Award Number: DAMD17-97-1-7096

TITLE: Training in Support of a Research Project Entitled "Study an ER Variant Identified from Breast Hyperplasia"

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REPORT DATE: July 2000

TYPE OF REPORT: Final

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

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Training in Support of a Research Project Entitled "Study an ER Variant Identified from Breast Hyperplasia"

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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, and that breast tumor tissue expresses elevated levels of ERα as compared to adjacent normal tissue. Previous studies suggest that ER expression is partly regulated at the transcriptional level. The research funded by this grant has focused on investigating the factors that regulate the expression of ERα in breast cancer cells as potential targets for clinical therapy.

The ERα minimal promoter is contained within the −245 bp to +212 bp region of the gene, and contains an E box, a GC box, and a CA rich region that are critical for ERα promoter activity. A multi-protein complex containing Sp1/Sp3, ERα and USF-1 interacts with the ERα minimal promoter. Independently, Sp1 and USF-1 are able to each transactivate the ERα minimal promoter, but together have an additive, if not synergistic, effect on transactivation. Additionally, a short sequence (AACT) located at −203 bp to −200 bp appears to be essential for functional activity since mutation of these nucleotides results in loss of all transcriptional activity of the promoter. The results of these experiments could prove clinically important in identifying new treatment targets.

1. Estrogen receptor
2. Breast Cancer
3. Transcription

Study an ER Variant Identified from Breast Hyperplasia

17. SECURITY CLASSIFICATION OF REPORT  
Unclassified
18. SECURITY CLASSIFICATION OF THIS PAGE  
Unclassified
19. SECURITY CLASSIFICATION OF ABSTRACT  
Unclassified
20. LIMITATION OF ABSTRACT  
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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, and that breast tumor tissue expresses elevated levels of ERα as compared to adjacent normal tissue. Previous studies suggest that ER expression is partly regulated at the transcriptional level. The research funded by this grant has focused on investigating the factors that regulate the expression of ERα in breast cancer cells as potential targets for clinical therapy.

The ERα minimal promoter is contained within the −245 bp to +212 bp region of the gene, and contains an E box, a GC box, and a CA rich region that are critical for ERα promoter activity. A multi-protein complex containing Sp1/Sp3, ERα and USF-1 interacts with the ERα minimal promoter. Independently, Sp1 and USF-1 are able to each transactivate the ERα minimal promoter, but together have an additive, if not synergistic, effect on transactivation. Additionally, a short sequence (AACT) located at −203 bp to −200 bp appears to be essential for functional activity since mutation of these nucleotides results in loss of all transcriptional activity of the promoter. 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, 2, 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:
I have made excellent progress in this final year of my investigation, focusing predominantly upon Specific Aims 2 and 3 and am on schedule in the grant. The work done in the first year of this study led to the identification of three transcriptional regulatory regions within the ERα minimal promoter, including a GC box, a flanking imperfect E box and a CA rich region, as well as the finding of Sp1/Sp3 as critical regulatory factors for the expression of ERα in breast cancer cells (Appendix A). The work done in the proceeding two years has focused on identifying other transcription factors that regulate ERα expression in breast cancer cells.
To identify other transcription factors that interact with the ERα minimal promoter (Specific Aim 2), we used a combined EMSA, Western blot analysis technique. Briefly, we probed whole cell extracts from MCF-7 breast cancer cells with a $^{32}$P-labeled oligonucleotide that spanned the ERα minimal promoter from nucleotides -245 to -182 (8). After separation through a 5% acrylamide gel and exposure to x-ray film, the protein/DNA complex (box, Fig. 1A) was excised from the gel and resolved (Fig 1B, lane A), along with whole cell extract from MCF-7 cells as a positive control (Fig 1B, lane B), through a 10% SDS-polyacrylamide gel. A standard Western blot protocol was followed as described previously (9). We probed the Western blot containing the minimal promoter binding proteins with antibodies to Sp1 (Fig 1B, Sp1) and Sp3 (Fig 1B, Sp3), which we had previously identified as critical for ERα transcription. A non-consensus E-box at -231 to -226 bp, which is a potential binding site for the basic helix-loop-helix transcription factor USF-1 (10), had been identified in the first year of the study as critical for full transcriptional activity of the ERα promoter, so we also probed the blot with an antibody to USF-1 (Santa Cruz Biotechnology, Santa Cruz, CA) (Fig 1B, USF-1). In addition, previous work done in the lab had identified the ER itself as able to autoregulate itself through the minimal promoter (11). We therefore also probed the blot with an antibody to ER (Fig 1B, ER). Antibodies for AP2 and GATA-3 were also used, but were negative in the EMSA lane, but positive in the MCF-7 lane (data not shown). This demonstrates that Sp1, Sp3, ERα and USF-1 all interact with the ERα minimal promoter.

In order to better understand the dynamics of the interaction of the multi-protein complex containing Sp1/Sp3, USF-1 and ERα and the ERα minimal promoter, we performed a series of EMSAs using the minimal promoter probe described earlier, and non-radioactive in vitro translated (IVT) proteins for Sp1, Sp3, USF-1 and ERα (Fig 2.). IVT products were prepared using the TNT® Coupled Reticulocyte Lysate System (Promega, Madison, Wi) as per manufacturer’s instructions. The expression plasmid for the ER α has already been used in our lab for EMSA (11). We obtained a CMV-driven expression plasmid for Sp3 from
Invitrogen (San Diego, Ca). The expression plasmid for USF-1 (pCX-USF-1) was kindly provided by Robert G. Roeder (12), while an Sp1 mammalian expression plasmid was kindly provided by Paul D. Gardner. In Figure 2, lanes A and B are controls, containing only the ERα minimal promoter probe, and probe with rabbit reticulocyte, respectively. Lanes C-G contain the USF-1 IVT product. In lane C a specific DNA/protein complex is formed (star) which is disrupted by the addition of an antibody to USF-1 (Santa Cruz Biotechnology) (lane D), but not by the addition of rabbit pre-immune serum (lane E). The addition of 10-fold molar excess of a cold consensus USF-1 oligonucleotide (5’ CACCCGGTCACGTGGCCTACACC 3’) in lane F is able to compete with the complex, but the same molar excess of an Sp1 consensus sequence (5’ AATTGATCGGGGCGGGCGGAGC 3’) in lane G is not. Lanes H–L contain the Sp1 IVT product. Again, a specific complex is formed in lane H (star), which can be supershifted (arrow) by the addition of an Sp1 antibody (lane I). The complex is not disrupted by the addition of the rabbit pre-immune serum (lane J) or the USF-1 consensus oligonucleotide (lane L), but is competed by a 10-fold molar excess of the cold Sp1-consensus oligonucleotide (lane K). The same is true for the Sp3 IVT product in lanes M–Q. A specific complex is formed (star, lane M) which can be supershifted with an antibody to Sp3 (lane N, arrow), or competed with by the Sp1 consensus oligonucleotide (lane P). The complex is unaffected by the addition of either rabbit pre-immune serum (lane O), or the USF-1 consensus oligonucleotide (lane Q). In contrast, the ERα IVT product is unable to bind to the ERα minimal promoter probe (lanes R–S), as we have seen previously in this lab (11). The ERα IVT product is able to bind to a consensus ER sequence (11) (free probe, lane T), since incubation of the ERα product with this oligonucleotide results in the formation of a specific complex (lane U, star), which can be supershifted by the addition of an ER antibody (lane V, arrow).

