GRANT NUMBER DAMD17-97-1-7096

TITLE: Predoctoral Fellowship to Study an ER Variant Identified from Breast Hyperplasias

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REPORT DATE: July 1999

TYPE OF REPORT: Annual

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

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Predoctoral Fellowship to Study an ER Variant Identified from Breast Hyperplasias

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Studies have shown that elevated expression levels of estrogen receptor α (ERα) in benign breast epithelium is a risk factor for progression to invasive breast cancer. We have isolated a variant form of the ERα from breast hyperplasias which displays a 100-fold increase in estrogen sensitivity and increases cell proliferation. Expression of a variant ER that confers a proliferative advantage as well as a heightened sensitivity to estrogen might predispose breast hyperplasias to malignant progression. The research funded by this grant is focused on investigating the factors that regulate the expression of ERα and its variant’s expression in breast cancer cells as potential targets for clinical therapy.

We have previously shown that the basal promoter of the ERα gene lies within the first 245 bp of the 5' flanking region of the gene, and displays unusually high transcriptional activity in transient transfection assays. We now demonstrate that there are three elements within the ERα gene 5' flanking region that are critical for full promoter activity, and that the transcription factors Sp1 and Sp3 can each bind to this region, and are independently able to transactivate the ERα promoter. Inhibition of DNA binding by Sp1 to the ERα minimal promoter results in both a decrease of ERα promoter activity as well as ERα expression in breast cancer cells. Therefore, we conclude that Sp1 and/or Sp3 are critical for ERα expression in breast cancer cells, and are investigating other potential interacting factors in the regulation of ERα expression in breast cancer. The results of these experiments could prove clinically important in identifying new treatment targets.
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MAIN REPORT:

ABSTRACT:

Studies have shown that elevated expression levels of estrogen receptor α (ERα) in benign breast epithelium is a risk factor for progression to invasive breast cancer. We have isolated a variant form of the ERα from breast hyperplasias which displays a 100-fold increase in estrogen sensitivity and increases cell proliferation. Expression of a variant ER that confers a proliferative advantage as well as a heightened sensitivity to estrogen might predispose breast hyperplasias to malignant progression. The research funded by this grant is focused on investigating the factors that regulate the expression of ERα and its variant's expression in breast cancer cells as potential targets for clinical therapy.

We have previously shown that the basal promoter of the ERα gene lies within the first 245 bp of the 5' flanking region of the gene, and displays unusually high transcriptional activity in transient transfection assays. We now demonstrate that there are three elements within the ERα gene 5'flanking region that are critical for full promoter activity, and that the transcription factors Sp1 and Sp3 can each bind to this region, and are independently able to transactivate the ERα promoter. Inhibition of DNA binding by Sp1 to the ERα minimal promoter results in both a decrease of ERα promoter activity as well as ERα expression in breast cancer cells. We therefore conclude that Sp1 and/or Sp3 are critical for ERα expression in breast cancer cells, and are investigating other potential interacting factors in the regulation of ERα expression in breast cancer. The results of these experiments could prove clinically important in identifying new treatment targets.
INTRODUCTION:

Understanding the molecular changes that occur during the progression of breast disease will provide clinicians with both earlier markers for detection of the disease, as well as potential targets for early intervention. One well-recognized target is the estrogen receptor α (ERα). We know that ERα is expressed at elevated levels in many tumors as compared to the adjacent normal tissue (1-3). In fact, studies have shown that elevated levels of ERα in benign breast epithelium is itself a risk factor for progression to invasive breast cancer (4,5). The ER is a nuclear transcription factor and a well known mitogen for breast cancer cells (6), thus its overexpression may play a permissive role in the progression of breast cancer (7). We hypothesize that the delineation of the mechanisms regulating ERα expression is clinically important and may identify new treatment targets. We plan to address the identification of these potential new targets with the following Specific Aims:

1. To identify regions within the ERα promoter regulating ERα transcription in breast cancer cells (months 1-12).

2. To identify factors binding to transcriptional regulatory regions of the ERα promoter (months 12-30).

3. To determine the relevance of candidate proteins in the regulation of ERα transcription in breast cancer cells (months 18-36).

