammary tumor virus
MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NEAA, nonessential amino acids
PBS, phosphate buffered saline
PCR, polymerase chain reaction
PMS, phenazine methosulfate
poly A, polyadenylation
s.e., standard error
TAE, tris(hydroxymethyl)aminomethane acetate and ethylenediaminetetraacetic acid
WAP, whey acidic protein
wt, wild type

PUBLICATIONS AND MEETING ABSTRACTS

"Independent pathways for estrogen and epidermal growth factor signaling in breast cancer cell lines." Poster presented at 10th International Congress of Endocrinology in San Francisco, CA, June 1996. Abstract: B.D. Gehm, J.M. McAndrews, V.C. Jordan & J.L. Jameson (1996) *ICE '96 Poster & Abstracts* v.1, p. 607.

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REPLY TO
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MCMR-RMI-S (70-1y) 6 May 98

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2. Point of contact for this request is Ms. Betty Nelson at DSN 343-7328 or email: betty_nelson@ftdetrck-ccmail.army.mil.

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