These sets of experiments demonstrate that Sp1, Sp3 and USF-1 are all able to bind to the ERα minimal promoter, yet the ERα itself is not. This suggests that ERα must be
part of the complex through protein:protein interactions. This has been demonstrated on other promoters, most notably through interaction with Sp1 at Sp1-binding sites (13) (14).

To evaluate the effect of USF-1 on ERα promoter transcriptional activity (Specific Aim 3), we performed co-transient transfections assays into MCF-7 breast cancer cells using the ERα minimal promoter luciferase reporter described in earlier work (see Appendix A, fragment A), the pCX-USF-1 expression plasmid, and an expression plasmid for a dominant-negative USF-1 which is void of the DNA-binding domain (ΔbTDU1), which was generously provided by Anne-Marie Le Francois-Martinez (15). The plasmid pNull-Renilla (Promega) was co-transfected for transfection normalization. All transfections are reported as fold activity over control after normalization for Renilla expression; with the control being the ERα minimal promoter luciferase reporter co-transfected with the pCDNA3.1 vector (Invitrogen) (Fig 3, column A). As shown in Figure 3, increasing exogenous expression of wild-type USF-1 increased promoter activity two to five fold over control, (Fig 3, columns B-D, respectively). Co-transfection with the dominant-negative USF-1 reduced wild-type USF-1 transactivation of the minimal promoter in a dose-dependent manner, 30% (column E) to 41% (column F). This data demonstrates an important role for USF-1 in the regulation of ERα transcription.

To further understand the significance of Sp1, USF-1 and ERα on transcriptional activity of the ERα minimal promoter (Specific Aim 3), we again performed co-transient transfection assays into MCF-7 breast cancer cells. We transfected the ERα minimal promoter luciferase reporter along with either the expression plasmid for Sp1, the expression plasmid for USF-1, the expression plasmid for ERα, or a combination of the expression plasmids, along with the pNull-Renilla plasmid for transfection normalization. The results are reported as fold over control after normalizing for Renilla expression (Fig 4.). Expression of Sp1 alone (Fig 4, column B) had little effect on promoter activity, as is commonly seen with mammalian cell transfection of Sp1. Expression of USF-1 (column C) resulted in a six-fold increase of activity over control. ERα alone had little effect on activity
(column D). When USF-1 and Spl are combined, as in column E, a synergistic effect is observed, with a thirty-fold increase in activity. The combination of ERα with either Spl (column F) or USF-1 (column G) appears to augment the activity of these factors, with activity at three- and fourteen-fold levels over control, respectively. When all three are expressed together (column H), activity is at its greatest, with a forty-eight-fold level of activity over control. This would suggest that while important individually, it is in their combination that these factors are able to most affect transcriptional activity of the ERα minimal promoter.

The CA rich region of the ERα minimal promoter, located between nucleotides -203 to -192 was critical for ERα transcriptional activity in earlier studies (see Appendix A, Fig 2B, fragment E). EMSA using a probe specifically to this area (-212 bp to -182 bp (16)) failed to detect any binding, and mutation of this region did not interfere with binding by the Spl family members or USF-1 to their upstream binding sites (data not shown). Therefore, to better understand the functional significance of this region, we introduced sequential 4 bp mutations into the CA rich region of the promoter. These reporter constructs were then transfected into MCF-7 cells and the results were expressed as fold activity compared to control, which was the pGL3-Basic vector (Promega), after normalizing for Renilla expression (Fig 5). The wild-type ERα minimal promoter fragment showed about a six-fold level of activity over control (Fig 5, A). The EcoRI-mutated -203 to -192 fragment (Fig 5, E) demonstrated an 80% loss of activity compared to the wild-type fragment. Mutation of nucleotides -203 to -200 (AACC) also resulted in complete loss of promoter activity (Fig 5, E-1), while mutation of the flanking 3' sequences had little effect on activity (Fig 5, E-2 and E-3). Earlier work (Appendix A, Fig 2B, fragment D) demonstrated that mutation of the flanking 5' sequence also has little effect. Ongoing DNase I footprinting analyses of this region will hopefully demonstrate whether the functional importance of this element is due to binding by transcription factors that are not directly recruited to the promoter at this
specific sequence, but are recruited as part of a protein complex. Whatever the reason, the integrity of nucleotides −203 to −200 is essential for ERα promoter transcription.

Transcriptional regulation of any promoter is usually complex. This appears to also be the case with the regulation of the ERα promoter. The data contained within this study lay a strong foundation for further work in understanding the mechanisms regulating ERα expression in breast cancer and developing potential clinical targets focusing on the transcriptional events which are involved in the progression of the disease.
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.
- Demonstrating that ERα transcription is regulated by a multi-protein complex which includes, Sp1/Sp3, USF-1 and ERα, and showing that when expressed together, have a synergistic effect on ERα promoter activity.
- Defining a critical function for the nucleotides located between −203 and −200 of the ERα gene in the regulation of ERα transcription.
REPORTABLE OUTCOMES:

Manuscripts:

SP1 IS ESSENTIAL FOR ESTROGEN RECEPTOR α GENE TRANSCRIPTION. Linda A. deGraffenried, Susan G. Hilsenbeck, and Suzanne A. W. Fuqua. Journal of Steroid Biochemistry and Molecular Biology (submitted)

Abstracts:


Degrees obtained:

Institution: Molecular Medicine program
University of Texas Health Science Center, San Antonio

Degree: Ph.D.