BODY:

While the original focus of this grant was on the study of an ER variant with estrogen hypersensitivity, the tremendous success that we have had in the characterization of the transcriptional regulation of ERα expression in breast cancer cells has led us to focus on this aspect of the study, as was introduced in last year’s progress report. I have made excellent progress in this second year of my investigation, focusing predominantly upon Specific Aims 2 and 3 and am on schedule in the grant. In the first year of this study, we used ERα promoter deletion constructs (Fig 1A) that were subcloned into the luciferase reporter vector pGL3-Basic (Promega, Madison, Wi) in transient transfection assays in a panel of both ER-positive and negative breast cancer cells, and found that a majority of the ERα promoter activity lies within the first 245 bp of the 5'flanking region of the gene (Fig. 1B, A). Because
the majority of ERα promoter activity in all of the breast cancer cell lines resided within the first -245 bp, we next focused our attention to this region of the promoter with the goal of identifying those factors critical for regulating its transcription. I generated an ERα promoter construct deleting the region between -245 and -200 bp and subcloned this into the pGL3-Basic vector, because an initial search for potential transcription factor binding sites (8) identified a number of sites within this region. This promoter construct was then examined in the ERα-positive MCF-7 breast cancer cell line using transient transfection (Fig. 2A), with transfection efficiency corrected using β-galactosidase expression. The -245 fragment (Fig. 2A, second bar) exhibited a 17-fold increase in activity over the control vector alone. The removal of the -245 to -200 bp region in the -200 fragment (Fig. 2A, third bar) results in the loss of all promoter transcriptional activity. These results suggest that elements critical for transcriptional activity of the ERα promoter lie between nucleotides -245 and -200, and thus contains the ERα minimal promoter.

To more precisely define the functional elements within the ERα minimal promoter that confer transcriptional activation, eight to twelve bp EcoRI linker-scanner mutations directed at known regulatory elements, such as a non-consensus E-box at -231 to -226 bp, a GC box at -223 to -214 bp, and a C/A rich box at -203 to -192 bp, were introduced into ERα promoter reporter fragment A (Fig. 2B, starred regions). These elements are potential binding sites for basic helix-loop-helix transcription factors and Sp1 transcription factor family members, respectively (9-12). These reporter constructs were then transfected into MCF-7 cells and the results were expressed as fold activity compared to vector control, after normalizing for β-gal expression (Fig. 2B). Mutation of the region between -235 and -224 bp (Fig. 2B, B) resulted in a 37% loss of promoter activity. Even more significant activity losses of 63% and 81% respectively, were seen with fragments C and E. In comparison, a negligible decrease in activity was seen when the region between bases -211 and -204 was mutated (Fig. 2B, Fragment D). These results suggest that there are critical elements contained within Fragments C (the region between -223 and -212 bp) and E (-203 to -192 bp). Furthermore, the significant reduction also seen when the region in Fragment B is
mutated suggests that multiple elements, and their cognate binding factors, may potentially cooperate for full ERα transcription activity of this region.

To determine what transcription factors might be binding to the critical elements within fragment A (Specific Aim 2), a radiolabeled oligonucleotide probe spanning the ERα promoter region from -245 to -182 bp was used in an EMSA to detect potential binding factors. Seven specific DNA-protein complexes were detected with MCF-7 protein extracts (Fig. 3, lane A, arrows labeled 1 through 7). All seven of these complexes were specifically competed by a 25-fold excess of unlabeled probe (cold competitor, lane B), or an excess of an oligonucleotide containing a consensus binding site for Sp1 family members (lane C). The specificity of these competition assays was also confirmed using an oligonucleotide with a mutated Sp1 binding site (data not shown).

The identity of the protein-DNA complexes was next investigated with polyclonal antibodies to Sp1 and Sp3 (Santa Cruz Biotechnology, Santa Cruz, Ca.). It appears that most of the seven complexes indeed contain Sp1 and/or Sp3 (lanes D, E and H). The most demonstrable difference in the supershifted complexes was seen with complex 7. This complex clearly contains Sp3, but not Sp1 (compare lanes D and E). Incubation of the MCF-7 extract with an antibody to either Sp4 (lane F) or AP2 (lane G) (both Santa Cruz Biotechnology) prior to addition of probe had no effect on any of these complexes. When both Sp1 and Sp3 antibodies are preincubated with the extract, all seven of the complex bands are affected (lane H). These data suggest binding by both Sp1 and Sp3 to the minimal ERα promoter region.