Year: expected completion date 10/00
CONCLUSION:

The work that has been accomplished by this study is significant. The ERα minimal promoter is located within the −245 bp to +212 bp region of the gene and contains a majority of the activity of the promoter in breast cancer cells. A multi-protein complex, containing at least Sp1/Sp3, USF-1 and ERα interacts with the minimal promoter and is important for the regulation of ERα transcription in breast cancer cells. Additionally, the integrity of a short 4-bp sequence within the minimal promoter is essential for transcription of the promoter. Together, these data present a strong foundation for the advancement of further studies that target specific transcriptional events that occur in the progression of breast disease.
REFERENCES:

APPENDIX A

SP1 IS ESSENTIAL FOR ESTROGEN RECEPTOR α GENE TRANSCRIPTION

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Running title: Sp1 Expression and ERα Gene Transcription
For Journal of Steroid Biochemistry and Molecular Biology
Influence of Sp1 on ERα Gene Transcription

SUMMARY

The exact molecular mechanisms regulating estrogen receptor (ER) α expression in breast tumors are unclear, but studies suggest that the regulation is at least partly transcriptional. We therefore undertook a detailed analysis of ERα promoter activity in a number of breast cancer cell lines. We find that the majority of ERα promoter activity lies within the first 245 bp of the 5'-flanking region of the gene. Three elements essential for full ERα promoter transcriptional activity were identified within the -245 bp to -192 bp region in transient transactivation assays using linker-scanner mutation analysis. These three elements include two binding sites for the Sp1 family of transcription factors as well as a non-consensus E box. We show that both Sp1 and Sp3 bind to this region using electrophoretic mobility shift assays. Exogenous expression of Sp1 or Sp3 in Sp1/3-negative Drosophila Schneider SL2 cells results in transactivation of the -245 bp to +212 bp fragment of the ERα promoter. These data demonstrate that transcription of ERα is dependent upon the expression of members of the Sp1 family.
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1. Introduction

Understanding the molecular changes that occur during the progression of breast disease could provide clinicians with earlier markers for detection of the disease, as well as potential targets for early intervention. One well-recognized target is 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 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], so that its overexpression may play a permissive role in the progression of breast cancer [7].

ERα expression is regulated, in part, at the mRNA level both in human breast cancer cell lines and in tumors [8-12]. The mechanisms regulating this expression so far remain unclear. There are at least two major transcription start sites residing within the upstream promoter region that have been identified in human breast cancer cells [13,14]. One of these, the P1 start site, is located at the +1 nucleotide relative to the cap site, and a less utilized P0 site is located at -3090 bp relative to the major transcription start site. Recently, a number of other potential start sites have been reported [15,16]. However, the relevance of these multiple start sites to the elevated levels of ERα expression seen in breast tumors is not understood at present.

Other investigators have identified potential regulatory regions in the ERα promoter [17,18]. One enhancer region, termed ER-EH0, contains an AP-1 binding site as described by Tang et al [17]. Although it has not yet been shown whether AP-1, or any other factor, binding to this enhancer region can directly transactivate the ERα promoter, it has been demonstrated that this region contains stronger promoter activity in ER-positive than in ER-negative breast cancer cells. Another candidate regulatory element has been identified within the 5' untranslated region [18], but mutation of this proximal region suggests that it has a minimal effect on the ER-EH0 enhancer activity [17]. It has also been shown that this proximal region binds the factor ERF-1, now known to be AP2γ [19]. AP2γ appears to be more highly expressed in ER-positive cell lines [18], but studies investigating how expression of AP2γ affects ERα promoter activity have not been detailed [20]. Recent studies in tumors would suggest that while there is a positive correlation between AP2γ expression and ERα expression, ERα expression is not solely dependent upon AP2γ expression, as evidenced by the fact that some ER-positive tumors fail to express AP2γ [21]. Obviously, further work is required to fully determine AP2γ's role in the regulation of ERα transcription.

In the current study, we have identified key regulatory elements essential for ERα transcription by examining the activity of ERα promoter fragments from -4100 bp to +212 bp in a panel of breast cancer cell lines.
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We show that the majority of ERα promoter activity lies within the -245 bp to +212 bp region. We also demonstrate important roles for three distinct elements within this region of the promoter and the 5' untranslated region. Two of these are potential sites for binding of Sp1 transcription factors, and we show that exogenous expression of either Sp1 or Sp3 increases ERα promoter activity. Furthermore, inhibition of DNA binding of these two transcription factors results in a decrease of both ERα promoter activity and ERα expression in breast cancer cells. We conclude that the Sp1 family of transcription factors play an essential role in ERα gene transcription.
2. Materials and Methods

2.1. Plasmid Construction

To isolate a genomic clone containing the 5' flanking region and first exon of ERα from a bacterial artificial chromosome library of human male fibroblasts CCD-978SK (ATCC No. CRL 1905) [22], Touch polymerase chain reaction (PCR) amplification [23] using primers \(5'\)TGAGCAGACA GCAAGTCTCC\(\text{2126}^{-}\) and \(5'\)TGCTCAGTG TGTAAGTCTAGG\(\text{1962}^{-}\) to the ERα gene [14] was used. For Touch PCR, the genomic DNA was amplified in a 25-µl reaction volume containing 100 pmoles of each primer as well as 5 units of AmpliTaq polymerase (Perkin-Elmer, Foster City, CA), 320 µM dNTPs, and 3 mM MgCl₂, using the following parameters: 15 sec at 96°C, 30 sec at 55°C and 3 min at 72°C for 35 cycles followed by an extra cycle with a 10-min extension step at 72°C. DNA was then prepared using a QIAfilter Plasmid Maxi Kit (QIAGEN Inc., Santa Clarita), and the ER genomic insert was confirmed by dideoxy sequencing [24].

The ERα genomic DNA was then used as a template to generate a series of 5'-deletion fragments. First, another set of PCR primers were designed with an added Kpn I site to facilitate subcloning into the luciferase reporter vector pGL3-Basic (Promega, Madison, WI). The 5' primers were ERP-0.245 \(5'\)GCCGTACCCC TTAGCTGAC TCTGCGTGC\(\text{217}^{-}\), ERP-0.735 \(5'\)GCCGTACCCC TAATGGGACC AAGTACAG\(\text{708}^{-}\), ERP-2.7 \(5'\)GCCGTACCCA TCCATGTGAA CGCCACTGG\(\text{2740}^{-}\), ERP-1.0 \(5'\)GCCGTACCG AGAAATGCGA GTTGTGACG\(\text{970}^{-}\) [14], and ERP-4.1 \(5'\)CCCGTACCGG ATCTGAAGCG AGTA\(\text{4077}^{-}\) [17]. All PCR fragments were generated with the same 3' primer, ERP+0.212 \(5'\)CGCTCAGTG CAGACCGTGG CCCCCGAGG\(\text{183}^{+}\), which included an added Xho I site for cloning purposes (see Fig. 1A, A-E respectively). PCR amplification was carried out using the Expand High-Fidelity polymerase (Boeringer Mannheim, Indianapolis, IN), with the same parameters described previously.