In order to determine whether Sp1 and Sp3 are able to transactivate the ERα minimal promoter (ER promoter reporter fragment A), we performed a series of transient transfections using pPac expression plasmids for Sp1 and Sp3 in Drosophila SL2 cells, which do not express any of the Sp1 family members. The plasmid pNull-Renilla (Promega) was cotransfected for transfection normalization. We also treated the transiently transfected cells with the Sp1 DNA-binding inhibitor mithramycin A (Sigma, St. Louis, Mo.) to see if transactivation of the ERα promoter fragment by exogenous Sp1 and Sp3 was affected. As shown in Fig. 4, both Sp1 and Sp3 are independently able to transactivate the ERα minimal
promoter in a dose-dependent manner. Exogenous expression of Sp1 was able to increase activity of the minimal promoter fragment 3 to 5-fold (Fig 4., lanes 2 and 3, respectively) and exogenous expression of Sp3 increased promoter activity 4 to 6-fold (Fig 4., lanes 8 and 9, respectively). Furthermore, this transactivation was inhibited in a dose-dependent manner by increasing concentrations of mithramycin A. Concentrations of drug at 5 x 10^{-8} M slightly inhibited promoter activity in Sp1 transfected cells (Fig 4, lane 4), but significantly affected transactivation of the minimal promoter in Sp3 transfected cells (Fig 4, lane 10). This affect was enhanced with increasing concentrations of drug in both Sp1 and Sp3 transfected cells. A concentration of mithramycin A at 5 x 10^{-6} M resulted in ERα promoter activation levels actually below vector control cells (Fig 4, compare lane 1 to lane 6, and lane 7 to lane 12). These results indicate that Sp1 and Sp3 are independently able to transactivate the ERα minimal promoter in Drosophila SL2 cells, and that this transactivation is due to binding of these proteins to the promoter since this binding can be inhibited by mithramycin A.

The significance of binding by Sp1 and/or Sp3 to the ERα minimal promoter for transcriptional activity was confirmed in data shown in Fig. 7 (Specific Aim 3). MCF-7 cells transiently transfected with ER promoter fragment A, and treated with increasing concentrations of mithramycin A, demonstrate a dose-dependent loss of both promoter (Fig. 5, A) as well as a decrease in endogenous ERα protein levels (Fig. 5, B). ERα promoter activity was decreased approximately 60% in cells treated with 10^{-6} M drug, 70% in cells treated with 10^{-8} M drug, and almost 80% in cells treated with 10^{-7} M drug, as compared to the promoter activity in cells treated with ethanol alone. The observed effects on promoter activity are specific to Sp-dependent transactivation, since an ERE-tk promoter was actually activated at the same drug concentrations (Fig. 5 A). The effects on ERα promoter activity by mithramycin A were correlated to the effects on ERα expression levels (Fig. 5 B). ERα protein levels decreased in cells treated with 10^{-6} M mithramycin A as compared to control. This decrease in expression was more dramatic in cells treated with 10^{-4} M drug. In cells treated with 10^{-7} M drug, expression of ERα was below detectable levels. Taken together with the SL2 transactivation data, these results demonstrate an essential role for Sp1/3 in transcription of the ERα gene.
In the next year I will continue to identify other transcription factors which are involved in the transcriptional regulation of ERα expression in breast cancer using co-immunoprecipitation assays with Sp1, Southwestern blot analysis of MCF-7 extract with the -245 to -182 bp probe, and Western blot analyses of the different EMSA protein/DNA complexes seen in Figure 3 (Specific Aim 2). We have already shown that ERα itself is able to autoregulate the minimal promoter region (13), and will expand upon this observation to determine the mechanism by which ER autoregulates itself.

By the end of the upcoming year I plan on completing the studies identifying the transcription factors involved in the regulation of ERα transcription, as well as discerning the biological significance of the interaction of these factors with the minimal ERα promoter (Specific Aim 3). I will use transient transfection assays to evaluate the effects of exogenous expression of candidate transcription factors on ERα minimal promoter activity, as was done for Sp1 in Figures 4 and 5. Finally, I will perform a correlative study on a statistically significant number of clinical samples, comparing the expression levels of ERα with expression levels of those transcription factors found to be critical for ERα transcription.