To create EcoRI-linker mutations scanning the -235 bp to -192 bp region of the ERα promoter, a modified version of the three-step PCR mutagenesis strategy described by Li and Shapiro [25] was used. These were then subcloned into the Kpn I-Xho I sites of the pGL3-Basic luciferase reporter plasmid. The PCR primers were designed to substitute an EcoRI-linker sequence (underlined below) for the wild-type ERα promoter sequence as well as to include a 5' Kpn I site for subcloning purposes. These primers include: an EcoRI site at -235 \(5'\)-

```plaintext
\(\text{CGCTCAGTG CAGACCGTGG CCCCCGAGG}\)
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ACCTTACGCA CGCGATTCC GGCCCCCGCC CC^{214}, an EcoRI site at -223 (5'-ACCTTACGCA
GATCCTCGTG CGCGCGATT CCGGGGCGTG GAAA^{202}, an EcoRI site at -211 (5'-ACCTTACGCA
GATCCTCGTG CGCCCCCGCC CCCTGAATT CCAACTCAGC CT^{194}), and an EcoRI site at -203 (5'-
ACCTTACGCA GATCCTCGTG CGCCCCCGCC CCCTGAATT CCCTGATCCAG CAGC^{182}). Briefly, the PCR reactions (25 μl) contained 100 ng of the luciferase reporter construct containing the -245 to +212 bp fragment and 5 units Amplitaq DNA polymerase (Perkin-Elmer, Foster City, CA). The first PCR amplification was carried out with 20 pmole of each mutation primer paired with 10 pmole of the GL3-2 downstream vector primer (Promega, Madison, WI). The reactions were carried out at 96 °C for 15 sec, 45 °C for 30 sec, and 72 °C for 3 min for 35 cycles, followed by an additional cycle with a 10-min 72 °C extension. Five μl of the products from the first round of amplification were then used directly in a second round of asymmetric amplification with the same primers and volumes used in the first round. The second round PCR conditions were 96 °C for 15 sec, 55 °C for 30 sec, and 72 °C for 3 min for 35 cycles with the additional extension cycle. The products from the second round were then gel-purified and 5 μl used as the template in a third round of amplification using the same conditions as the second round. The products were gel purified, digested with Kpn I and Xho I restriction enzymes, and again gel-purified. All constructs and mutations were confirmed by dideoxy sequencing. Plasmid DNA was again prepared using a QIAfilter Plasmid Maxi Kit.

2.2. Cell Culture and Transfection-

MCF-7C cells were kindly provided by Dr. Powel Brown and maintained in Improved Minimal Essential Medium (IMEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, Mo.) and 6 ng of bovine insulin (Sigma, St. Louis, MO) per ml. All of the other cell lines (T47-D, ZR-75-1, MDA-MB-231, and MDA-MB-435) were obtained from American Tissue Culture Collection (Rockville, MD) and maintained in complete Minimal Essential Medium (MEM) (Gibco/BRL, Gaithersburg, Md.) supplemented with 10% FBS. Cells were incubated at 37 °C in 5% CO₂.

Drosophila Schneider SL2 cells (American Type Culture Collection, Rockville, MD) were maintained in Schneider's Drosophila Medium (Gibco/BRL, Gaithersburg, MD) supplemented with 10% FBS,
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penicillin/streptomycin at 100 U/ml, and Amphotericin B fungazole (Cellgro, Herndon, VA) at 2.5 μg/ml. Cells were incubated at room temperature without CO₂.

Transient transfections with the human cell lines were performed two or more times in triplicate wells. Cells were seeded in 6-well cluster plates (Falcon, Franklin Lakes, NJ) at a density of 2 x 10⁵ cells/well 24 hours prior to transfection. For each well, 8 μl of Lipofectamine (Gibco/BRL, Gaithersburg, MD) were combined with 100 μl of OptiMEM media (Gibco/BRL), and then added to 0.5 μg of ER luciferase reporter construct plus 0.1 μg of pCMV-β-galactosidase (Promega, Madison, WI) to correct for transfection efficiency. Cells were incubated overnight with the DNA/lipofectamine mixture, and then washed in phosphate buffered saline. Fresh growth medium was applied and the cells were incubated for 24 additional hours before harvesting for luciferase determinations. Luciferase activity was measured using the Promega Luciferase Assay System (Promega, Madison, WI) based on the method of Rouet et al [26]. Luciferase values were normalized using the β-galactosidase values.

Transient transfection assays with the SL2 cells were carried out as described above with the following modifications: Cells were seeded 24 hours prior to transfection at a concentration of 2 x 10⁵ cells/well. 4.5 μl of FuGene 6 transfection reagent (Boeringher Mannheim, Indianapolis, IN) was used to transfect 0.5 μg of the ER luciferase reporter construct containing the −245 to +212 bp fragment, along with 0.5 μg of vector DNA (pPac) or increasing concentrations of expression plasmids for either Sp1 (pPacSp1 [27], a kind gift of Dr. Robert Tjian (University of California at Berkeley), or Sp3 (pPacUSp3 [28], generously provided by Dr. Guntram Suske, Philipps-Universitat Marburg, Germany). Both expression plasmids contain the coding sequence of Sp1 or Sp3 inserted downstream of the actin 5C promoter. The plasmid pNull-Renilla (Promega), an expression plasmid for the Renilla luciferase gene void of eukaryote promoter or enhancer sequences, was co-transfected for transfection normalization. Luciferase activity was measured using the Dual Luciferase kit from Promega. Mithramycin A (Sigma, St. Louis, MO), an inhibitor of Sp1 DNA binding [29], was also added 24 hours after transfection in some experiments at concentrations of 5 x 10⁻⁸ M to 5 x 10⁻⁶ M.

For Mithramycin A studies, MCF-7 cells were seeded 24 hours prior to transfection at a concentration of 1 x 10⁵ cells/well. Again, 4.5 μl of FuGene 6 transfection reagent (Boeringher Mannheim) was used to transfect 0.5 μg of the ERα promoter luciferase reporter construct containing the −245 to +212 bp fragment. The plasmid pNull-Renilla (Promega) was co-transfected for transfection normalization. Mithramycin A (Sigma) was added 24 hours
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after transfection at concentrations of $10^{-9}$ M, $10^{-8}$ M, and $10^{-7}$ M. Cells were harvested 18 hours after treatment and luciferase activity was measured using the Dual Luciferase kit from Promega.

2.3. Electrophoretic Mobility Shift Assay (EMSA)

EMSA's were carried out as previously published [30], with a few modifications. A probe was generated using PCR as previously described with ERα promoter primers 5'-ACCTTAGCAG ATCCTCGT-3' (245) and 5'-GCTGCTGGAT AGAGGCTGA-3' (182). The PCR fragment was then 32P-end-labeled using T4 kinase as previously described [30]. The radiolabeled probe (20,000 cpm) was added to 30 µg of high salt cellular extract [30] from MCF-7 cells, in a final reaction buffer of 10 mM HEPES (pH 7.9), 100 mM KCl, 5 mM MgCl2, and 10% glycerol, along with 3 µg of poly dIdC in a final reaction volume of 20 µl. Reactions were incubated for 30 minutes at room temperature. Oligonucleotides for competitive binding studies were preincubated with the MCF-7 extract for 5 min at room temperature before the addition of radiolabeled probe. The sequence for the consensus Sp1 oligonucleotide was 5'-ATT CGA TCA GGG CGG GGC GAG C-3'. For supershift assays, cell extracts were preincubated with 2 µg of either Sp1 (PEP2), Sp3 (D-20), Sp4 (V-20), or AP2 (C-18) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min on ice. The reactions were then separated on a 5% polyacrylamide gel with 0.5X TBE buffer electrophoresed for 5 hours at 150 volts at 4° C. The gel was dried and exposed to film (Kodak X-OMAT).

2.4. Western Blot Analysis

MCF-7 cells were seeded in 6-well plates at a density of 2 x 10^5 cells per well 24 hours prior to treatment. Cells were then treated with Mithramycin A (Sigma, St. Louis, MO) for 18 hours, harvested in a 5% SDS solution, and 10 µg of protein extract were resolved on a polyacrylamide gel as previously described [31]. These were then transferred from the gel to nitrocellulose membranes and subjected to immunodetection [31] with the ER-specific 6F11 monoclonal antibody (Novocastra Ltd., England), the Sp1 and Sp3 antibodies, or tubulin (Chemicon, Temecula, CA) for a loading control, S and the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL). Densitometry analysis was done using NIH Image 1.62 software.

2.5. Statistical Analysis-Promoter Activity
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For each of 5 human breast cancer cell lines, luciferase activity from triplicate wells was used to measure promoter activity of 5 promoter fragments and vector alone. Each experiment was completely replicated. Luciferase data were log-transformed prior to analysis, as indicated by a Box-Cox analysis [32]. Two-way analysis of variance, with promoter construct as one factor and experiment as the other, was used to evaluate differences among constructs after adjusting for experiment-to-experiment differences. Pairwise comparisons of least-squares estimated means for each construct were used to determine which constructs had similar activities. P-values were adjusted for multiple comparisons by the Sidak method, which is similar to but less excessively conservative than the Bonferroni method [33]. For purposes of presentations, means and 95% confidence intervals were back-transformed to give estimates of average and 95% confidence intervals of fold-increases in relative luciferase activity.

Linker-Scanner Mutated Fragments: Luciferase activity from triplicate wells was analyzed using one-way analysis of variance. Box-Cox analysis did not indicate a need for transformation. Pairwise comparisons were used to determine which fragments had similar activities. P-values were adjusted for multiple comparisons by the Sidak method. Analyses were performed using SAS (Version 6.12, SAS Institute, Cary, NC).

All other statistical analysis were performed using the Student T test, comparing the difference between control and experimental values.
3. Results

The Majority Of ERα Promoter Activity Resides Within The Most Proximal 245 Bases Of 5'-Flanking DNA-To identify regulatory regions involved in ER promoter transcriptional activity, five overlapping ER promoter deletion constructs, -245 bp to +212 bp, -735 bp to +212 bp, -1000 bp to +212 bp, -2769 bp to +212 bp, and -4100 bp to +212 bp (Fig.1, fragments A-E respectively), all relative to the first transcriptional start site (Fig. 1, P1), were subcloned into the luciferase reporter vector pGL3-Basic. [INSERT FIG 1 HERE] Since we have previously shown that the region between -245 bp to -9 bp contains low basal activity [34], we designed our smallest fragment (Fig. 1, fragment A) to include this region, as well as the two binding sites for AP2α identified by deConinck, et al [18], within the 5’ untranslated region. We extended the next fragment (Fig. 1, fragment B) out to -735 bp to include a 1/2 estrogen response element (ERE) at -420 bp [35]. Fragment C was then extended 5’ to include two other 1/2 EREs located at -860 and -888 bp. Fragments D and E were designed to include the P0 transcription start [13]; fragment E also contains the ER-EH0 enhancer [17].

These five constructs were transiently transfected into a panel of breast cancer cell lines that included the ER-positive MCF-7, T47D, and ZR75-1, as well as the ER-negative MDA-MB-231 and MDA-MB-435 (Fig 2) cell lines. [INSERT FIG 2 HERE] Data is shown as fold increase in luciferase units over vector control values. Fragment A exhibited high levels of activity in all the cell lines (Fig. 2A, panel A), displaying a 7 to 20-fold increase compared to vector alone, depending on the cell line. This supports our earlier observation [34] that this region contains important regulatory elements necessary for ERα transcription.

The activity profiles of the ER-positive cell lines were essentially the same. The activity of fragments B and C did not significantly change in MCF-7, T47D, or ZR75-1 cells, but was slightly increased (2-fold) relative to fragment A in ZR75-1 cells. The activity of fragment D significantly decreased (p<0.04) in all of the ER-positive cell lines relative to the activity seen with the A fragment, suggesting the presence of negative regulatory elements between -1000 to -2769 bp. In contrast, the activity of fragment E increased (p<0.003) in all three ER-positive cell lines, consistent with the data of Tang et al [17] who has previously characterized the ER-EH0 enhancer element within this region of the ERα promoter.

The activity profiles of the longer ERα promoter fragments were different in the ER-negative breast cancer cell lines compared the ER-positive cells. The activity of fragments B-E was significantly reduced compared to fragment A in the two ER-negative cell lines (p<0.005) (Fig. 2A). Since fragments B-E all have the region between -245 to -735 bp, it is possible that this region of the ER promoter contains potential negative regulatory elements
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that are utilized in ER-negative cells, but not ER-positive cells for reasons that we do not yet know. This will be pursued in a future study.

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. To do this we generated a promoter construct deleting the region between -245 and -200 bp, because an initial search for potential transcription factor binding sites [36] identified a number of sites within this region. This promoter deletion construct was then examined in MCF-7 cells using transient transfection analysis (Fig. 2B). Because the activity of fragment A in MCF-7 cells was representative of all the ER-positive cell lines, we chose this cell line for the next series of experiments. The -245 fragment (Fig. 2B, second bar) exhibited a 10-20 fold increase in activity, depending on the experiment, over the control vector alone. The removal of the -245 to 200 bp region (the -200 fragment, Fig. 2B, third bar) invariably resulted in the loss of all promoter transcriptional activity. These results suggest that elements critical for transcriptional activity of the ERα promoter lie within the -245 and -200 bp region; this region thus contains the ER minimal promoter.

Multiple Elements Are Required For Full ERα Promoter Activity: 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 the non-consensus E-box at -231 to -226 bp, the GC box at -223 to -214 bp, and a C/A rich box at -203 to -192 bp (Fig. 3) were introduced into the ERα promoter reporter fragment A (Fig. 4, starred regions). [INSERT FIG. 3 AND 4 HERE] These elements are potential binding sites for basic helix-loop-helix transcription factors and Sp1 transcription factor family members, respectively [37-40]. The linker-scanner mutation reporter constructs were then transfected into MCF-7 cells and the results expressed as fold activity compared to vector control (Fig. 4). Mutation of the region between -235 and -224 bp (Fig. 4, Fragment A1) resulted in a 37% loss of promoter activity. Even more significant activity losses of 63% and 81% respectively, were seen with fragments A2 and A4. In comparison, a negligible decrease in activity was seen when the region between bases -211 and -204 was mutated (Fig. 4, Fragment A3). These results suggest that there are critical elements contained within Fragments A2 (the region between -223 and -212 bp) and A4 (-203 to -192 bp). Furthermore, the significant reduction also seen when the region in Fragment A1 is mutated suggests that multiple elements, and their cognate binding factors, may cooperate for full ERα transcription activity of the minimal promoter.
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**Sp1 and Sp3 Interact with the ER Proximal Promoter Region**-To determine which transcription factors might be binding to the critical elements within fragment A, a radiolabeled oligonucleotide probe spanning the ERα promoter region from -245 to -182 bp (Fig. 3) was used in an EMSA to detect potential binding factors. Seven specific DNA-protein complexes were detected with MCF-7 protein extracts (Fig. 5, lane A, arrows labeled 1 through 7). [INSERT FIG. 5 HERE] 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 investigated with supershift analysis with antibodies to Sp1 and Sp3. 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) prior to addition of probe had no effect on any of these complexes. However, when both Sp1 and Sp3 antibodies are preincubated with the extract, all seven of the complex bands were affected (lane H). These data suggest that both Sp1 and Sp3 bind to the minimal ER promoter region.