CONCLUSION:
As is evidenced by this year's report, I have made significant progress toward my goal. I anticipate that I will successfully complete all of my stated Specific Aims by the end of Year 3 of this proposal. I will continue in my efforts at examining those factors that regulate not only the expression of the “super-active” ER variant, but of the wild-type ER as well. Finally, once I have determined some of the regulating factors of both expression and function, I will attempt to determine their usefulness as targets in therapy for some women with breast disease.
REFERENCES:


FIGURE LEGENDS:

Fig. 1. Deletion fragments of the ERα gene promoter and their activity in a panel of breast cancer cells. A, ER promoter fragments spanned from (A)-245 to +212 bp, (B)-735 to +212 bp, (C)-1000 to +212 bp, (D)-2769 to +212 bp, and (E)-4100 to +212 bp. Fragment coordinates are expressed relative to the primary transcription start site. B, Fragments cloned into a pGL3-Basic reporter vector were transiently transfected with pCMV-βgal (to correct for transfection efficiency) into a panel of ER-positive (MCF-7, T47D, and ZR75-1) or ER-negative (MDA-MB-231 and MDA-MB-435) breast cancer cells. Promoter activity was expressed as corrected fold luciferase activity relative to that obtained for the vector alone. Shown was the mean ± S.D. of two experiments combined, performed in triplicate.

Fig. 2. Identification of important regulatory elements in the ERα promoter. A., ER promoter deletion fragments -245/+212 (see fragment A) or -200/+212 subcloned into pGL3-Basic were transiently transfected with pCMV-βgal into MCF-7 cells. Promoter activity was expressed as corrected fold luciferase activity relative to vector alone and was representative of two independent experiments performed in triplicate wells. B, Luciferase reporter constructs containing the wild type ER minimal promoter fragment or linker-scanner mutants of the minimal promoter were transfected with pCMV-βgal into MCF-7 cells. Left panel, schematic presentation of the ERα promoter fragments and the potential transcriptional elements and the linker-scanner mutations (star) between nucleotides -235 and -224 (B), nucleotides -223 and -212 (C), nucleotides -211 and -204 (D), and nucleotides -203 and -192 (E). Right panel, promoter activity was expressed as corrected fold luciferase units over vector control and was representative of two independent experiments done in triplicate.

Fig. 3. Sp1 and Sp3 interact with the -245 to -182 bp region of the ER promoter. EMSA were performed using MCF-7 extracts. An end-labeled (20,00 cpm) oligonucleotide which
spanned the ER promoter from -245 bp to -182 bp (Fig. 3) was incubated with 10 μg of extract alone (lane A), or in the presence of 25-fold molar excess of cold oligonucleotide (lane B), 25-fold molar excess of a cold oligonucleotide containing an Sp1 consensus binding site (lane C), anti-Sp1 antiserum (lane D), anti-Sp3 antiserum (lane E), anti-Sp4 antiserum (lane F), anti-AP2 antiserum (lane G), or both anti-Sp1 and anti-Sp3 antisera (lane H). Complexes are indicated by numbered arrows with supernoviated complexes indicated by SS.

Fig. 4. Sp1 transcription factors transactivate the ERα minimal promoter in Drosophila cells. Drosophila SL2 cells were co-transfected with the ER minimal promoter luciferase reporter construct and the pPac vector or increasing concentrations of either an Sp1 or Sp3 expression vector. Transfected cells were also treated with increasing concentrations of an Sp1 binding inhibitor, mithramycin A at concentrations ranging from $5 \times 10^{-8}$ M to $5 \times 10^{-6}$ M. Luciferase values were normalized to Renilla luciferase expression. Fold induction was calculated relative to the normalized luciferase activity obtained by transfecting the minimal promoter reporter with the pPac vector alone and was representative of two independent experiments done in triplicate.

Fig. 5. Inhibition of Sp1 DNA binding results in a loss of both ER promoter activity and ERα protein expression in MCF-7 cells. A, MCF-7 cells were transiently transfected with either the ER minimal promoter luciferase reporter construct (Fig 1A, A) or an ERE-tk-luciferase reporter. Transfected cells were treated with increasing concentrations of an Sp1 binding inhibitor, mithramycin A at concentrations ranging from $10^9$ M to $10^{-7}$ M. Luciferase values were normalized to Renilla luciferase expression. Fold induction was calculated relative to the normalized luciferase activity obtained from the untreated control cells. B, MCF-7 cells were treated for 18 hours with concentrations of 0 M, $10^9$ M, $10^8$ M, or $10^7$ M mithramycin A and measured for ERα expression.
conc. DNA
control 0.1 ug 0.5 ug 0.5 ug 0.5 ug 0.5 ug
conc. Mithramycin A
- - $5 \times 10^{-8}$ $5 \times 10^{-7}$ $5 \times 10^{-6}$
Appendices

1. Key research accomplishments

- Defining a critical regulatory region of the ERα promoter responsible for a majority of the transcriptional activity of the gene.
- Determining that Sp1 and/or Sp3 are critical for ERα transcription in breast cancer cells. These are the first transcription factors, other than the ER itself, which have proven to have any regulatory effect on ERα expression.