**Sp1 and Sp3 are Able To Directly Transactivate the ER Minimal Promoter**-In order to determine whether Sp1 and Sp3 are capable of transactivating the ERα minimal promoter (ER promoter reporter fragment A), we performed a series of transient transfections in *Drosophila* SL2 cells. These cells do not express any of the Sp1 family members. In this experiment, we also treated the transiently transfected cells with the Sp1 DNA-binding inhibitor Mithramycin A (29) to determine its effect on exogenous Sp1 and Sp3 activity. As shown in Fig. 6, both Sp1 and Sp3 were capable of transactivating the ERα minimal promoter in a dose-dependent manner. [INSERT FIG 6 HERE] Exogenous expression of Sp1 was able to increase activity of the minimal promoter fragment 3 to 5-fold (Fig. 6, lanes 2 and 3), and expression of Sp3 increased promoter activity 4 to 6-fold (Fig. 6, lanes 8 and 9). Furthermore, this transactivation was inhibited in a dose-dependent manner by increasing concentrations of Mithramycin A. Concentrations of drug at $5 \times 10^{-8}$ M slightly inhibited promoter activity in Sp1 transfected cells (Fig. 6, lane 4), but significantly affected transactivation of the minimal promoter in Sp3 transfected cells ($p \leq 0.001$, Fig 6, lane 10). This effect was further enhanced with increasing concentrations of drug in both Sp1 and Sp3 transfected cells.
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These results show that Sp1 and Sp3 are independently capable of transactivating 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 could 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. [INSERT FIG 7 HERE] MCF-7 cells transiently transfected with the ER minimal promoter fragment A, and treated with increasing concentrations of Mithramycin A, demonstrated a dose-dependent loss of promoter activity (Fig. 7, panel A, p ≤ 0.006) as well as a decrease in endogenous ERα protein levels (Fig. 7, panel B). ERα promoter activity was decreased approximately 60% in cells treated with 10⁻⁶ M drug, 70% in cells treated with 10⁻⁴ 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 effects on ERα promoter activity were correlated with the effects on ERα expression levels (Fig. 7, panel B). ERα protein levels decreased 20% in cells treated with 10⁻⁹ M Mithramycin A as compared to control. This decrease in expression was more dramatic in cells treated with 10⁻⁸ M drug (62%). In cells treated with 10⁻⁷ M drug, expression of ERα was below detectable levels. The levels of Sp1 and Sp3 were unaffected by Mithramycin A (Tubulin levels were used as a control for loading in the Western blot, Fig. 7, panel B). Taken together with the SL2 transactivation data, these results demonstrate an essential role for Sp1 family members in transcription of the ERα gene in breast cancer cells.
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4. Discussion

Exploring the factors influencing ERα expression has been the focus of a number of studies [12-14,16-18,34,35]. These studies provide strong evidence that ERα expression in breast cancer is regulated at both the transcriptional as well as the translational level. To date, few definitive transcriptional regulatory regions have been identified within the ERα gene, and except for the ER itself [34,35], and possibly AP2γ [20], no transcription factor has been identified which can directly transactivate the ERα promoter. A better understanding of the specific factors that can influence ERα expression in breast cancer cells is critical for future strategies attempting to regulate its expression.

In this study, we first analyzed the activity of fragments spanning +212 to -4100 bp of the ERα promoter and 5’ untranslated region in a panel of breast cancer cell lines, thus identifying regions of the promoter to focus upon for closer study. Previous literature suggested regions with potential regulatory activity [17,18,34,35], upon which we based a strategy for construction of reporters with ERα promoter deletions. The results of these deletion experiments suggest that while there appears to be enhancer activity located between nucleotides -2769 and -4100, as was originally proposed by Tang et al. [17], the majority of ERα promoter activity lies within the first 245 bp of the 5’-flanking region of the ER gene. We also found a statistically significant loss of activity (p< 0.005) in ER-negative cells with the longer ER promoter fragments, which was not evident in the ER-positive cells. Thus the overall profiles of ER-positive and ER-negative cells were distinct. This might indicate the presence of a negative regulator within the -1000 to -2769 bp region of the promoter. This observation will be a focus for future studies.

We chose to first identify those factors that were essential for ERα expression. Since the majority of ERα promoter activity was located within the first 245 bp of the proximal promoter, we more closely analyzed this region. Our deletion studies indicated that this region of the promoter contained elements critical for transcriptional activity, thus defining this region as the minimal ER promoter. We then used linker-scanner analysis of this smallest defined minimal promoter region to identify potential regulatory regions. We demonstrated that two GC/CA rich boxes, as well as a non-consensus E box, were essential for full transcriptional activity of the ERα minimal promoter. GC/CA rich boxes classically bind members of the Sp1 family of transcription factors [37,39,41], and these factors have proven critical for full promoter activity of many genes. In this study we found that both Sp1 and Sp3 can bind to the minimal promoter region. In addition, these two proteins significantly transactivated the minimal promoter when exogenously expressed in Drosophila SL2 cells. Sp3 was once believed to be solely a
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repressor factor [28,42], but recently an activation role for Sp3 has also been demonstrated [41,43]. We conclude that one, if not both, of these two transcription factors are important for transcriptional regulation of ERα.

One potential mechanism how Sp1 and Sp3 affect ERα expression could involve a direct protein:protein interaction between Sp1 and ERα. We have previously shown that one mechanism by which ERα autoregulates this promoter region is by a protein:protein interaction with an as yet unidentified factor [34]. There is also precedence for ER-Sp1 interactions for transactivation of other promoters [44-47]. We are currently investigating the possibility of such an interaction on the ERα promoter.

Our results also suggest that Sp1 and Sp3 do not function alone in increasing ERα expression. Many studies indicate that Sp1 can participate in multi-protein complexes with diverse interacting partners to regulate transcription of certain genes [48,49]. Our linker-scanner analyses suggest that regions bordering the putative binding sites for the Sp1 transcription factors are also important for full promoter activity. The formation of a multi-protein, DNA-binding complex containing Sp1 and Sp3, as well as other as yet unidentified factors, is consistent with our detection of seven protein/DNA complex bands. Identifying the additional components within these complexes could prove critical for understanding the precise mechanism involved in regulating ERα expression.

In summary, we have demonstrated that the majority of ERα promoter activity in breast cancer cell lines lies within a minimal promoter located in the first 245 bp of the 5'-flanking region of the ERα gene. We have localized elements required for full ERα transcriptional activity to at least two potential binding sites for the Sp1 family of transcription factors, and have demonstrated that both Sp1 and Sp3 present in cellular extracts from breast cancer cells are capable of binding to this region. We have also shown that both of these factors are able to transactivate the ERα minimal promoter in Drosophila SL2 cells, and that interference with Sp1/Sp3 DNA binding results in a loss of ERα promoter activity as well as ERα protein expression in MCF-7 cells. These data strongly suggest an important role for these two proteins in transcription of the ERα gene. We anticipate that the results of this study will be a basis for the identification of other factors involved in the regulation of ERα transcription.

Acknowledgements

The authors wish to thank Drs. Torsten Hopp, Steffi Oesterreich, and Rachel Schiff for their invaluable input, and William Friedrich, M.S., and Irma Parra, M.S., for their skilled technical contributions. This work was supported by funds provided by NIH grant CA30195 to Suzanne A. W. Fuqua, and Department of Defense grant DAMD17-97-1-7096 to Linda deGraffenried.