2. Manuscripts

- **Sp1 is Essential for the Regulation of Estrogen Receptorα Gene Transcription.** deGraffenried LA, Hilsenbeck SG, and Fuqua SAW. *J Biol Chem* (submitted)

Abstracts


THE SPI TRANSCRIPTION FACTOR IS INVOLVED IN TRANSCRIPTIONAL REGULATION OF THE ESTROGEN RECEPTOR GENE IN HUMAN BREAST CANCER CELLS.
deGraffenried LA and Fuqua SAW, The University of Texas Health Science Center, San Antonio Texas, 78284.

Elevated levels of ER are expressed in the hyperplastic mammary epithelium as compared to adjacent normal tissue and this elevated level of ER may be a permissive event for the progression of the disease. Understanding the regulation of ER expression therefore, is of extreme clinical significance. We hypothesize that transcriptional regulation of specific regions of the ER promoter might be involved in the overexpression of ER during breast carcinogenesis. The regulatory regions of the ER promoter have not yet been fully defined, but transgenic mouse models suggest that several regions of the promoter may be important for cell-type specific expression. We therefore have employed both classical promoter deletion mapping and gel-retardation assays to study the differential binding of putative transcription factors to a series of overlapping regions of the ER promoter. ER promoter fragments analyzed ranged from -4100 bp to +212 bp, all relative to the first transcriptional start site. We have used both ER-positive and ER-negative breast cancer cells for these assays. Based upon rigorous statistical analysis, we have been able to categorize promoter activity into two groups: those without repressor activity and those with potential repressor activity within the -1000 bp to -2769 bp region. These groupings correlate with the ER status of the cell lines. In both ER-positive and ER-negative cells, the majority of the promoter activity of the ER gene resides within the first 245 bp of the promoter. We found that SPI elements located within this region are essential for full promoter transcriptional activity in transient transfection assays. Gel shift assays demonstrated that the Sp1 transcription factor binds to these elements. Other investigators have provided evidence for a participation of Sp1 with other factors in the regulation of gene transcription. We are therefore currently analyzing Sp1 and other potential interacting factors in the regulation of ER expression in breast cancer, as well as continuing to define the regions of the ER promoter involved in the differential expression of this gene.


An apparent paradox is that expression of the estrogen receptor (ER) gene is very low in normal breast epithelial cells, yet over 70% of human breast tumors express high levels of the ER. We hypothesize that transcriptional regulation of specific regions of the ER promoter might be involved in the overexpression of ER during breast carcinogenesis. In order to test this hypothesis, we have chosen as our model system a series of MCF-7 breast cancer cell sublines which have progressively lost ER expression. This system provides us with genetically matched environments that differ only the expression levels of ER and the consequences thereof. Previous studies suggest that there are several different transcriptional start sites upstream of the ER gene which regulate expression, however the regulatory regions of the ER promoter have not yet been fully defined. To identify regulatory regions, we have employed classical promoter deletion mapping using a series of overlapping regions of the ER promoter which include fragments from -4100 bp to +212 bp, -2769 bp to +212 bp, -1000 bp to +212 bp, -735 bp to +212 bp, -245 bp to +212 bp and -200 bp to +212 bp, all relative to the first transcriptional start site. We have determined that the minimal promoter of the ER gene resides within the first 245 bp of the promoter. We also found that two GC/GT boxes as well as an E box located within this region are essential for promoter transcriptional activity in transient transfection assays. Gel shift assays demonstrated that both Sp1 and Sp3 transcription factors bind to the minimal promoter. Treatment of MCF-7 cells with the Sp1-
inhibitor, mithramycin A, inhibits expression of ER in a dose dependent manner. Other investigators have provided evidence for a participation of Sp1 with other factors in the regulation of gene transcription. We are therefore currently analyzing Sp1 and other potential interacting factors in the regulation of ER expression in breast cancer, as well as continuing to define the regions of the ER promoter involved in the differential expression of this gene.