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References


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FIGURE LEGENDS

Fig. 1. Construction of deletion fragments of the ERα gene promoter. ERα promoter fragments spanning 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. Fragments were designed to include the P1 transcriptional start site as well as the putative binding sites for AP2γ (fragment A), one _ERE (fragment B), two additional _EREs (fragment C), the secondary transcriptional start site P0 (fragment D), and the ER-EH0 enhancer (fragment E). All the fragments include the ATG at +233 bp. Fragment coordinates are expressed relative to the primary transcription start site.

Fig. 2. Promoter activity of the ERα 5'-flanking region. A, Constructs described in Fig. 1 were transiently transfected with pCMV-βgal (to correct for transfection efficiency) into a panel of ERα-positive (MCF-7, T47D, and ZR75-1) or ERα-negative (MDA-MB-231 and MDA-MB-435) breast cancer cells. Shown are the geometric mean relative fold luciferase (RFL) units over control, and 95% confidence intervals, summarizing two experiments each performed in triplicate. B, ERα promoter deletion fragment A-245/+212 and -200/+212 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.

Fig. 3. Schematic representation of the ERα promoter region between nucleotides -245 and -182 bp. Schematic diagram of the potential protein-binding sites located within the ERα minimal promoter region. The non-consensus E box (CTCGTG) located between nucleotides -231 and -226 deviates from the consensus E box at one nucleotide (CANNTG), and potentially binds members of the bHLH family of transcription factors [50]. The GC box located between nucleotides -223 and -214 and the CA rich box located between nucleotides -203 and -192 are potential binding sites for the Sp1 family of transcription factors.

Fig. 4. Identification of important regulatory elements in the ERα promoter by linker-scanner mutagenesis. Luciferase reporter constructs containing the wild type ERα minimal promoter fragment A, or linker-scanner mutations of this fragment, were transfected with pCMV-βgal into MCF-7 cells. Left panel, schematic representation of the ERα promoter fragments and the potential transcription elements and the linker-scanner mutations generated (star) between nucleotides -235 and -224 (B), nucleotides -223 and -212 (C), nucleotides -211
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and -204 (D), and nucleotides -203 and -192 (E). Right panel, promoter activity is expressed as relative fold luciferase units over vector control and is representative of two independent experiments done in triplicate.

Fig. 5. Sp1 and Sp3 interact with the -245 to -182 bp region of the ERα promoter. EMSA were performed using MCF-7 extracts. An end-labeled (20,000 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 a 25-fold molar excess of cold oligonucleotide (lane B), a 25-fold molar excess of a cold oligonucleotide containing an Sp1 consensus binding site (lane C), Sp1 antibody (Ab) (lane D), Sp3 Ab (lane E), Sp4 Ab (lane F), AP2 Ab (lane G), or both Sp1 and Sp3 Abs (lane H). Complexes are indicated by numbered arrows and supershifted complexes indicated by SS.

Fig. 6. Sp1 transcription factors transactivate the ERα minimal promoter in Drosophila cells. Drosophila SL2 cells were co-transfected with the ERα minimal promoter luciferase reporter construct and the pPac control vector or increasing concentrations (0.1-0.5 μg) of either an Sp1 or Sp3 expression vector. Transfected cells were also treated with increasing concentrations of an Sp1 DNA binding inhibitor, Mithramycin A, at concentrations ranging from $5 \times 10^{-6}$ M to $5 \times 10^{-8}$ 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. * p≤0.001 compared to control, # p≤0.001 compared to 0.5 μg untreated.

Fig. 7. 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 the ERα minimal promoter luciferase reporter construct (Fig 1A, A), and were treated with increasing concentrations of an Sp1 DNA binding inhibitor, Mithramycin A, at concentrations ranging from $10^{-9}$ 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, $5 \times 10^{-9}$, $5 \times 10^{-8}$, or $5 \times 10^{-7}$ Mithramycin A and measured for Sp1, Sp3, ERα expression by Western blot analysis. Tubulin staining was used as a control for protein loading.
Fig. 1
Fig. 3
Fig. 4
Fig. 6
Fig. 7
APPENDIX B

FIGURE LEGENDS:

Fig. 1. Sp1, Sp3, USF-1 and ERα interact with the -245 to -182 bp region of the ERα promoter. A, EMSA was performed using MCF-7 extracts. An end-labeled (20,000 cpm) oligonucleotide which spanned the ER promoter from -245 bp to -182 bp (Appendix A, Fig. 3) was incubated with 30 μg of extract alone. The complex is indicated by a box. B. ERα promoter-binding proteins isolated by EMSA (A) and MCF-7 extract (B) were resolved by SDS-PAGE and probed with antibodies to Sp1, Sp3, USF-1 and ERα.

Fig. 2. Sp1, Sp3, and USF-1 but not ERα are able to bind to the -245 to -182 bp region of the ERα promoter. EMSAs were performed using in vitro translated products. An end-labeled (20,000 cpm) oligonucleotide which spanned the ER promoter from -245 bp to -182 bp was incubated alone (lane A), or in the presence of rabbit reticulocyte (lane B), USF-1 IVT product (lane C), USF-1 IVT with anti-USF-1 antiserum (lane D), USF-1 with rabbit pre-immune serum (lane E), USF-1 IVT with 10-fold molar excess of a cold oligonucleotide containing a consensus USF-1 binding site (lane F), USF-1 IVT with 10-fold molar excess of a cold oligonucleotide containing an Sp1 consensus binding site (lane G), Sp1 IVT product (lane H), Sp1 IVT with an anti-Sp1 antiserum (lane I), Sp1 IVT with rabbit pre-immune serum (lane J), Sp1 IVT with 10-fold molar excess of a cold oligonucleotide containing an Sp1 consensus binding site (lane K), Sp1 IVT with 10-fold molar excess of a cold oligonucleotide containing a consensus USF-1 binding site (lane L), Sp3 IVT product (lane M), Sp3 IVT with an anti-Sp3 antiserum (lane N), Sp3 IVT with rabbit pre-immune serum (lane O), Sp3 IVT with 10-fold molar excess of a cold oligonucleotide containing an Sp1 consensus binding site (lane P), Sp3 IVT with 10-fold molar excess of a cold oligonucleotide containing a consensus USF-1 binding site (lane Q), ERα IVT (lane R), ERα IVT with anti-ER antiserum (lane S). A 32P-radiolabeled consensus ERE probe was incubated alone (lane T).
with the ERα IVT product (*lane U*) or with the ERα IVT product and anti-ER antiserum (*lane V*). Complexes are indicated by *stars* with supershifted complexes indicated by arrows.

Fig. 3. **USF-1 transactivates the ERα minimal promoter in MCF-7 breast cancer cells.** MCF-7 cells were co-transfected with the ER minimal promoter luciferase reporter construct and pCDNA3.1 vector (column A) or increasing concentrations of either USF-1 alone (columns B-D) or USF-1 with increasing concentrations of a dominant-negative USF-1 expression vector (columns E and F). 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 pCDNA3.1 vector alone and was representative of two independent experiments done in triplicate.

Fig. 4. **Multiple factors transactivate the ERα minimal promoter in MCF-7 cells.** MCF-7 cells were co-transfected with the ER minimal promoter luciferase reporter construct and the pCDNA3.1 vector (A), an Sp1 expression vector (B), a USF-1 expression vector (C), an ERα expression vector (D) or a combination of Sp1 and USF-1 (E) Sp1 and ERα (F), USF-1 and ERα (G) or Sp1, USF-1 and ERα expression vectors. 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 pCDNA 3.1 vector alone and was a combination of three independent experiments done in triplicate.

Fig. 5. **Identification of important regulatory nucleotides in the ER promoter by mutagenesis.** 

*Left panel,* schematic presentation of the ERα minimal promoter fragments and the potential transcriptional elements (A), the linker-scanner mutation (star) between nucleotides -203 and -192 (E), and the 4 bp mutations within the -203 to -192 bp region (E-1 – E-3).

*Right panel,* promoter activity was expressed as corrected relative fold luciferase units over control and was representative of two independent experiments done in triplicate.
Fig. 5